Investigating Mechanisms of Environmental Chemical Tolerance and Toxicity in Brown Trout using RNA-seq

Submitted by

Tamsyn Uren Webster

To the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences, November 2013

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

…….. (Tamsyn Uren Webster)

Abstract

Brown trout (*Salmo trutta*) are an ecologically and economically important native European species, known to be sensitive to environmental stressors. Compared to other model species, there is little information available on the toxicological responses of this species to environmental pollutants. High-throughput RNA-sequencing (RNA-seq) is emerging as a sensitive and accurate tool for conducting transcriptomics, but is yet to be widely used in ecotoxicology. A major advantage of RNA-seq is that it can be used to conduct non-biased, global gene expression analysis in species without existing genomic sequence information. Therefore, during this PhD I set out to investigate global mechanisms of toxicity for a selection of the most environmentally relevant chemicals likely to impact upon natural brown trout populations. By using RNA-seq, I also aimed to demonstrate the potential application of this technology as a valuable tool in ecotoxicology.

To address these objectives, I conducted transcriptomic profiling, both on wild brown trout and on those exposed to agricultural pollutants in a laboratory setting. Using RNA-seq in combination with analysis of tissue metal concentration I found evidence of a high degree of metal tolerance in a chronically exposed wild population of brown trout from the river Hayle. The main molecular mechanisms responsible for this metal-tolerance included regulation of metal- and ion-homeostasis pathways. In the laboratory exposures, I found evidence of considerable transcriptomic changes in male brown trout exposed to 34.38 ng/L E2, including up-regulation of typical oestrogen-responsive transcripts (vitellogenins, zona pellucida proteins and estrogen receptor 1), as well as hepatic processes that can be associated with vitellogenesis such as lipid metabolism, cell proliferation and ribosome biogenesis. This concentration is within a range measured in sewage effluent and, more occasionally, in surface waters. I also exposed male brown trout to linuron, a widely used pesticide, and observed a striking down-regulation of enzymes involved in the cholesterol biosynthesis pathway and up-regulation of transcripts involved in cellular stress response following exposure to 250 µg linuron/L. There was also some evidence of similar responses occurring at the lower, environmentally relevant concentration (2.5 µg/L). I then compared the mechanisms of toxicity of glyphosate, the most widely used herbicide in the world, and its commercial formulation Roundup in juvenile brown trout. I found evidence of a cellular stress response consistent with

generation of oxidative stress at concentrations of 10 µg/L and above which, importantly, is within the range of concentrations measured in the environment. To investigate the potential reproductive toxicity of these compounds I also conducted an exposure of breeding zebrafish to glyphosate and Roundup, and found evidence of reproductive toxicity, but only at a very high concentration (10 mg/L). This work therefore provides valuable information on the toxicological effects of these environmentally relevant chemicals in brown trout, which can potentially be used to assess the risk they pose to natural populations and therefore contribute to the sustainable management of this species.

I have successfully employed RNA-seq to achieve the main objective of this PhD and, in so doing, have demonstrated the value of this technology in ecotoxicology. Specifically, we have demonstrated the ability of RNA-seq to identify conserved responses typically associated with oestrogen exposure. We also highlight the importance of optimising the experimental design and strategy for RNA-seq data analysis to improve the quality of transcript expression analysis. Throughout the course of this work we have benefited from improvements in sequencing technology and the tools available for data analysis. This technology is continuing to develop rapidly, and it is likely that RNA-seq will become the dominant tool for conducting transcriptomics in ecotoxicology in the future.

Acknowledgments

First I would like to thank my supervisor Eduarda Santos, whose enthusiasm for ambitious and progressive science has truly inspired me. Eduarda has always been extremely supportive and encouraging, and I really appreciate the huge amount of effort and time she has invested in me. She has helped me develop confidence in my own ability as a scientist, and played a massive part in making this PhD such an enjoyable experience.

I also thank Ronny van Aerle for all the help he has given me with the bioinformatics, making it not quite such a daunting task as it might have been. He gave me invaluable advice, patiently helped decipher endless error messages, put up with me crashing his computer and was always enthusiastic about talking code!

Next I would like to acknowledge everyone who made this work possible, including Karen Moore, Audrey Farbos, Alex Moorhouse and Konrad Paszkiewicz who conducted the sequencing and offered technical advice, and Hannah Florance for the analysis of water samples. I also thank Nic Bury, our collaborator from Kings College and Charles Tyler for valuable advice on experimental design.

I'd particularly like to thank Jan Shears who helped me look after the brown trout, and shared several moments of the associated despair, and also Lauren Laing, who was just as enthusiastic about counting zebrafish eggs on the 41st straight day as the first.

There are many other people from the lab group that I would like to thank, but especially Patrick and Shelly for good scientific discussion and challenging questions. Also, Jenny and Victoria, who put up with me sitting next to them, provided biscuits and pretended to be interested in my favourite genes and coding highs and lows. Various sporting activities have unquestionably kept me sane at times during my PhD, so I'd like to thank Lisa, Rhys, Jo, Greg and Steve for the competition and company.

Finally I'd like to thank the Sivell Place crew and, especially, my mum and dad who have always been incredibly supportive and encouraging of my 'colouring in'.

Table of Contents

List of Figures and Tables

Research Papers and Author's Declaration

Research paper 1. Tamsyn M. Uren Webster, Nic Bury, Ronny van Aerle and Eduarda M. Santos (2013) Global Transcriptome Profiling Reveals Molecular Mechanisms of Metal Tolerance in a Chronically Exposed Wild Population of Brown Trout. *Environmental Science and Technology*; 47; 8869−8877.

Research paper 2. Tamsyn M. Uren Webster, Janice A. Shears and Eduarda M. Santos (2014) Identification of conserved hepatic transcriptomic responses to estrogen using high-throughput sequencing in brown trout. *Manuscript in preparation.*

Research paper 3. Tamsyn M. Uren Webster and Eduarda M. Santos (2014) Global transcriptome profiling reveals down-regulation of cholesterol biosynthesis and up-regulation of cellular stress response in brown trout (*Salmo trutta*) exposed to linuron. *Manuscript in preparation.*

Research paper 4. Tamsyn M. Uren Webster and Eduarda M. Santos (2014) Global transcriptomic profiling demonstrates induction of oxidative stress and compensatory stress responses in brown trout exposed to glyphosate and Roundup. *Manuscript in preparation.*

Research paper 5. Tamsyn M. Uren Webster, Lauren V. Laing, Hannah Florance and Eduarda M. Santos (2014) Effects of glyphosate and its formulation, Roundup, on reproduction in zebrafish (*Danio rerio*). *Manuscript provisionally accepted by Environmental Science and Technology.*

Appendix:

Tamsyn M. Uren Webster, Ceri Lewis, Amy L. Filby, Gregory C. Paull and Eduarda M. Santos (2010) Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. *Aquatic Toxicology;* 99; 360-369.

Statement: I, Tamsyn Uren Webster, made the following contributions to the research papers presented in this thesis. I sampled the fish from the field locations, prepared tissue samples for metal analysis, prepared the libraries for sequencing, conducted the bioinformatics analysis with Ronny van Aerle, and wrote the manuscript for **paper 1**. Nic Bury sampled fish from field locations, contributed to experimental design and performed the measurement of tissue metal concentrations for **paper 1.** For **papers 2, 3 and 4**, I planned and conducted the exposure experiments with some technical support from Jan Shears, prepared the libraries for sequencing, performed the bioinformatics analysis of sequence data and wrote the manuscript. I planned the experiments for **paper 5**, conducted the exposure and histological analysis with support from Lauren Laing, performed the RT-QPCR analysis and wrote the manuscript. Hannah Florance performed the water chemistry measurements for **paper 5**. For all papers, Eduarda Santos supervised all aspects of the experimental work and the manuscript preparation.

For the paper in the appendix, I performed to the exposure experiment and histological analysis during my MSc, and this work contributed to the award of this degree. I performed the molecular analysis and wrote the manuscript during the first part of my PhD.

List of General Abbreviations

11KT	11-ketotestosterone
AChE	acetylcholinesterase
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
AR	androgen receptor
a.e.	glyphosate acid equivalent
ARE	antioxidant response element
ATP	adenosine tripgosphate
BCV	biological coefficient of variation
bp	base pair
BPA	bisphenol A
cDNA	complementary DNA
DDT	dichlorodiphenyltrichloroethane
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
dpf	days post fertilisation
E1	oestrone
E2	17β-oestradiol
EE2	ethinylestradiol
ER	oestrogen receptor
ERCC	External RNA Controls Consortium
ERE	oestrogen response element
FDR	false discovery rate
FPKM	fragments per kilobase of exon per million fragments mapped
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GO	Gene Ontology
GPX	glutathione peroxidases
GR	glutathione reductase
GSH	Reduced glutathione
GSI	gonad-somatic index
GSSH	oxidised glutathione
GST	glutathione S-transferase
hpf	hours post fertilisation
HPG	hypothalamic-pituitary-gonadal axis
HPI	hypothalamic-pituitary-interrenal axis
HPT	hypothalamic-pituitary-thyroid
HSI	hepatosomatic index
HSP	heat shock protein
IGF	insulin-like growth factor
LH	luteinising hormone
MAPK	mitogen-activated protein kinase
MDS	multidimensional scaling

MRE	metal response element
mRNA	messenger RNA
MT	metallothionein
MYC	myelocytomatosis oncogene
N50	N50 statistic
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OECD	Organisation for Economic Co-operation and Development
OP	organophosphorous
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCR	polymerase chain reaction
POEA	polyethoxylated tallow amine
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
QSAR	Quantitative Structure-Activity Relationship
RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RT-QPCR	quantitative real time polymerase chain reaction
SCAP	SREBP-cleavage-activating proteins
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
SRE	sterol response elements
SREBP	sterol regulatory element binding protein
StAR	steroidogenic acute regulatory protein
VTG	vitellogenin

List of Species Names

Atlantic croaker	*Micropogonias undulates*
Brown trout	*Salmo trutta*
Cod	*Gadus morhua*
Fathead minnow	*Pimephales promelas*
Flounder	*Platichthys flesus*
Glanville fritillary butterfly	*Melitaea cinxia*
Gudgeon	*Gobio gobio*
Gulf killifish	*Fundulus grandis*
Human	*Homo sapiens*
Japanese medaka	*Oryzias latipes*
Mouse	*Mus musculus*
Rainbow trout	*Oncorhynchus mykiss*
Rainbowfish	*Melanotaenia duboulayi*
Roach	*Rutilus rutilus*
Silver catfish	*Rhamdia quelen*
Stickleback	*Gasterosteus aculeatus*
Tilapia	*Oreochromis niloticus*
Yeast	*Saccharomyces cerevisiae*
Yellow perch	*Perca flavescens*
Zebrafish	*Danio rerio*

CHAPTER 1

General Introduction

CHAPTER 1: GENERAL INTRODUCTION

1.1 Brown trout and potential chemical exposure

The brown trout (*Salmo trutta*) is a native European species, more closely related to Atlantic salmon (*S. salar*) than north American salmonids from the *Oncorhynchus* genus, which include rainbow trout (*O. mykiss*). Brown trout inhabit lakes and rivers, and tend to prefer fast-flowing, well-oxygenated, cold waters which are often characteristic of smaller, upland rivers. In England and Wales, populations of brown trout were found in 70 % of rivers surveyed by the Environment Agency in 2001, and were more common in Wales, and South-West and Northern England, than in the larger, slower flowing rivers associated with the South-East of England (Environment Agency 2004). Brown trout spawn annually in the headwaters of streams of rivers between October-December, where females dig indentations (or redds) in gravel stream beds. After spawning the fertilised eggs are covered with gravel, the embryos hatch in the spring and the alevins stay closely associated with the gravel beds until yolk-sac re-absorption. Fry and parr tend to seek sheltered areas with plenty of cover and food supply, and gradually establish wider territories within the river. After 1-3 years as parr, adult brown trout return to the headwaters to spawn. Brown trout generally grow to be between 20-50 cm in length, and have been reported to live for up to 20 years (Elliott 1994, Klemetsen et al. 2003). A significant number of brown trout are anadromous, and known as sea trout, although the exact proportions are unclear and vary in different populations. These fish undergo smoltification and migrate to sea before returning to spawn in their natal rivers in a similar way to Atlantic salmon, but there appears to be no definitive underlying genetic distinction between sea trout and resident brown trout. The factors influencing migration have been hypothesised to involve environmental cues and the energetic cost-benefit balance determined by food availability (Wysujack et al. 2009).

Brown trout feed upon zooplankton, invertebrates, crustaceans, worms and molluscs, and larger individuals also eat amphibians and other fish such as minnow and stickleback. During the fry and parr stages, they are also important prey for piscivorous fish and birds (Elliott 1994, Klemetsen et al. 2003). Therefore, brown trout have an essential role in the food web of freshwater ecosystems. In addition to their ecological importance, brown trout also have a considerable social and

economic value. Angling is a common recreational activity, and was estimated to involve 3.9 million people with a total annual expenditure of £3 billion/year in 2001. Although, coarse fisheries make up the majority of this figure, angling for brown trout can make significant contributions to local economies, particularly in rural areas. In 2001 the total value of brown trout fisheries in England and Wales was estimated to be £525 million, with £545 million associated expenditure by anglers, and this provided employment for 700 people (Environment Agency 2004). Sea trout fisheries have a considerable additional value, although this is difficult to distinguish from the figures for salmon. Brown trout aquaculture, for food and re-stocking, was worth an additional £21.5 million (Environment Agency 2004).

Brown trout, along with other fish species, are potentially exposed to over 100,000 different chemical pollutants in surface waters (e.g. Desbrow et al. 1998, Erickson et al. 2008). Point sources of chemical pollution include wastewater treatment work effluents, industrial and mining outflows, while diffuse sources include urban and agricultural runoff. For brown trout, which are typically found in headwaters and smaller rivers, chemicals originating from agricultural activities are arguably the most environmentally relevant. Agricultural pollution typically occurs via direct input of steroidal oestrogens and pharmaceuticals from livestock waste entering streams, and via run-off from fields spread with manure (Shore and Shemesh 2003, Shappell et al. 2010), and applied with pesticides and fertilisers (Racke 2003, Blanchoud et al. 2007). Such agricultural pollution is unlikely to result in a constant input of contaminants and instead may consist of peaks in contamination, for example in runoff when rainfall follows pesticide/fertiliser application. Upper regions of streams typically have low and variable flow rates which can make them especially vulnerable to acute pollution events.

Many chemical contaminants of freshwater environments are potentially toxic to fish, and induce toxicity via a range of different mechanisms. Compared to some other species, including zebrafish (*Danio rerio*) and rainbow trout, there has been far less research on chemical toxicity in brown trout. In particular, previous research investigating molecular mechanisms of toxicity has been limited due to the lack of genomic resources available for this species. While evidence from model species is invaluable, it is also very important to specifically investigate potential chemical

toxicity in more environmentally-relevant, native species. For example, brown trout are known to be more sensitive to a number of chemical pollutants, and also other environmental stressors including temperature change and hypoxia, than rainbow trout (e.g. Elliott 1994, Molony 2001, Klemetsen et al. 2003), and therefore may also be more susceptible to these pressures in the environment.

In the introduction to this thesis, first I discuss the mechanisms of toxicity by which chemical pollutants are likely to affect natural populations of brown trout, in particular highlighting endocrine disruption and oxidative stress, and giving special emphasis to their underlying molecular basis. Next, I discuss the value of investigating molecular mechanisms of toxicity, and the approaches commonly employed to do this. I then describe the development of high-throughput RNA sequencing and its potential application as a valuable tool in ecotoxicology, especially for species lacking existing genomic sequence information like the brown trout. Finally, I present the aims of this thesis and a summary of the approach taken.

1.2. Mechanisms of chemical toxicity

Mechanism (or mode) of action has been defined as the common physiological or behavioural response characterising an adverse biological effect of chemical exposure, while mechanisms of toxicity generally refer to the underlying biochemical processes affected (Rand et al. 1995). However, these terms have been defined in different ways and are often used interchangeably (Escher and Hermens 2002). Mechanisms of toxicity can be assessed and described at different levels of biological organisation, including effects at a gene, protein, cellular, tissue and individual level. In this introduction ‘molecular mechanisms of toxicity’ refer to changes at a transcriptional level.

Mechanisms of toxicity can be classified in a number of ways. Escher and Hermens (2002) recommend classification based on both the target site and type of interaction of a chemical with the target. They categorise three main types of chemicals; those which induce baseline toxicity; those with specific mechanisms of toxicity; and those with multiple mechanisms of toxicity. Baseline toxicity (or narcosis) refers to the non-specific partitioning of chemicals in biological membranes, which subsequently

disrupts their integrity and function (Schultz 1989, Wezel and Opperhuizen 1995, Escher and Hermens 2002). Baseline toxicity can be induced by a diverse range of chemicals, including many lipophilic organic compounds such as polycyclic aromatic hydrocarbons (PAHs) (Schultz 1989, Wezel and Opperhuizen 1995, Escher and Hermens 2002, Smith et al. 2013a). In contrast, chemicals with specific mechanisms of toxicity include those that stimulate transcriptional changes through receptor binding, those that bind and interfere with the function of enzymes, channel proteins and other cellular components, and those that generate reactive oxygen species (ROS) which subsequently damage cellular components (Escher and Hermens 2002, Di Giulio and Meyer 2008, Hahn and Hestermann 2008). Additionally, many chemicals possess multiple mechanisms of specific and/or non-specific toxicity.

The mechanism(s) of toxicity of a chemical depends on its chemical structure and properties, specifically the functional groups that govern the type and degree of interactions with biological molecules (Russom et al. 1997, Escher and Hermens 2002). Therefore, chemicals with structural similarities may share mechanisms of toxicity, and are often classified together. For example, Russom et al. (1997) describe the categorisation of ~600 chemicals based on their chemical structure, toxicodynamic profiles and acute toxicity data from fathead minnow (*Pimephales promelas*), from which they infer eight general mechanisms of toxicity. Chemical groups that contain the same basic functional structure generally share similar mechanisms of toxicity. For example, organophosphorous (OP) insecticides all contain derivatives of phosphoric acid or thiophosphoric acid, and their target mechanism of action in invertebrates is neurotoxicity through the direct phosphorylation and inhibition of acetylcholinesterases (AChEs) (Pope 1999). In fish the major mechanism of toxicity of OP insecticides is also AChE inhibition, although there are also other mechanisms of toxicity, including generation of oxidative stress, which vary depending on chemical structure (Pope 1999, Fulton and Key 2001). Modification of the active functional group can widely change the specificity and potency of chemical-biological interactions. In OP pesticides, for instance, substituting a '=0' group with a '=S' group (thiono moiety) on the phosphorous atom increases toxicity to both target and non-target species (Fulton and Key 2001). Such modification of functional groups is crucial in the design of pesticides and drugs, and is also crucial when considering potential toxic effects of exposure in fish. For

example, the relationship between chemical structure and mechanism of toxicity forms the basis of Quantitative Structure-Activity Relationships (QSARs), which are widely used by regulatory bodies to predict toxicological effects of exposure (Russom et al. 1997, Escher and Hermens 2002).

1.3 Specific mechanisms of toxicity discussed in this thesis

1.3.1 Endocrine disruption

The endocrine system consists of a network of glands and/or cells which secrete chemical messengers (hormones) into the bloodstream. Hormones bind specific receptors in target cells, effecting specific biological responses. In this way the endocrine system is responsible for regulating many physiological processes including reproduction, metabolism, homeostasis, growth and development. Chemical pollutants have been shown to disrupt many hormone regulated processes including via interference with the hypothalamic-pituitary-gonadal (HPG) axis, the hypothalamic-pituitary-interrenal (HPI) axis and the hypothalamic-pituitary-thyroid (HPT) axis (Young et al. 2005, Thomas 2008). The disruption of the endocrine control of reproduction via the HPG axis has been the subject of extensive research and will be the focus of this discussion.

1.3.1.1. Endocrine control of reproduction

The endocrine regulation of reproduction is highly conserved in vertebrates, including in teleosts, and is controlled principally via the HPG axis. The hypothalamus integrates a series of environmental stimuli, including temperature, photoperiod and social cues, with signalling associated with the physiological status of the individual, and responds by secreting gonadotropin-releasing hormone (GnRH). GnRH binds membrane-bound receptors in the pituitary inducing the release of gonadotropins, follicle-stimulating hormone (FSH) and luteinising hormone (LH), into the bloodstream. FSH and LH bind specific membrane-bound gonadotropin receptors in ovarian and testes somatic cells where they play a key role in the regulation of gonad development and steroidogenesis. The role of FSH is mainly associated with stimulating gonadal development and vitellogenesis in females and spermatogenesis in males, while LH is primarily associated with the regulation of final oocyte

maturation and ovulation in females, and spermiation in males (Thomas 2008, Zohar et al. 2010).

There are three primary classes of steroid hormones involved in the control of reproduction; androgens, oestrogens and progestins (Young et al. 2005, Thomas 2008). All steroids are synthesised from cholesterol and a simplified schematic of steroidogenesis is shown in Figure 1. The key rate-limiting step in steroidogenesis is the transport of cholesterol to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), the production of which is regulated by gonadotropins. Cholesterol is converted to pregenolone, and then to progestins and androgens by a series of steroidogenic enzymes. Androgens are converted to oestrogens by aromatase. These sex steroids have a number of roles in the regulation of reproduction, both in the gonads and in distant target tissues. In males, androgens are responsible for controlling all stages of spermatogenesis and sexual development, development of secondary sexual characteristics and are important regulators of reproductive behaviour. The principle androgen in fish is 11-ketotestosterone (11KT) (Borg 1994). In females, oestrogens (principally 17β-oestradiol; E2) are similarly involved in the regulation of sexual development and oogenesis. An additional, essential, role of E2 in fish, and other oviparous vertebrates, is to regulate the production of the egg yolk precursor proteins, vitellogenins, and vitellin envelope proteins (zona pellucida) in the liver (Thomas 2008). The predominant role of progestins in fish is to promote the final maturation of gametes in both males and females. Sex steroids also feedback to the hypothalamus and pituitary, which regulate gonadotropin secretion and further steroidogenesis (Young et al. 2005).

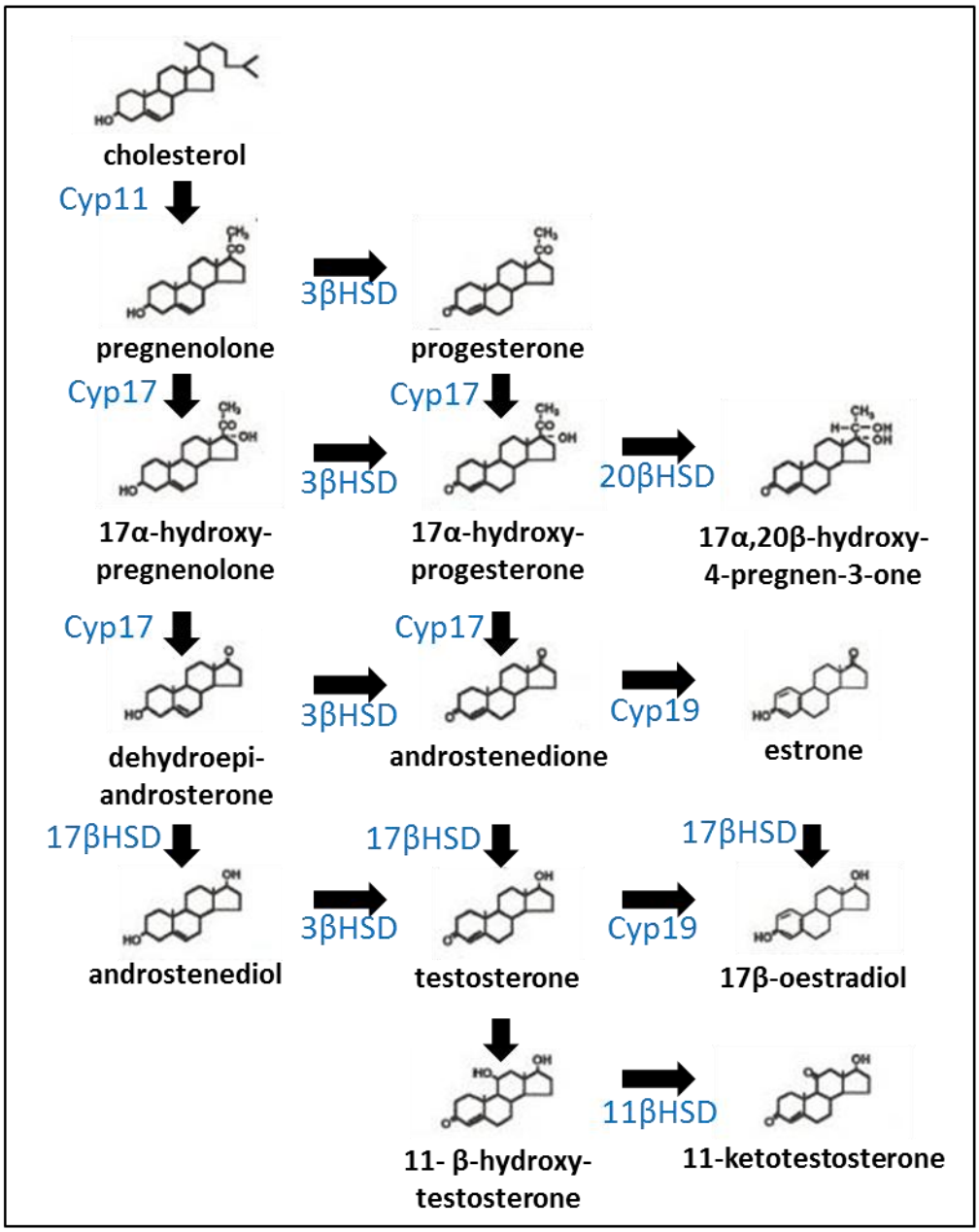


Figure 1. Steroidogenesis in fish. Adapted from Young et al. 2005. Cyp11: cholesterol side-chain cleavage enzyme, Cyp17: 17-alpha hydroxylase, Cyp19: aromatase, 3βHSD: 3-beta hydroxysteroid dehydrogenase, 11βHSD: 11-beta hydroxysteroid dehydrogenase, 17βHSD: 17-beta hydroxysteroid dehydrogenase, 20βHSD: 20-beta hydroxysteroid dehydrogenase.

The action of sex steroids in target cells is primarily mediated via intracellular nuclear receptors, which are ligand-dependent transcription factors (Aranda and Pascual 2001). Steroids diffuse across cellular membranes before binding and activating

nuclear receptors present in the cytosol. Activated nuclear receptors undergo conformational changes, including dissociation from molecular chaperones and dimerization, and translocate to the nucleus where they bind to hormone response elements in the promoter regions of target genes, regulating their transcription. In fish, three subtypes of nuclear oestrogen receptors (ESR1, ESR2a and ESR2b) have been identified in a number of species including zebrafish (Menuet et al. 2002), while a second distinct alpha isoform has also been identified in rainbow trout (Nagler et al. 2007). Multiple androgen receptors (ARα and ARβ) have also been identified in salmonids (Takeo and Yamashita 1999) and several other species, although only one AR has been identified in zebrafish (Jargensen et al. 2007). Steroid receptor isoform localisation shows a tissue-specific pattern and the concentration of steroid receptors primarily determines the sensitivity of target tissues to steroid hormones. Additionally, steroid receptor transcription is regulated by steroid hormones and therefore varies in response to changes in steroidogenesis at different stages in the reproductive cycle (Thomas 2008). In addition to nuclear receptors, membrane-bound receptors for oestrogens, androgens and progestins have also been identified, which induce rapid, non-genomic reproductive effects mediated by second messenger signalling. For example, membrane bound progesterone receptors (mPRs) are thought to have a major role in oocyte maturation and also sperm mobilisation (Thomas et al. 2002).

1.3.1.2 Chemically-induced endocrine toxicity

Chemical pollutants can potentially target and disrupt the HPG axis at any of the levels discussed above. There is some evidence of chemically-induced neuroendocrine disruption in fish. For example, a PCB mixture (Arochlor® 1254) was found to reduce GnRH secretion by the hypothalamus, lessen sensitivity of pituitary GnRH receptors and decrease gonadotropin production in Atlantic croaker (Khan and Thomas 2001). Disruption of steroidogenesis in the gonads has also been demonstrated, and a wide range of mechanisms have been reported to contribute to this. For example, β-sitosterol, which is found in bleached pulp mill effluent, inhibited steroidogenesis by reducing the supply of available cholesterol (Leusch and MacLatchy 2003). Additionally, chemical pollutants have been found to alter the activity and/or transcription of a number of steroidogenic enzymes including aromatase (Cheshenko et al. 2008). However, interference with classical (genomic)

steroid action via the interaction with nuclear steroid receptors has been the most widely investigated and demonstrated mechanism of endocrine toxicity. A diverse range of chemical pollutants, from various classes, have been shown to agonistically and/or antagonistically interact with steroid receptors, in particular with nuclear ERs. Agonistic interactions occur when a chemical binds and activates the receptor in a way analogous to the endogenous hormone, while antagonistic binding does not activate the receptor and can block the activity of endogenous hormones (Brzozowski et al. 1997, McDonnell 2003). In this way, chemical pollutants that activate or block nuclear ERs are categorised as oestrogenic or anti-oestrogenic chemicals, respectively, and a similar distinction is made for chemicals that interact with the other nuclear steroid receptors. The nature of these interactions depends on chemical structure, which determines the type of receptor conformational change induced by binding, and also the strength of interaction. These factors, in turn, affect the potency of the chemical and its ability to cause adverse effects in exposed organisms.

A large proportion of oestrogenic activity in rivers is attributable to steroidal oestrogens. These include the natural steroids E2 and oestrone (E1), which enter rivers in sewage effluent and from agricultural effluent and runoff, as well as the synthetic oestrogen ethinylestradiol (EE2), which is used in the contraceptive pill and hormone replacement therapy. Additionally, many chemicals have structural similarity to steroidal oestrogen, and can therefore activate the ER. These xenoestrogens include many industrial chemicals and pesticides such as Bisphenol A, DDT, phthalates and alkylphenols, and also several plant flavonoids (phytoestrogens) (Thomas 2008). While these xenoestrogens typically interact less strongly than E2 and are therefore less potent, EE2 has been shown to have a higher binding affinity for rainbow trout and fathead minnow ERs than E2 (Denny et al. 2005), and is also more resistant to biological degradation, which makes it considerably more potent than E2 (11-27 fold in rainbow trout) *in vivo* (Thorpe et al. 2003).

Oestrogenic activity has been widely demonstrated amongst chemical contaminants of surface waters, and this has been facilitated in part due to the use of vitellogenin (VTG) as a convenient and specific indicator of oestrogen exposure (Sumpter and Jobling 1995). Genes encoding vitellogenin include oestrogen response elements (EREs) in their promoter region, and their transcription is highly inducible by

oestrogen exposure. In male and juvenile fish, in which *vtg* genes are normally expressed at very low levels, an increase in *vtg* transcription and elevated VTG protein concentrations in the plasma have been extensively used to demonstrate oestrogenic exposure. Typically concentrations of ~50 ng/L E2 and ~1 ng/L EE2 have been found to induce vitellogenin in a range of species (e.g. Thorpe et al. 2001, Thorpe et al. 2003), which are within the range of environmental concentrations of these steroids in the most contaminated rivers (Desbrow et al. 1998).

Disruption of the endocrine system, predominantly by oestrogenic chemicals, has also been extensively associated with reproductive toxicity. For example, exposure to steroidal oestrogens have been reported to impair spermatogenesis, reduce sperm quality, induce the development of ovarian cavities in the testes (intersex) and in some cases cause complete sex reversal and population collapse (e.g. Panter et al. 1998, van Aerle et al. 2002, Schultz et al. 2003, Brion et al. 2004, Nash et al. 2004, Kidd et al. 2007, Lange et al. 2008). Additionally, disruption of the endocrine system and reproductive toxicity have been demonstrated for a number of other oestrogenic chemicals, for example Bisphenol A (Sohoni et al. 2001), which are less potent than steroidal oestrogens but are often found at higher concentrations in rivers (Harries et al. 1996, Desbrow et al. 1998). Importantly, some evidence of intersex, impaired sperm quality and fertility, together with elevated vitellogenin levels, have been found in wild male fish, raising concerns for the health of natural populations (e.g. Jobling et al. 1998, Jobling et al. 2002a, Jobling et al. 2002b).

In brown trout specifically, disruption of the endocrine system and reproductive toxicity have been reported on several occasions. For example, BPA delayed male and female gametogenesis (Lahnsteiner et al. 2005), while E2 was found to induce VTG, alter circulating steroid levels and impair egg fertilisation success (Bjerregaard et al. 2008, Schubert et al. 2008). Additionally, elevated VTG levels were detected in wild male brown trout, associated with waste-water treatment work effluent outflows in Irish rivers (Kelly et al. 2010), while some evidence of intersex was found in brown trout populations inhabiting Swiss rivers (Körner et al. 2005).

There is also some evidence from laboratory studies that anti-androgenic chemicals cause reproductive toxicity in male fish. For example, flutamide impaired typical male reproductive behaviour and the production of the nest-building protein spiggin, which

is under direct control of androgens, in stickleback (Sebire et al. 2008), while vinclozolin impaired sperm production, reproductive behaviour and fertility in guppies (Bayley et al. 2002, Bayley et al. 2003). However, the de-masculinising effects associated with anti-androgens may be phenotypically similar to the feminising effects induced by oestrogenic chemicals. Corresponding with this, statistical modelling studies suggested that anti-androgens significantly contribute to widespread feminisation of male fish in UK rivers (Jobling et al. 2009). However, the specific transcriptional expression profiles characterising oestrogenic and anti-androgenic exposure are distinct, for example as demonstrated by (Filby et al. 2007), highlighting the value of using molecular profiling to determine the mechanistic basis of chemical toxicity.

Gene expression analysis can also help distinguish complex mechanisms of toxicity of endocrine disrupting chemicals that involve multiple, interacting signalling pathways. For example phthalates appear to induce reproductive toxicity in male fish by acting as both oestrogens and peroxisome proliferators by interacting with both ER and PPARs (Harris et al. 1995, Corton and Lapinskas 2005, Uren-Webster et al. 2010). Furthermore, apart from reproductive toxicity, endocrine disrupting chemicals can induce a diverse range of other effects. For example, oestrogenic chemicals are known to target the cardiovascular system and immune system, and have also been associated with the generation of oxidative stress (Milla et al. 2011, Thilagam et al. 2011). Understanding the molecular mechanism(s) of endocrine toxicity is therefore important in assessing the potential effects of exposure on individuals and populations, particularly because complex mixtures of chemicals, which may interact, often occur in the environment.

1.3.2 Oxidative stress

1.3.2.1 Endogenous sources of ROS and the antioxidant system

The redox potential of oxygen is critical for its biological role in numerous cellular processes, including as an electron acceptor in oxidative phosphorylation. However, the propensity of oxygen to undergo electron transfer also yields toxic forms of oxygen, termed reactive oxygen species (ROS) (Halliwell and Gutteridge 2007, Di

Giulio and Meyer 2008). ROS are generated by a series of reductive reactions of oxygen firstly generating the superoxide anion radical, then hydrogen peroxide, then the hydroxyl radical (Figure 2). The hydroxyl radical is the most reactive of these ROS, and indiscriminately reacts with cellular components causing oxidative damage. Other species that contribute to generation of oxidative stress include singlet oxygen, ozone, alkoxyl and peroxyl radicals and reactive nitrogen species (Halliwell and Gutteridge 2007, Di Giulio and Meyer 2008). ROS are continually generated at a low level in the cell as a by-product of a number of cellular reactions, for example through leakage of electrons from the mitochondrial electron transfer chain. Additionally, while ROS can be damaging to cells they also have a number of crucial biological roles including as signalling molecules in various cellular pathways, and play a key role in phagocytosis (Forman and Torres 2002). To control the potentially damaging effects of these endogenous sources of ROS, cells have developed a complex, and highly inducible, cellular antioxidant system (Di Giulio and Meyer 2008).

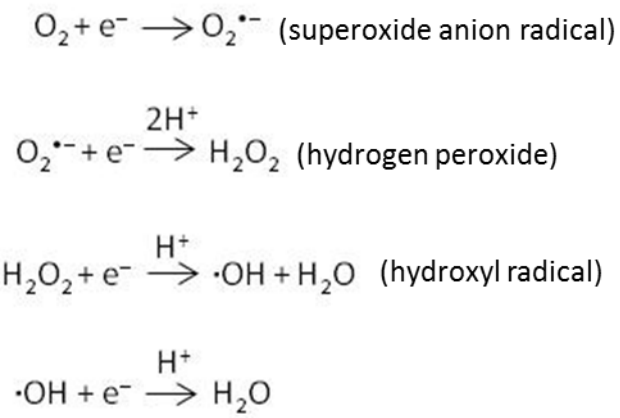


Figure 2. Generation of ROS through successive reduction of molecular oxygen.

Glutathione (GSH) is a major cellular antioxidant, and also has a number of vital roles in metabolic and signalling processes. It is a tripeptide (L-cysteine, L-glutamate and L-glycine) containing a thiol group (R-SH) on the cysteine residue that it readily oxidised. Two reactive oxidised glutathione molecules form a stable disulphide molecule (GSSH). GSH therefore serves as an important reducing agent for the

neutralisation of ROS (including $O_2^{\bullet-}$, ·OH, RO·, ROO· and ONOO-) via direct non-enzymatic conjugation (Halliwell and Gutteridge 2007). When associated with the enzymatic peroxidases, GSH is also responsible for reducing a range of organic peroxides, including H_2O_2, which removes their damage-causing potential. Glutathione peroxidases (GPXs) catalyse the reduction of both lipid peroxides and H_2O_2, while one of the roles of glutathione S-transferases (GSTs) is to reduce lipid peroxides (Hayes and McLellan 1999). The thiol moiety of GSH also directly binds free metal ions, and some other reactive metabolites, facilitating their sequestration away from sensitive parts of the cell. In cells experiencing oxidative stress the ratio of GSH:GSSG, which is usually ~100:1, is reduced. Synthesis and restoration of reduced glutathione is therefore essential to maintain antioxidant capacity. GSH is synthesised in a two-step process by glutamate cysteine ligase and GSH synthetase, both of which can be induced by increased oxidative stress (Hayes and McLellan 1999). Glutathione reductase (GR) is responsible for the restoration of GSH from GSSG, using NADPH as an electron supplying cofactor. Glucose-6-phosphate dehydrogenase (G6PDH), which catalyses the formation of NADPH, is therefore also essential in this process.

Other essential components of the antioxidant system include the antioxidant enzymes superoxide dismutase (SOD) and catalase, which are independent of glutathione. SOD enzymes increase the rate of reaction of superoxide anion radicals ($O_2^{\bullet-}$) to form hydrogen peroxide, while catalases largely catalyse the dismutation of hydrogen peroxide to molecular oxygen and water as follows:

$$O_2^{\bullet-} + O_2^{\bullet-} \rightarrow H_2O_2 + O_2$$

$$2H_2O_2 \rightarrow O_2 + H_2O$$

The antioxidant system also includes a diverse range of associated proteins with redox potential, and a number of non-enzymatic antioxidants that directly scavenge ROS. The former group includes peroxiredoxins, sufiredoxin and thyrodoxin, while the latter includes ascorbic acid, vitamin E, carotenoids, ubiquinol, uric acid and metallothioneins (Hayes and McLellan 1999, Halliwell and Gutteridge 2007).

1.3.2.2 Chemically induced oxidative stress

An extensive number of chemicals pollutants, from a range of different classes, have been shown to disrupt cellular redox homeostasis and induce oxidative stress. The mechanisms responsible for these effects involve either an increase in ROS generation, which can overwhelm cellular antioxidant capacity, and/or interference with the antioxidant defence system (Di Giulio and Meyer 2008). ROS are most commonly generated through redox cycling, whereby a chemical compound accepts an electron from a reduced cofactor, such as NADH, forming a radical metabolite. This metabolite then reacts with oxygen (is oxidised) forming ROS in the process. Chemicals that generate ROS in this way include many pesticides (e.g. paraquat) and industrial pollutants (e.g. benzene derivatives). The redox activity of metal ions is also responsible for generation of oxidative stress, which is one of the major mechanisms of metal toxicity. The ability of metal ions to be reduced allows them to catalyse the reaction of hydrogen peroxide with superoxide to generate more reactive hydroxyl radicals, in a process known as the Fenton reaction. Other chemicals, including cadmium and some PAHs, specifically disrupt the mitochondrial electron transport chain, leading to excess ROS generation and accumulation. In addition, chemical pollutants can cause oxidative stress via disruption of the antioxidant defence system. These effects include inhibition of antioxidant gene transcription or direct enzyme binding and interference (e.g. Zhang et al. 1997).

Generation of oxidative stress is a specific mechanism of toxicity that induces characteristic effects in fish. However, the number of possible measureable responses is extremely broad and these can vary based on the degree and duration of stress. Additionally, it can be difficult to distinguish the effects of oxidative stress from those of other mechanism of toxicity (Di Giulio and Meyer 2008). Therefore, it is often beneficial to combine multiple measures at multiple levels of biological organisation to provide a comprehensive assessment of chemical-induced oxidative stress (Halliwell and Gutteridge 2007).

Direct measurements of oxidative damage are probably amongst the most commonly employed techniques. ROS, particularly the most damaging hydroxyl radicals, tend to react indiscriminately with cellular components, especially DNA, lipids and proteins. Direct oxidation by ROS constitutes a major form of DNA damage, which can lead to

mutagenesis and tumourgenesis, if unrepaired. Oxidative DNA damage is often assessed using the Comet assay, which measures single or double strand DNA breakage, while the micronucleus test is another commonly employed measure of genotoxicity in fish (e.g. Belpaeme et al. 1996). Lipids, particularly polyunsaturated fatty acids, are also prone to oxidation by ROS. This initiation step forms reactive lipid radicals which subsequently induce the free radical chain reaction that constitutes lipid peroxidation. This has many potentially damaging consequences in cells, particularly the impairment of membrane structure and function. Lipid peroxidation is commonly assessed in fish by measuring the reaction of thiobarbituric acid with malondialdehyde, a product of lipid peroxidation, using the TBARs assay. Proteins are also subject to oxidation by ROS, which can have various adverse consequences including disruption of signalling pathways and enzymatic functions. Oxidative damage of proteins has been measured in fish through measurement of carbonyl formation (e.g. Almroth et al. 2005). Various components of the antioxidant system have also been measured extensively in fish, including assessment of catalase, SOD, GPX, GST and GR activity, as well as cellular GSH concentrations and GSH:GSSG ratios. The antioxidant system has been shown to be induced by chemical-induced oxidative stress, although responses often vary overtime (Di Giulio and Meyer 2008). Cellular concentrations of ROS can also be measured directly, although they are typically only active for a very short period of time (Halliwell and Gutteridge 2007). In brown trout, generation of oxidative stress has been reported on several occasions. For example, the herbicide paraquat was found to increase protein carbonylation and reduce GSH:GSSG ratio (Carney Almroth et al. 2010), while both the transcription and activity levels of a suite of antioxidant enzymes were found to be induced by exposure to copper (Hansen et al. 2006) and cadmium (Hansen et al. 2007).

Molecular changes induced by oxidative stress can be complex, often reflecting a broad cellular stress response, which can vary depending on the degree of oxidative stress. ROS can activate a number of eukaryote transcription factors, generally via a series of interacting upstream signalling pathways, which include MAPK signalling and calcium signalling (Martindale and Holbrook 2002, Di Giulio and Meyer 2008). A wide array of genes are regulated by ROS-activated transcription factors, and exposure to oxidative stress-inducing chemicals can cause extensive and diverse

molecular changes. These genes include those encoding catalase, SOD, GPXs and GSTs, although the transcriptional responses of these classical antioxidants to oxidative stress have often been reported to be inconsistent (Halliwell and Gutteridge 2007). Many other transcriptional changes reflect induction of an integrated cellular stress response, including the regulation of cellular proliferation and growth, inflammatory response and various metabolic processes. It is thought that in fish, as in mammals, activated transcription factors bind antioxidant response elements (AREs) on target genes, although there has been limited definitive characterisation of their presence and function (Hayes and McLellan 1999, Di Giulio and Meyer 2008).

1.4 Application of molecular ecotoxicology

As previously discussed with regard to endocrine disruption and oxidative stress, investigating molecular mechanisms of toxicity can be a valuable, sensitive measure of chemical exposure. Transcriptional changes can occur rapidly following chemical exposure and can be sensitive to low concentrations. Understanding the molecular mechanisms of toxicity of environmental pollutants can therefore contribute important information to the protection and sustainable management of natural fish populations.

Combining molecular mechanisms of toxicity with effects at other levels of biological organisation (including biochemical, physiological and morphological changes), can be used to build a comprehensive signature of exposure to a chemical, or group of chemicals with a shared mechanism of action (Hinton et al. 2005). In short-term laboratory exposures, high treatment concentrations are often employed in order to induce measureable adverse effects, for example on reproduction, behaviour, growth or survival. Investigating molecular mechanisms of toxicity have a potentially important role in ensuring such studies retain environmental relevance. For example, evidence of the same molecular mechanisms occurring at environmentally relevant concentrations as those underlying adverse health effects at higher concentrations may potentially indicate similar adverse effects following chronic exposure. This may also allow identification of molecular ‘early warning signs’ of exposure and help identify suitable biomarkers in wild fish populations. Such tools are important in identifying the need for management of a population before more damaging effects occur, which are likely to be more difficult to remediate.

Fish are often exposed to a complex mixture of chemicals in the aquatic environment, which often interact. Additive effects can occur between chemicals sharing a common mechanism of toxicity. For example, the total oestrogenic activity of a mixture of steroidal oestrogens (E1, E2 and EE2) was accurately predicted using the sum of the individual chemical activities (Thorpe et al. 2003). In contrast, antagonistic or synergistic interactions can occur when the toxicity of one chemical is modulated by the action of another. For example, a mixture of organophosphate and carbamate pesticides synergistically increased AChE inhibition in Pacific salmon (Laetz et al. 2009). Therefore, characterising the molecular mechanisms of toxicity of likely components of a mixture can help in the understanding and prediction of the biological outcome of exposure (Escher and Hermens 2002). Interactive effects can also occur between chemicals and other environmental stressors, which can also share mechanisms of toxicity. Generation of oxidative stress, in particular, is a very common mechanism of toxicity induced by other environmental stressors, including temperature and UV radiation, as well as many chemical pollutants (Di Giulio and Meyer 2008). Furthermore, the cellular response to chemical and other environmental stressors is often similar. For example, the transcription of heat shock proteins (HSPs), which are molecular chaperones that bind and stabilise damaged proteins, is induced in response to exposure to a number of chemicals as well as temperature stress (Basu et al. 2002). Another example is hypoxia inducible genes, which can also induced by chemical exposure (Kalmar and Greensmith 2009). Considering the possible interactive effects of chemical exposure with other environmental stressors, and characterisation of their shared mechanisms of toxicity, is therefore important, particularly when considering potential effects of future climate change. For brown trout specifically, a major additional environmental pressure is siltation of spawning redds following changes in land use and agricultural run-off (Environment Agency 2004). This can limit water flow, cause hypoxia and reduce embryo survival. Additionally, smaller, upland streams tend to be more susceptible to freshwater acidification and temperature fluctuations, and these stressors may increase susceptibility of brown trout populations to chemical stress (or vice versa).

1.5 Investigating molecular mechanisms of toxicity

Methods that have been used to investigate molecular mechanisms of toxicity at the transcript level can be divided into those that use a targeted gene approach or a global approach. Currently, the most commonly used method for quantification of individual transcript profiles is quantitative real time PCR (RT-QPCR). This technique involves the selective amplification of cDNA sequences using specific primer sequences from the target gene, and it is often used to quantify small suites of genes representing one or more pathways. The concentration of the amplified cDNA is quantified in real-time, most commonly through the use of double-stranded DNA binding fluorescent dyes, or fluorescent reporter probes (Bustin 2000). Expression levels between samples can be normalised using a control gene, and fold changes between treatments calculated. RT-QPCR allows sensitive and reproducible measurements of gene expression, and is extensively used in ecotoxicology (Filby and Tyler 2007). This targeted gene approach is ideal for investigating specific hypotheses about the mechanisms of toxicity of a given chemical or mixture. For example, expression profiling of a suite of oestrogen-responsive genes is commonly used to investigate whether observed reproductive effects are associated with an oestrogenic mechanism of toxicity (e.g. Filby et al 2007). Targeted gene approaches can also be relatively quick and inexpensive to perform, and the results easy to interpret.

The transcriptome consists of all mRNA transcripts present in cells at a given time under specific developmental and physiological conditions (Wang et al. 2009). Compared to targeted gene approaches, global transcriptome profiling can potentially allow a more comprehensive assessment of the molecular mechanism(s) of toxicity of a given chemical or mixture, including by revealing novel or unexpected effects. The application of transcriptomics in ecotoxicology may be especially beneficial in identifying comprehensive molecular signatures of exposure, and early warning signs of potential adverse effects. A number of transcriptomics approaches have been developed and used in ecotoxicology in the last 15 years and, of these, microarrays have been the most extensively employed. The use of microarray technology rapidly increased from the late 1990s, and evolved into the dominant transcriptomic tool used in both ecotoxicology and human toxicology (Nuwaysir et al. 1999, Schirmer et al. 2010). Briefly, microarrays consist of thousands of oligonucleotide or cDNA

probes for individual genes immobilised on a solid platform. Hybridisation of labelled sample cDNA with the array allows for the measurement of the relative expression of each represented transcript (Lettieri 2006). This can provide a sensitive measure of global gene expression changes following chemical exposure, allowing the characterisation of mechanism(s) of toxicity. For example, microarrays have been widely used to characterise transcriptional response following exposure to oestrogens (e.g. Kishi et al. 2006, Gunnarsson et al. 2007, Moens et al. 2007), as well as a number of other types of chemical contaminant including metals (e.g.Sheader et al. 2006, Walker et al. 2008, Santos et al. 2010) and PAHs (e.g. Williams et al. 2009). However, a major disadvantage of microarray technology is that probe design relies on existing genomic or transcriptomic sequence information, which is often lacking in non-model species. Recently RNA-seq has emerged as a very useful tool for transcriptomics which is not dependent on existing genomic resources, therefore resolving a major hurdle for global gene expression profiling in environmentally relevant species. The potential application of RNA-seq in a (eco)toxicological context is discussed below.

1.5.1 RNA seq

RNA sequencing (RNA-seq) was first described in 2006 (Bainbridge et al. 2006) and utilises recently developed high-throughput DNA sequencing technologies to sequence complementary DNA transcribed from mRNA, extracted from a given cell, tissue or organism. In 2008, RNA-seq gained wider recognition when the transcriptomes of the yeast *Saccharomyces cerevisiae* and the mouse (*Mus musculus*) were characterised and quantified by Nagalakshmi et al. (2008) and Mortazavi et al. (2008), while Vera et al. (2008) were the first to characterise the transcriptome of a non-model species (the Glanville fritillary butterfly, *Melitaea cinxia*). Since then, particularly in the last two years the use of RNA-seq has rapidly increased in a wide array of fields. In environmental biology, RNA-seq is predominantly employed to conduct transcript expression analysis through sequencing of mRNA, and this provides the focus of this discussion. However, RNA-seq has many other potential applications. These include sequencing of non-coding RNAs and small RNAs which can provide vital information on gene structure and

regulation (McGettigan 2013). Additionally, identification of novel SNPs, splice variants and rare mutations using RNA-seq has been extensively employed, particularly in medical and cancer research (e.g. Cowper-Sal et al. 2012, Shah et al. 2012, Ren et al. 2013). It is also often used in conjunction with genomic sequencing, and to improve existing gene annotations (e.g. Trapnell et al. 2010, Kapushesky et al. 2012).

A generalised schematic of RNA-seq experiments is shown in Figure 3. Briefly, following extraction of high quality total RNA, mRNA is purified, fragmented and converted to cDNA. Barcode adaptors are ligated to the ends of cDNA fragments, which are then amplified with PCR. cDNA is then sequenced, producing millions of short reads which are used to assemble a transcriptome. Transcriptome assembly can be guided by a good quality reference genome, or employ a *de novo* approach which relies solely on sequence read overlaps to construct individual transcripts. In both cases, the depth of sequencing coverage is essential for accurate assembly and inclusion of rare transcripts. All of the sequence reads are then re-mapped against the transcriptome to conduct expression analysis, whereby the coverage of a transcript is directly proportional to its expression level.

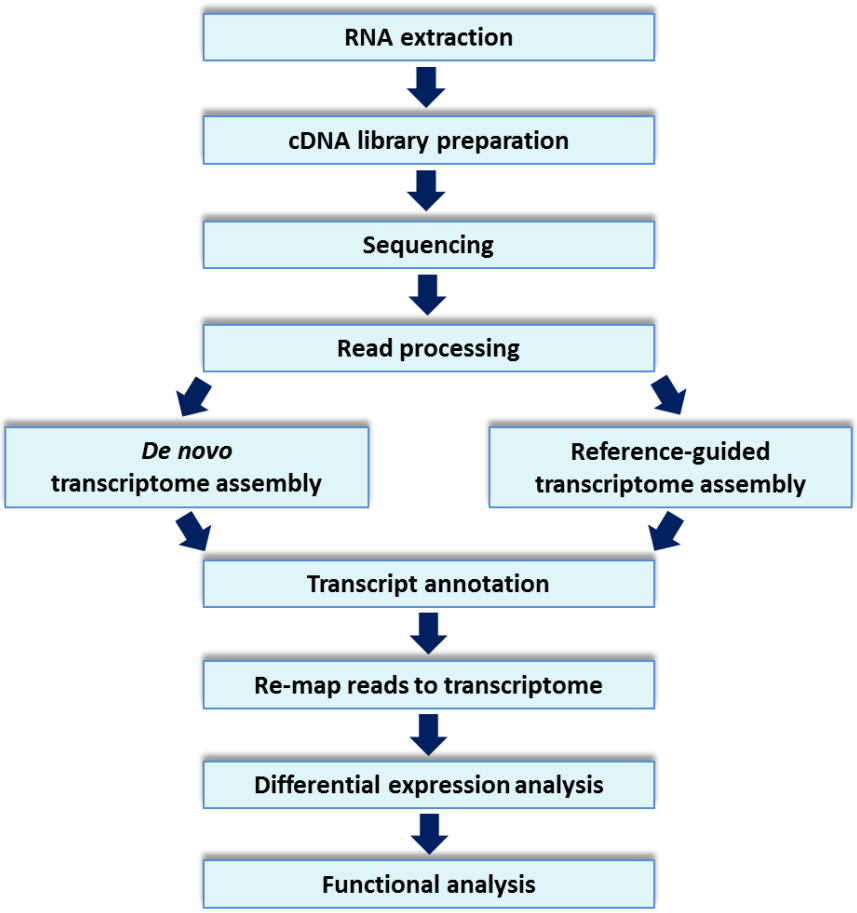


Figure 3. A summary of the steps involved in a typical RNA-seq experiment.

Sequencing technologies, and associated bioinformatics methods for data analysis, have experienced rapid development and improvement over the last five years. A number of different sequencing technologies, and bioinformatics tools, are available. Depending on the experimental aims, selection of the most appropriate methods should be an important factor in RNA-seq experimental design. Several of the key factors to consider (sequencing technology, transcriptome assembly strategy and differential expression analysis) are outlined in the discussion below.

1.5.1.1 Sequencing technology

The most commonly employed sequencing technologies currently used for gene expression analysis in RNA seq experiments are Illumina and Roche 454, while others include Ion Torrent, Solid and Pacific Biosciences. Illumina (originally known as Solexa) is based on a sequencing by synthesis chemistry. Briefly, single stranded

cDNA molecules firstly bind to the glass flow cell via the adaptor molecules on both ends. Free nucleotides and DNA polymerase are added, and each cDNA strand is copied in a process known as bridge amplification. The double stranded DNA molecules are then denatured, the single strands reform bridges and this amplification cycle is repeated. This process generates distinct clusters of copies of single stranded, primed DNA fragments (>1 million strands). Sequencing by synthesis of each of the strands then proceeds one nucleotide at a time. Fluorescently labelled nucleotides and DNA polymerase are added to the flow cell, and a single nucleotide binds to each of the primed DNA molecules by complementary base pairing. Chemical blocking of the 3' -OH group prevents additional nucleotide incorporation. The flow cell is then washed to remove spare nucleotides, and the flow cell is imaged. The colour of each cluster of DNA molecules indicates the first base in the sequence. The 3' block and dyes on the incorporated nucleotide are then removed by laser excitation, and the cycle is repeated for the length of the read. A base calling algorithm assigns the sequence and associated quality scores for each read (Mardis 2008, Illumina 2013).

Roche 454 was the first commercial high-throughput DNA sequencing platform, introduced in 2004, and employs pyrosequencing chemistry. Each cDNA fragment binds an agarose bead via adaptor sequences, and then is immersed in an emulsion of PCR reactants, which amplify the DNA fragments until there are approximately 1 million copies per bead. The beads are then arrayed in individual wells on a picotitre plate with an enzyme mix, and the sequencing reactions occur in parallel as one type of nucleotide is added at a time. Each incorporated nucleotide releases pyrophosphate, which in turn stimulates light emission (Mardis 2008, Rothberg and Leamon 2008).

These differences in sequencing chemistry mean that Illumina generates many more reads, which are shorter in length, while 454 generates fewer, longer reads. These characteristics confer different advantages and disadvantages on both the transcriptome assembly and differential expression. Longer reads are advantageous for transcriptome assembly, especially when a reference genome is not available, because there is greater certainty in the read overlaps. During the early development of RNA-seq there was a clear advantage for the 454 technology in this respect. In

2008, Illumina sequencing generated single-end reads that were typically 25-35 bp long, while 454 produced reads of 400-500 bp (Mardis 2008). The subsequent advent of paired-end sequencing provided a considerable advantage for transcriptome assembly, particularly using shorter Illumina reads, by providing additional scaffolding of reads that originated from the same cDNA fragment. Additionally there have been improvements in sequencing chemistry that have allowed longer read lengths for both technologies. Today, the standard expected output of an Illumina HiSeq 2500 is up to 400 million, 100 bp paired reads (although up to 250 bp are possible) per lane (Illumina 2013), and of a 454 GS FLX Titanium XL+ is expected to be 1 million reads of up to 1000 bp in length (Roche 2013). There have been no direct comparisons between the quality of the transcriptomes assembled using these most recent Illumina and 454 sequencing platforms, but they are expected to be largely equivalent.

The other major difference between the Illumina and 454 platforms is the number of reads that can be obtained per sequencing run, which ultimately determines the cost of the experiment. Increased number of reads improves sequencing coverage depth, which improves assembly of rare transcripts. Different samples can be multiplexed (labelled with specific barcodes) and sequenced together. Greater sequencing coverage also facilitates a higher degree of multiplexing, which allows for an increase in the number of experimental replicates. Therefore a major advantage of the Illumina platform is the considerably higher number of reads generated per sequencing run. Reflecting this, Illumina is now often considered the sequencing technology of choice for transcriptomics (Vijay et al. 2013).

More recently '3rd generation sequencing' has been developed, and seems likely to become more widely utilised in the future (for example the newly released Pacific Biosciences platform). This technology does not amplify or fragment cDNA libraries during library preparation, which avoids some of the concerns over the potential impact of PCR biases on gene expression analysis (McGettigan 2013). PacBio technology currently generates long reads of 1500 bp, and their newest chemistry promises to achieve average sequence reads of 8500 bp. This potentially allows single molecule sequencing, whereby an entire RNA molecule is sequenced in a

single read, and this was recently successfully achieved across the entire human transcriptome (Sharon et al. 2013).

1.5.1.2 De novo transcriptome assembly strategy

For species with a reference genome, transcriptome assembly can be relatively straightforward, and there are well-established bioinformatics pipelines available, such as the Tophat-Cufflinks pipeline (Trapnell et al. 2012). Reference-guided transcriptome assemblies inevitably tend to be of higher quality than those assembled *de novo*, especially in terms of accurately resolving alternative transcript isoforms, obtaining full-length transcripts and resolving rare transcripts (Lu et al. 2013, Vijay et al. 2013). As mentioned previously, when there is no reference genome the quality of the *de novo* transcriptome assembly is essential for the quality of downstream expression analysis and the biological interpretation of results. It is therefore important to carefully consider the most appropriate *de novo* transcriptome assembly strategy to employ. Optimised *de novo* assemblies can be of a quality approaching that of reference-guided assemblies, and have been shown to be robust for differential expression analysis (Schulz et al. 2012, Vijay et al. 2013).

Transcriptome quality has been previously assessed using a number of parameters (e.g. Sandmann et al. 2011, Lu et al. 2013, Vijay et al. 2013). However, there is a lack of consensus on which are the most important quality measures, and these are often defined by the aims of the experiment. Measures of assembled transcript length (N50, mean/median lengths) have been the most widely reported measure of a quality in *de novo* transcriptome assemblies in the literature. These values are used to indicate transcript completeness, in a similar way to those reported in genomic assemblies. However, while transcript completeness is certainly important, transcript length can be very variable so this measure can end up being fairly arbitrary. Genuine measures of transcript completeness tend to be difficult to achieve without a reference genome. Another measure of quality is the number of unique genes (including genuine transcript isoforms) represented in the assembly, which is assessed following transcript annotation. This is important for the biological interpretation of downstream expression analysis, and poorer-quality assemblies

tend to not resolve rarer transcripts. All *de novo* transcriptome assemblies have some degree of redundancy, where there are multiple fragments of the same transcript that are not true alternative isoforms. These redundant transcripts can result from the incorporation of sequencing errors and insufficient scaffolding support during assembly, and are difficult to unambiguously identify and remove. Transcriptome redundancy adversely affects downstream expression analysis, and is therefore a very important measure of assembly quality. Limiting the number of miss-assembled chimeric transcripts, that result from incorrect fusions of distinct transcript fragments, is also very important. However, accurate identification of chimeras is another challenge of transcriptome assembly without a reference genome.

The most important factor affecting the quality of the final transcriptome is the choice of assembly software, and its optimisation (Lu et al. 2013, Vijay et al. 2013). De Bruijn graph-based methods, which are used for both genomic and transcriptomic data, are generally used for the assembly of short reads generated by Illumina sequencing, which was the sequence technology used in this thesis. In contrast to the overlap-consensus layout assemblies generally used for long-read 454 data, the individual reads are split into shorter, linked, sections *k* nucleotides in length, which are known as k-mers. This reduces the time and memory constraints of aligning whole sequence reads to each other. The de Bruijn graph consists of a series of overlapping k-mers, or nodes, connected by edges, and transcript assembly is conducted by finding a directional path through the graph, visiting each edge between nodes only once (Zerbino and Birney 2008). K-mer length is the most influential parameter affecting the resulting assembly characteristics and quality. Graphs using higher k-mer values tend to have higher specificity and are better at constructing highly-expressed transcripts which have high coverage support for transcript assembly, and therefore tend to produce assemblies consisting of fewer transcripts and fewer miss-assemblies. The upper limit of *k* is at a length approaching read length, where assembly starts to break down. In contrast, lower k-mer values have higher-sensitivity, and are better at assembling rare transcripts with lower coverage support. However, too-short k-mers produce fragmented assemblies including more miss-assemblies, and also have greater memory requirements. Therefore, the best approach has been to combine assemblies using a range of k-mer lengths to produce an optimum assembly which strikes a balance between

assembly specificity and sensitivity, and this approach is widely used in different de Bruijn graph assemblers (Zerbino and Birney 2008, Grabherr et al. 2011, Schulz et al. 2012). The most widely used de Bruijn graph-based programs used for *de novo* transcriptome assembly are probably the Velvet-Oases pipeline (Zerbino and Birney 2008, Schulz et al. 2012) and Trinity (Grabherr et al. 2011), while others include Trans-AbySS (Simpson et al. 2009, Robertson et al. 2010), the commercial package CLC Bio Workstation (CLC Bio 2013) and, recently, SOAPdenovo-trans (Xie et al. 2013). Recent comparisons by Lu et al. (2013) and Vijay et al. (2013) using model organism and simulated RNA-seq datasets revealed broadly comparable performance between different assembly software programs, although each has different strengths and weaknesses. They also found that the complexity of a transcriptome, particularly in terms of size and degree of alternative splicing, strongly affected assembler performance. This highlights the importance of carefully considering the most appropriate assembly strategy to employ for individual RNA-seq datasets.

1.5.1.3 Differential expression analysis

A large number of different statistical methods have been developed to perform differential expression analysis on RNA-seq data. As with transcriptome assembly, there is no consensus on the best method to use, and different approaches may be most appropriate for different datasets (Vijay et al. 2013). Briefly, the key steps in differential expression analysis are the normalisation of the counts of mapped reads against each transcript, modelling of transcript expression and the statistical test for differential expression between groups (Bullard et al. 2010, Rapaport et al. 2013). Normalisation is essential to control for the differences in sequencing depth between samples, and allows accurate comparison between samples. Modelling of gene expression is generally performed assuming Poisson distribution or negative binomial distribution, but there are a number of variants of these methods employed in different software packages. Similarly, various statistical algorithms are employed to conduct differential expression analysis, and a crucial element of this analysis is to control for false positives by employing multiple test correction (usually Benjamini-Hochberg). Rapaport et al. (2013) recently conducted a comprehensive review of the performance of different statistical methods on several model RNA-seq datasets.

They found that no single method performed best across all performance measures, but that generally methods employing negative binomial modelling, including EdgeR, DeSeq and BaySeq, were the best and displayed good sensitivity and specificity. They found that Cuffdiff performed significantly less well than the other methods, and accounted for a large number of false positives. They also found that differential expression analysis sensitivity and specificity could be improved by increases in both sequencing coverage depth and number of replicates, particularly for rare transcripts. However, they also concluded that maximising number of biological replicates, which increases the power of statistical analysis, was more important than increasing coverage per sample. This highlights the importance of experimental design (number of replicates, sequencing method) as well as the choice of differential expression analysis method, when designing RNA-seq experiments.

1.6 Application of RNA-seq in ecotoxicology

The potential application of RNA-seq to ecotoxicology is very significant, in particular by enabling studies on non-model organisms in which gene expression studies were previously restricted to target gene approaches or reliant on cross-species microarrays, but the technology has so far been underused (Mehinto et al. 2012). There have been a number of studies that have sequenced the transcriptome of various fish species with the aim of characterising specific tissues or developmental stages (Fraser et al. 2011, Rhee et al. 2011, Cannon et al. 2012), to identify SNPs (Liu et al. 2011) and to design microarrays (Garcia-Reyero et al. 2008). However, only a limited number of studies have used RNA-seq to quantify gene expression changes following exposure to environmental stressors, with the aim of investigating mechanisms of toxicity. For example, Pierron et al. (2011) investigated mechanisms of metal toxicity in a chronically exposed population of yellow perch (*Perca flavescens*) using 454 sequencing and Whitehead et al. (2012) compared gene expression profiles in populations of gulf killifish (*Fundulus grandis*) affected by the Deepwater Horizon oil spill using Illumina sequencing. More recently, Smith et al. (2013b) used RNA-seq to identify differentially expressed genes in rainbowfish (*Melanotaenia duboulayi*) subjected to temperature stress.

Microarray technology is currently still the dominant transcriptomic tool used in ecotoxicology. Additionally, the design of microarray probes is often seen as the primary use of RNA-seq sequence data (Schirmer et al. 2010, Mehinto et al. 2012). However, there is growing evidence to suggest that RNA-seq performs better than microarrays on a number of technical criteria. The dynamic range of accurately measured gene expression level is potentially far higher in RNA-seq experiments because it is based on count-based detection, which is in theory unlimited. In contrast, microarrays have a finite dynamic range due limitations in fluorescence detection and saturation of probe binding sites (Black et al. 2013). Compared to the several hundred fold change typically expected of microarray experiments (Wang et al. 2009), the early RNA-seq experiments reported dynamic ranges in excess of 9000 fold (Nagalakshmi et al. 2008) and 10,000 fold (Mortazavi et al. 2008), and this has since increased (Vijay et al. 2013). Additionally, RNA-seq is associated with less technical variation due to batch effects (McGettigan 2013) and also requires less input material than microarrays. Currently Illumina technology can utilise as little as 100 ng total RNA per sample, with protocols for smaller amounts currently in development (Illumina 2013). For these reasons RNA-seq has become the transcriptomic tool of choice in many fields, particularly in medical research (Vijay et al. 2013).

1.7 Aims and objectives of this PhD

The brown trout is an ecologically and economically important native species in the UK and is known to be sensitive to environmental stressors. However, compared to other species (such as zebrafish and rainbow trout) there has been very limited previous research investigating mechanisms of chemical toxicity in brown trout. The major objective of my PhD was therefore to investigate the impact of environmentally relevant chemical stressors on brown trout. This species typically breeds in and inhabits fast-flowing water in smaller streams, therefore we selected chemicals associated with mining and agricultural pollution rather than those primarily found in industrial or sewage work effluent which typically impact larger, slower-flowing rivers in lowland areas. We used global transcriptomic profiling to investigate molecular mechanisms of toxicity for a selection of these environmentally-relevant chemicals, with the aim of providing important information on the risk they may pose to brown

trout populations and, ultimately, for the sustainable management of this species. To do this I used RNA-seq, which allows global transcriptomic profiling to be conducted in species without a reference genome. RNA-seq is still yet to be widely employed in ecotoxicology, therefore I also set out to demonstrate its potential application in this field. Throughout the course of my PhD there has been a rapid improvement in both sequencing technology and tools available for bioinformatics analysis. This has allowed us to improve our RNA-seq experimental design and data analysis, and this progression is documented throughout my thesis.

These objectives are addressed throughout the thesis in individual research papers as follows:

The aim of *Chapter 2* was to sequence and assemble the brown trout transcriptome using a *de novo* approach, and use it to conduct global transcript profiling in a population of wild brown trout from the river Hayle which are chronically exposed to a mixture of metals, in comparison to a control population (River Teign). In this work I combined RNA-seq analysis with measures of tissue metal concentration in order to investigate the molecular mechanisms which enable the Hayle population to tolerate extremely high concentrations of metals in this river.

The aim of *Chapter 3* was to investigate the mechanisms of toxicity of the natural steroidal oestrogen E2, a major agricultural pollutant, in reproductively mature male brown trout. In addition, transcriptomic profiling of this model oestrogen was conducted in order to demonstrate the potential application of RNA-seq in ecotoxicology.

The aim of *Chapter 4* was to investigate the hepatic transcriptional response in male brown trout exposed to a widely used herbicide, Linuron. This compound is known to be an anti-androgen, but its wider mechanistic effects have been rarely investigated, despite its importance as a contaminant in freshwater systems.

The aim of *Chapter 5* was to optimise *de novo* assembly strategy to improve the quality of transcriptomic analysis. I then investigated and compared the mechanisms

of toxicity of the most widely used herbicide worldwide, glyphosate, and its commercial formulation, Roundup.

The aim of *Chapter 6* was to address the hypothesis that glyphosate and Roundup cause reproductive toxicity in fish. It is not practical to investigate the potential effects of chemical exposure on reproductive output in brown trout, so we used zebrafish as a well-established, reliable model species for this experiment. RT-QPCR was used to investigate suspected molecular mechanisms of toxicity potentially linked to the reproductive effects seen.

As a whole, this thesis addresses knowledge gaps in the evaluation of potential effects of environmentally relevant chemical contaminants in brown trout. In doing so, I conducted some of the first assessments of the global mechanisms of toxicity of environmental contaminants in brown trout and also developed considerable transcriptomic resources for this species. By using RNA-seq to conduct this transcriptional profiling, I also demonstrated the value of this tool in ecotoxicology.

CHAPTER 2

Global Transcriptome Profiling Reveals Molecular Mechanisms of Metal Tolerance in a Chronically Exposed Wild Population of Brown Trout

Environmental Science and Technology (2013) **47**; 8869−8877

pages 32-40

supporting information (pages 41-69)

Environmental Science & Technology

Article

pubs.acs.org/est

Global Transcriptome Profiling Reveals Molecular Mechanisms of Metal Tolerance in a Chronically Exposed Wild Population of Brown Trout

T. M. Uren Webster,*[,†] N. Bury,[‡] R. van Aerle,[†] and E. M. Santos*[,†]

[†]Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

[‡]Diabetes & Nutritional Sciences Division, 3.83 Franklin-Wilkins Building, King's College London, London, SE1 9NH

Supporting Information

ABSTRACT: Worldwide, a number of viable populations of fish are found in environments heavily contaminated with metals, including brown trout (*Salmo trutta*) inhabiting the River Hayle in South-West of England. This population is chronically exposed to a water-borne mixture of metals, including copper and zinc, at concentrations lethal to naïve fish. We aimed to investigate the molecular mechanisms employed by the River Hayle brown trout to tolerate high metal concentrations. To achieve this, we combined tissue metal analysis with whole-transcriptome profiling using RNA-seq on an Illumina platform. Metal concentrations in the Hayle trout, compared to fish from a relatively unimpacted river, were significantly increased in the gills, liver and kidney (63-, 34- and 19-fold respectively), but not the gut. This confirms that these fish can tolerate considerable metal accumulation, highlighting the importance of these tissues in metal uptake (gill), storage and detoxification (liver, kidney). We sequenced, assembled and annotated the brown trout transcriptome using a *de novo* approach. Subsequent gene expression analysis identified 998 differentially expressed transcripts and functional analysis revealed that metal- and ion-homeostasis pathways are likely to be the most important mechanisms contributing to the metal tolerance exhibited by this population.

INTRODUCTION

Metal contamination of freshwater systems occurs worldwide, in some cases reaching concentrations known to cause acute toxicity, yet a few of these rivers and lakes support viable populations of fish. Yellow perch populations inhabiting lakes in North America contaminated through industrial and mining activity with a number of metals (particularly copper, cadmium and nickel) are an exceptionally well studied example. Gradients in contamination have been used to demonstrate correlations between chronic metal exposure and a number of physiological changes associated with metal toxicity and/or tolerance, including alterations in metabolic processes, the antioxidant system and metal transporting/sequestering pathways [e.g., refs 1−7]. Additionally, these metal exposures have resulted in impaired growth, reproduction and genetic diversity, with potentially adverse implications for the health of these populations.[5,6,8]

Brown trout (*Salmo trutta*) inhabiting the River Hayle in Cornwall (Southwest England) are another population of fish chronically exposed to elevated metal concentrations. Historically, mining in the surrounding area dates back to Neolithic times, peaking during the 1800s and drainage from the disused mines continues to contaminate the river with a mixture of metals.[9,10] The middle region of the river Hayle has extremely high metal concentrations, where little fish or invertebrate life is found, however this does not prevent brown trout migration and gene flow between the upper and lower sections.[10] Concentrations of metals in the lower region, where brown trout are readily found, have been documented to cause acute toxicity in metal-naive brown trout [e.g., refs 10−13], including total zinc, copper and iron which averaged 639, 42 and 200 μg/L respectively (data kindly provided from the Environment Agency, Supporting Information Table S1). Despite the persistent and significant levels of metal contamination, the River Hayle appears to support a sustainable population of brown trout that exhibits no evidence of reduced genetic diversity.[10] Therefore, it is expected that the brown trout population in the River Hayle may exhibit mechanisms allowing them to tolerate chronic metal exposure. Populations of brown trout inhabiting water systems contaminated with metals are not unique to the River Hayle and other examples include populations found in Norway and the USA.[14,15] Despite this, very little is known about the physiological and molecular

Received: March 29, 2013
Revised: June 16, 2013
Accepted: July 8, 2013
Published: July 8, 2013

adaptations that allow this species to survive high concentrations of metals in their environment.

We aimed to address this knowledge gap, using brown trout from the River Hayle as a case study. We adopted an integrative approach combining genomics with analysis of tissue metal accumulation, to understand the molecular mechanisms employed by fish from this population to tolerate the high concentrations of metals in their environment. There is relatively limited gene sequence information for brown trout, therefore our first goal was to sequence, assemble and annotate the transcriptome for this species, using the Illumina sequencing platform to perform RNA-seq. We then used this resource to investigate molecular pathways differentially regulated in fish from the River Hayle, compared to a metal naive brown trout population originating from a relatively unimpacted river within the same geographical region. Our data demonstrated the very significant metal accumulation in tissues of fish originating from the River Hayle and proposes a number of molecular mechanisms employed by this species to cope with the high concentrations of metals in their environment, including the regulation of metal and ion homeostasis pathways and activation of antioxidant systems.

MATERIALS AND METHODS

Sample Collection. To obtain a comprehensive sequence data set for assembly of the brown trout transcriptome, we collected five embryos at ten different stages of development and a range of tissues from adult fish. In a second phase, to investigate the mechanisms of tolerance to metals in the brown trout population from the River Hayle, five adult fish from this river and ten from a control river (River Teign) were sampled for analysis of tissue metal content and for transcriptomic analysis. A full description of the samples collected is presented in Supporting Information (Table S2).

Metal Analysis. Metal concentrations in the River Hayle and Teign were kindly provided by the Environment Agency and are presented in Supporting Information (Table S1). Portions of gill, gut (stomach and intestine), kidney and liver were dissected from fish obtained from the River Hayle and River Teign for determination of the metal content in these tissues. Samples were dried to a constant weight and digested with 1 mL of concentrated HNO_3 (for trace metal analysis, Fisher Chemicals) for 24 h at 60 °C and treated with 60 μL H_2O_2. The samples were diluted with 9 mL of Milli-Q water and analyzed for Cu, Pb, Zn, As, Cd, Fe and Ni by inductively coupled plasma mass spectrometry (ICP-MS; E:AN 6100DRC, Perkin-Elmer, Cambridge, U.K.). Cluster analysis was performed on the metal concentrations in individual fish tissues from the river Hayle (h1−h5) and river Teign (t1−t10) using Euclidean distance measure and heatmaps were produced using the pheatmap package in R/Bioconductor.[16] Associations between individual fish length/weight and tissue metal concentrations were tested using regression analysis.

RNA Extraction, Library Construction and Sequencing. Total RNA was extracted from all individual embryos and adult tissues using TRI reagent (Sigma-Aldrich), according to the manufacturer's instructions. The concentration and quality of RNA in each sample was determined using a NanoDrop ND-1000 Spectrophotometer *(NanoDrop Technologies, U.S.A.)* and an Agilent 2100 Bioanalyzer *(Agilent Technologies, Inc., U.S.A.)*. Equal amounts of RNA from individual samples were pooled to obtain the samples described in Supporting Information Table S2. cDNA libraries were prepared for each pooled sample using the Illumina TruSeq RNA Sample Preparation kit and sequencing was conducted using an Illumina GAIIx Genome Analyzer, generating 100 bp paired end reads for the embryonic library and 76 bp paired end reads for 12× multiplexed adult libraries. A detailed description of these methods is presented in Supporting Information.

Assembly and Annotation of the Brown Trout Transcriptome. Raw sequences were processed to remove Illumina adapter sequences and filter out sequences that did not meet the quality thresholds. Sequences less than 30 bp in length were removed. All paired reads of the adult tissue and embryo libraries were pooled and assembled *de novo* using Velvet (version 1.2.08; ref 17) and Oases (version 0.2.08; ref 18) using a range of *k*-mers (see Supporting Information for full details). The resulting transcripts were annotated using Blastn and Blastx and a selection of fish and mammalian nucleotide and protein databases and using an *e*-value cut off <1 × 10^{-15}. Gene expression was determined in the gill, gut, kidney and liver of fish inhabiting the metal-contaminated river Hayle and the reference river Teign using RSEM.[19] Reads were mapped against the brown trout reference transcriptome using the "--no_polyA" parameter and using default settings. Subsequent analyses in RSEM were conducted using a selection of scripts provided as part of the Trinity assembly package (version r2012−10−05;.[20] Statistical differences in gene expression levels between tissues of the two rivers were calculated using edgeR.[21] Genes were considered differentially expressed when FDR < 0.1 (Benjamini−Hochberg correction). A 4-way Venn diagram showing overlapping differentially expressed genes was produced using VennDiagram[22] in R/Bioconductor. All analyses were carried out on a local server running under the NEBC Bio-Linux 7 environment [23] unless stated otherwise. A flow diagram describing the transcriptome assembly and gene expression analysis is presented in Figure 1 and a full description of the methodology is presented in the Supporting Information.

The sequence data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE45637.

Transcriptomic Analysis. Functional analysis was then performed for differentially expressed genes from each tissue using the Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7; [24]), using the brown trout transcriptome as background list. Gene Ontology (GO) Fat terms for Biological Process, Cellular Component and Molecular Function were considered significantly over-represented when $P < 0.05$.

In order to validate the quantitative analysis of differential gene expression between Hayle and Teign trout, a selection of four transcripts (*mtb, gpx1b, cat, slc40a1*) were analyzed via real time quantitative PCR (RT-QPCR) on gill, gut, kidney and liver samples from all individual fish, according to previously described methods.[25,26] These transcripts encode proteins involved in metal homeostasis and oxidative stress response and are therefore potentially differentially regulated by metal exposure. They include transcripts that were found to be both differentially expressed and not differentially expressed in the RNA-seq data, to corroborate both of these scenarios. Transcript expression levels were normalized using the control gene Actin-related protein 2/3 complex 3 (*arpc3*) which was selected from the RNA-seq data set based on its consistent expression between Hayle and Teign fish in all tissues

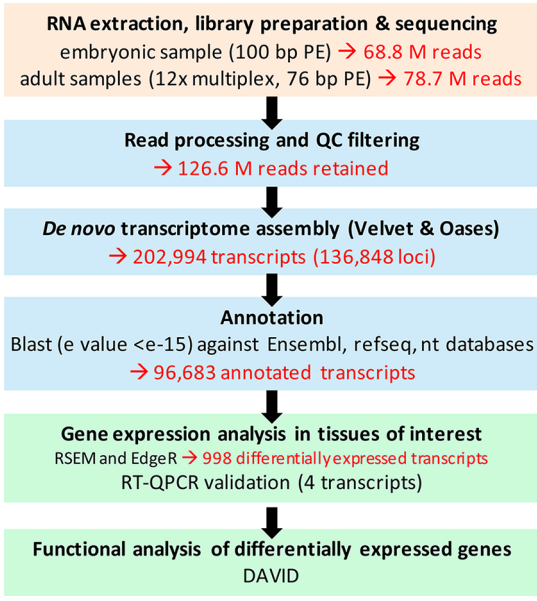


Figure 1. Flow diagram illustrating the workflow employed for sequencing, assembling and annotating the brown trout transcriptome and for determining changes in gene expression profiling between brown trout populations. Red text indicates results at each stage of the analysis pipeline.

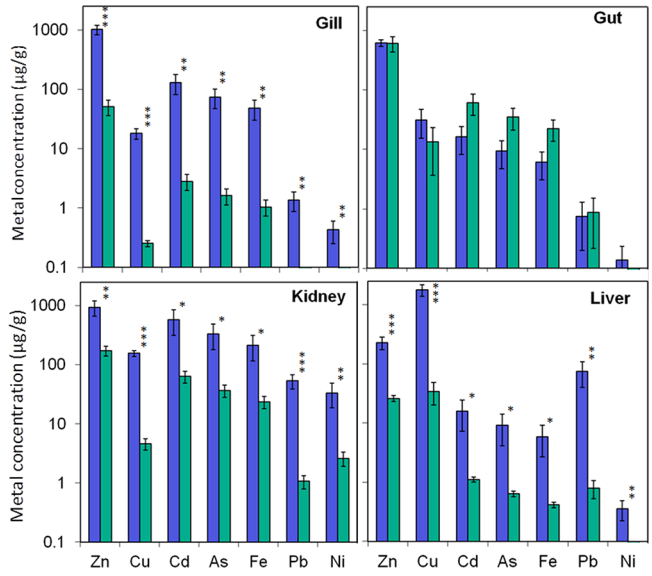


Figure 2. Concentration of six metals measured by ICP-MS, in the gill, gut, kidney and liver of fish from the rivers Hayle and Teign. Values are expressed as mean ± SEM. Blue bars represent data from fish originating from the metal contaminated river Hayle (n = 5) and green bars represent data from fish originating from the relatively unimpacted river Teign (n = 10). Asterisks indicate significant differences in concentration of each metal between fish from each population, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Supporting Information Table S3a). Full details are presented in the Supporting Information.

RESULTS AND DISCUSSION

Tissue Metal Accumulation. In the gills, liver and kidney the concentration of all seven metals measured (Cu, Pb, Zn, As, Cd, Fe, Ni) was significantly higher in the Hayle trout than the Teign trout. Across all metals the fold change was highest in the gill (mean 62.6-fold) followed by the liver (mean 33.7-fold), then the kidney (mean 18.5-fold). In contrast, in the gut there was no significant difference in the concentration of any of the metals measured (Figure 2). The considerable increase in Hayle gill metal concentration contrasts sharply with the lack of difference in the gut and suggests that the gills are the principal route of metal uptake in these fish. This is because of their large surface area in direct contact with water and abundance of metal specific carriers (e.g., for essential metals copper, zinc and iron), as well as other ion/metal transporters that allow uptake of a number of metals through ionic mimicry (e.g., Cu^{+} via Na^{+} uptake routes and Zn^{2+} and Cd^{2+} via Ca^{2+} uptake routes).[27,28]

After uptake, metals are transported in the bloodstream throughout the body. The considerable accumulation and greatest total concentration, of metals in the kidney and liver reflects the essential role of these tissues in metal processing, detoxification, storage and excretion. In both Hayle and Teign fish, zinc was the most abundant metal in the gill, gut and kidney, while copper was found at the highest concentration in the liver. Copper and zinc were also the metals that increased to the greatest extent, in terms of absolute concentration, in the gills, liver and kidney. Corresponding with this, water concentrations of zinc and copper were elevated to the greatest extent of all metals in the River Hayle compared to the Teign, by approximately 60- and 40-fold, respectively (Supporting Information Table S1). The tissue distribution and accumulation patterns of iron, cadmium and arsenic reveal some striking correlations between these three metals and this is supported by cluster analysis on individual fish (Supporting Information Figure S1). This strongly suggests that the uptake, storage and metabolism of these metals, in particular, are linked. No significant correlation was found between fish length or weight and metal concentration in any tissue, suggesting the difference in size/age of the sampled populations is unlikely to have influenced the metal accumulation patterns.

The levels of metal accumulation in the tissues of brown trout from the River Hayle were considerably higher than that measured in other chronically exposed fish from metal-contaminated regions. For example, Hayle trout had accumulated 156, 1800 and 18 μg/g copper and 929, 229 and 1020 μg/g zinc in the kidney, liver and gills respectively, compared with 4.88, 242 and 3.85 μg/g copper and 186, 47.9 and 73.7 μg/g zinc in the same tissues of brown trout in copper and zinc rich Norwegian rivers.[29] Highest recorded values of 256.6 μg/g copper and 157 μg/g zinc in liver of yellow perch from metal contaminated Canadian lakes [30] are also far lower than the concentrations of these metals in the liver of the Hayle brown trout (1800 and 229 μg/g for copper and zinc, respectively). This highlights both the extent of metal contamination in the River Hayle and the high degree of metal tolerance of its resident brown trout population.

Assembly of the Brown Trout Transcriptome. Sequencing generated 68.8 M 100 bp reads from the embryonic library and a total of 78.7 M 76 bp reads from the multiplexed libraries (ranging from 5.1 to 7.7 M reads per library). Following raw sequence read processing and quality filtering a total of 60.1 M (9.7% orphans) embryonic reads and 66.5 M (8.7% orphans) multiplexed reads were retained and input into the transcriptome assemblies. The *de novo* assembly consisted of 202,994 transcripts (136,848 loci), with an average length of 821 bp and an N50 of 1853 bp, 48% of which were annotated by Blast (*e*-value $<1 \times 10^{-15}$) (Figure 1). The final

dx.doi.org/10.1021/es401380p | Environ. Sci. Technol. 2013, 47, 8869−8877

transcriptome assembly provides a high quality template for global gene expression profiling in this study and also provides a valuable tool for wider research on brown trout, which is an ecologically and economically important fish species with limited existing genomic resources.

Transcriptome Profiling. Transcript profile analysis revealed that 73 881 transcripts were expressed in at least one of the eight Hayle and Teign libraries tested. The total number of genes present and their expression level distribution were generally consistent between libraries, although some tissue-specific differences were evident. For example, liver and gut tissues expressed a greater proportion of rare genes and fewer genes in total, than gill and kidney (Supporting Information Table S5). The gene expression patterns for the four selected tissues for the Hayle and Teign fish were examined to identify potential mechanisms of toxicity and/or tolerance to the metal exposure in the River Hayle. A total of 998 transcripts were differentially expressed in at least one tissue (Figure 3). The

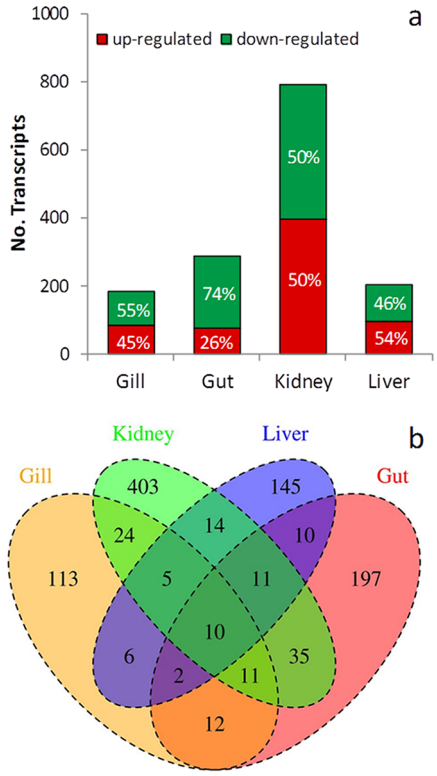


Figure 3. (A) Number of differentially expressed genes between populations obtained using EdgeR with a FDR <0.1 in each tissue. Numbers within bars represent the percentage of the total that were up/down-regulated in brown trout originating from the river Hayle compared to the river Teign. (B) Venn diagram displaying the number of differentially expressed genes in each tissue and the overlay between these gene lists across tissues.

greatest number of differentially expressed genes (792) occurred in the kidney. Perhaps surprisingly, given its role in metal uptake, substantial metal accumulation and known susceptibility to acute metal toxicity, fewest genes (183) were differentially expressed in the gill. In contrast, despite no increase in metal accumulation a considerable number of genes (288) were differentially expressed in the gut, but a large proportion of these genes are linked to digestion and likely to be related to dietary differences between the sites (see below). RT-QPCR analysis was in full agreement with the RNA-seq transcriptional profiling data, confirming the reliability of the quantitative data obtained from sequencing analysis on pooled samples (Supporting Information Table S6). Significantly over-represented GO FAT terms ($P < 0.05$), among the differentially expressed gene lists are shown in Supporting Information Table S7.

Metal Homeostasis. Fundamentally, metal homeostasis consists of ensuring an adequate supply of essential metals for metabolic processes and controlling the level of essential and nonessential free metal ions to prevent toxicity. This involves regulating uptake from the environment, distribution through the bloodstream and delivery to target organs, supply to metabolic pathways, biotransformation, storage and excretion.[28] Exposure to elevated metal concentrations in the River Hayle and the resulting increase in metal tissue accumulation, would therefore be expected to be associated with changes in the activity of components involved in this homeostatic system.

A number of cellular metal binding proteins serve to detoxify and store metal ions through binding and removal of their redox potential. Glutathione and metallothioneins (MTs) act as buffers for metal ions entering cells; both have very high affinity for most metals and glutathione is generally present at high concentrations. MTs are cysteine-rich, thiol-containing proteins and are widely acknowledged to account for a major portion of the cellular storage of zinc, copper, cadmium and to a lesser extent iron, lead and nickel in fish.[28] MT-bound metals contribute to the metabolically detoxified cellular fraction and can also be stored in metal rich granules for even more stable, long-term storage.[31] Increased MT synthesis has been extensively shown to occur in response to many metals, in both short-term laboratory exposures and in chronically exposed wild fish and it is the most consistent and sometimes only, mechanism of metal tolerance in fish [e.g., refs 14, 15 and 32−34]. A single MT isoform is predominantly induced by metals in fish; free metal ions bind *mtf1* transcription factors, which then bind metal response elements (MREs) in its promoter region, stimulating transcription.[35] Corresponding with this, we found that one MT (metallothionein b) was among the most strongly up-regulated genes in the Hayle trout (significantly up-regulated by 8.2-, 7.7- and 5.6-fold in the gill, gut and liver, respectively, as well as 2.2 fold in the kidney), indicating sequestration of metals by MT represents a very important mechanism of metal tolerance in this population. Another MT isoform was also present in the brown trout transcriptome assembly but only expressed at very low levels.

Information available in the existing literature shows that acute metal exposures alter the expression of genes encoding metal-specific transporting proteins in the gill, gut, kidney and liver. These include the main cellular transporters of copper (copper transporter 1 (*slc31a1*), divalent metal transporter 1 (*slc11a2*), copper-transporting ATPases (*atp7a, atp7b*)); zinc (various members of the ZnT (or *slc30*) and ZIP (or *slc39*) families); and iron (*slc11a2*, ferroportin (*slc40a1*)).[35−38] This suggests limiting uptake of metals from the environment, to slow onset of toxicity and increasing delivery to organs involved in metal metabolism, storage and excretion may be a mechanism of metal tolerance in short-term exposures. Less information is available on response to chronic exposure, but Xie and Klerks[39] found a reduced rate of cadmium uptake in a tolerant laboratory population of killifish and Gale et al.[40] showed a reduction in copper uptake rates in the gill

contributed to copper tolerance of rainbow fish. In contrast, other studies have found no evidence of altered metal uptake and distribution kinetics during acclimation [e.g., ref 34]. For Hayle trout, we hypothesized that gene pathways related to metabolism of copper and zinc were the most likely to be altered, given that concentrations of these metals were the most elevated in both river water and tissues. Of these genes, only the zinc transporter *slc39a2* was differentially expressed (down-regulated in the kidney). There were no apparent trends in altered regulation of the other copper and zinc specific transporting proteins listed above. However, potential changes in copper and zinc transporters at the protein level, for example through post-transcriptional modification or changes in tissue/cellular localization, should not be ruled out. In contrast, there were increasing trends in expression levels of iron transporters in Hayle trout; *slc11a2* was up-regulated 2.2 and 3.5 fold in the liver and kidney respectively, while *slc40a1* was up-regulated 4.3 fold in the kidney, although these differences were not statistically significant. A number of genes encoding proteins involved in wider iron transport and storage were differentially expressed, particularly in the kidney, liver and gut. These include transferrin, a precursor of serotransferrin, as well as transferrin receptor 1b, which is responsible for cellular uptake of metals from transferrin and a form of ferritin, the main cellular iron-binding protein. Additionally, hemopexin, heme-binding protein 2 and heme transporter, which are involved in wider iron homeostasis though hemoglobin regulation, were differentially expressed. Moreover, the main regulator of iron homeostasis, the hormone hepcidin, was down-regulated in the liver. These changes in iron-metabolism related genes are particularly marked in contrast to the lack of change in those specific to copper and zinc. Furthermore, these changes are occurring in the absence of long-term significant elevation in the concentrations of Fe in the Hayle, compared to the Teign, river water (Supporting Information Table S1), but in the presence of significant accumulation of Fe in the liver, kidney and gill of Hayle fish (Figure 2). We hypothesize that these iron-homeostasis genes may be regulated by other metals present in the water, or that iron-homeostasis is a target of metal toxicity. An alternate hypothesis may be that a peak in Fe in the river water occurred close to the sample collection and was not recorded in the water sampling conducted by the Environment Agency. It is impossible to ascertain if this was the case and, therefore, we cannot conclusively interpret the reasons for the alterations of iron related pathways in the Hayle fish. Despite this, the striking association between the concentrations of Fe and several other metals (particularly As and Cd), together with the alteration in the expression of genes involved in iron-homeostasis, suggest that these iron-handling pathways play an important role in the response of the Hayle fish to metal exposure. This is supported by previous reports suggesting an association of other metals, including Cd, Cu and Pb, with binding and regulation of transcription of various components of the iron homeostatic system.[41−44] Less is known about arsenic distribution pathways in fish, although arsenic is capable of being transported by transferrin in human plasma.[45]

Ion homeostasis. One major mechanism of toxicity common to a number of metals is disruption of ion homeostasis, particularly in the gills. Zn^{2+}, Cd^{2+} and Pb^{2+} inhibit Ca^{2+} uptake, through competition for Ca^{2+} uptake pathways and direct inhibition of Ca^{2+} ATPase, leading to hypocalcaemia. Cu^{+} reduces Na^{+} uptake, both competitively and by interference with Na^{+}/K^{+} ATPase. Copper, zinc, cadmium and lead also inhibit carbonic anhydrase, reducing supply of H^{+} and HCO_3^{-} ions for Na^{+} and Cl^{-} uptake exchange. Lower plasma NaCl levels lead to increased blood viscosity and can cause circulatory collapse.[46−49] Several laboratory studies have demonstrated acclimation of fish to metals, including copper, cadmium and lead, following chronic exposure, characterized by a restoration of plasma ionic balance and physiological condition. Increased synthesis of Na^{+}/K^{+} ATPase to restore total cellular Na^{+}/K^{+} ATPase activity, as well as morphological changes in the gill contribute to acclimation to copper.[50−52]

Although not significant, in the kidney our results show a trend of up-regulation for both of these ATPases known to be inhibited by metals, particularly for the most highly expressed (and therefore probably functionally most important) isoforms in this tissue. Na^{+}/K^{+} ATPases *atp1a* and *atp1b1a* were both up-regulated by 2 fold, while Ca^{2+} ATPase *atp2b1a* was up-regulated by 3.3 fold. Additionally there was a significant up-regulation of carbonic anhydrase in the liver. This suggests that the up-regulation of these enzymes in the Hayle brown trout may be employed to counter their inhibition by metals. A number of other genes encoding proteins important in maintaining ion balance were differentially expressed. In the kidney Na^{+}-Cl^{-} cotransporter (*slc12a3*), which reabsorbs NaCl from urine, was significantly up-regulated by 4.8 fold. There was also a general trend of up-regulation in the kidney of a number of other transporters including those in the *slc12* family, although a low-expressed isoform *slc12a9* had significantly reduced expression, as well as those in the *slc4* (sodium-bicarbonate transporter) and *slc9* ($Na^{+}H^{+}$ exchanger) families. However, *slc24a6*, a $Na^{+}Ca^{2+}K^{+}$ exchanger was significantly down-regulated in both the kidney and liver. Several other genes responsible for ion transport were down-regulated including chloride intracellular channel related proteins in the kidney and serum/glucocorticoid regulated kinase, which has a role in activating ion channels, in the liver. Three aquaporins, which contribute to maintenance of osmotic balance, were also differentially expressed in the kidney and gut. Additionally, a number of genes with a role in maintaining calcium homeostasis were differentially expressed, predominantly being down-regulated in the kidney, gut and liver. These include two calcium binding proteins and two s100-calcium binding proteins, calmodulin and calmodulin binding transcription activator, which are involved in calcium signaling and three isoforms of stanniocalcin, which regulates calcium flux. Additionally, two chemokine receptors which are related to calcium flux and signaling, together with calcium binding proteins with specific roles in muscle contraction (calponin, calsequestrin and caldesmon) were also down-regulated. Overall, these results indicate an integrated response of the ion-homeostatic system, which may contribute to the metal tolerance of this population through compensation of metal-induced ion balance disturbance. The most pronounced response appears to be in the kidney, reflecting the important role of this organ in regulating plasma ion and water balance. However, it is surprising that so few genes related to ion homeostasis were differentially expressed in the gill given that it is the main target of metal-disrupted ion balance.

Markers of Oxidative Stress and Cellular Damage. Metals induce cellular oxidative stress by several different mechanisms. Redox-active metal ions, including copper and iron, generate reactive oxygen species (ROS) via Fenton chemistry. Several

metals, including copper, cadmium, arsenic, nickel and lead, can deplete and inhibit the cellular antioxidants. Copper and cadmium can also generate ROS via disruption of the electron transport chain.[28,53] Oxidative stress can lead to lipid peroxidation, DNA and protein damage with associated adverse health effects at the cellular level. To counteract this, increased cellular ROS stimulate an up-regulation of the cellular antioxidant defense system, comprised of reduced glutathione (GSH) and a suite of enzymes, to limit oxidative damage. There is evidence from gene expression studies that various metals stimulate a response of the antioxidant system during short-term exposures [e.g., refs 11, 36 and 43]. In chronically exposed wild fish populations there is some evidence of damage caused by oxidative stress, including lipid peroxidation in brown trout,[15] but gene expression profiling has found inconsistent changes of the antioxidant system.[1,14]

Although gene expression profiling revealed that one isoform of the antioxidant superoxide dismutase was significantly down-regulated in all tissues, other, more highly expressed isoforms showed no change in expression level. No significant difference in expression was found for any other antioxidants, although in the gill, there was >2-fold apparent up-regulation of GSH-related antioxidants glutathione peroxidase (*gpx1b*) and glutathione-S-transferases (*gsta5, gsto1, gstz1*). There were no differences in expression level of glutathione synthetase or reductase, which are essential for GSH synthesis and restoration from its oxidized form, although a number of oxidoreductase enzymes, involved in supplying NADPH for restoring GSH, were significantly up-regulated. We found significant up-regulation of cytochrome C oxidases (*cox4i2, cox7a*) in the liver and kidney, which have been linked with oxidative stress generation and response, but are also associated with metal-induced metabolic changes (see below). 70 kD heat shock protein (*hsp70*), which temporarily binds and stabilizes damaged proteins was also significantly up-regulated in the gill and gut, while other heat shock proteins (*hsp40, hsp27*) were differentially expressed in the liver and gut, respectively. Overall, this suggests a modest response of the antioxidant system of Hayle trout, particularly in the gill. This is perhaps because after uptake from the environment and before being bound and/or transported elsewhere, the concentration of toxic free ions is likely to be higher in the gills compared to other tissues, leaving it more susceptible to oxidative stress. However, the response of the antioxidant system as a whole is much less pronounced than expected to occur following acute metal exposures, suggesting that other mechanisms of tolerance are likely to play a key role in reducing oxidative stress caused by metal ions in the Hayle brown trout population. These mechanisms may include increased synthesis of MTs, which have a high affinity for ROS and are therefore powerful cellular antioxidants and may offer significant protection against oxidative stress.[46] This mechanism is likely to be a consistent and significant contributor to the metal tolerance of this brown trout population. Corresponding with this, we found no evidence of changes in pathways related to cellular repair mechanisms or evidence of cellular toxicity, such as changes in expression of apoptotic gene markers.

Metabolic Processes. A large proportion of the genes differentially expressed in the gut are related to digestion. This is particularly obvious from the GO term analysis; various *peptidases* dominate molecular function over-representation and *proteolysis* is the only over-represented biological process in this organ. Protein metabolism, including proteolysis specifically, has been shown to be altered in response to both short-term and chronic metal exposures. Furthermore, it has been suggested that this facilitates enhanced protein turnover which is important in the replacement of damaged proteins and proteins involved in metal detoxification, repair and storage.[1−3,54] However, in the present study differentially expressed genes related to proteolysis were predominantly proteases, carboxypeptidases, chymotrypsins and elastases, which are more specifically associated with digestion and almost exclusively occurred in the gut. This strongly suggests that these differences are most likely related to diet. Dietary differences between the Hayle and Teign fish may be influenced by many environmental variables, although may not be entirely unrelated to metal contamination because metal exposure is well-known to alter food webs through changes in river species assemblage [e.g., ref 55].

The mechanisms of metal tolerance employed by the Hayle trout are likely to result in increased energetic demand. Metal exposure has induced metabolic changes that have been suggested to be compensatory, in order to facilitate metal tolerance and detoxification. For example, chronically exposed wild yellow perch show some evidence of enhanced aerobic respiration, potentially increasing ATP production.[1,56] Conversely, metal exposure has also been associated with down-regulation of various metabolic processes, due to the energetic demands of metal detoxification and/or through impairment of metabolic enzymes. In chronically exposed wild yellow perch there is evidence of lower aerobic capacity [3] and down-regulation of various components of lipid synthesis and transport, which have been associated with depletion of lipid reserves leading to adverse impacts on fish health and condition.[2,5]

In the Hayle trout, there was some evidence of changes in aerobic respiration, in particular down-regulation of genes with a role in oxidative phosphorylation including down-regulation in all tissues of several NADH dehydrogenases (*mt-nd1, mt-nd1b, mt-nd4, mt-nd5*) and up-regulation of cytochrome c oxidases (*cox4i2, cox7a*) in the liver and kidney respectively. With regard to other metabolic processes, in particular there were changes in expression of a number of genes involved in lipid, fatty acid and steroid synthesis and transport, mainly in the liver and kidney. These include peroxisome proliferator-activated receptor alpha, low-density lipoprotein receptor, lysophosphatidylglycerol acyltransferase, Cyp46, stearoyl-CoA desaturase and five apolipoproteins. In contrast to previous studies, these genes were almost exclusively up-regulated. A likely explanation for the up-regulation of lipid metabolism in the Hayle trout is that the sample population of five fish consisted of three reproductively mature females and two immature fish, while the Teign population sample was dominated by reproductively immature fish with no maturing females. Lipid metabolic pathways are essential for the synthesis of the egg yolk precursor protein vitellogenin and other egg shell proteins, in the liver of females as they undergo gonadal development and maturation. The energetic costs of reproductive maturation are also likely to influence other metabolic processes (i.e., energy generation through aerobic respiration and diversion from proteolysis), therefore the differences in metabolic processes observed can by no means be exclusively attributed to metal exposure. Consistent with this, genes associated with reproductive development in females were strongly up-regulated, including of genes encoding

vitellogenin and egg outer membrane zona pellucida proteins in the liver of Hayle fish.

Immune system. Impairment of immune function has been extensively demonstrated in previous studies to be associated with exposure to metals. This effect has been attributed, in part, to disruption of energy budget as well as to a direct inhibition of immune function and commonly results in increased susceptibility to bacterial and viral infection.[57−59] Gene expression analysis revealed a predominant down-regulation of a number of components of the complement system in the liver, gut and kidney of Hayle trout. These include complement components 1qa, 1qb, 1q2l, 3, 4 and 8; complement factors H, H1 and D and Fanconi anemia, complementation group C. This suggests an impairment of the immune system and is consistent with the findings of Pierron et al.[2] and Reynders et al.[60] who found inhibited expression of genes in the immune system in fish exposed to metals, especially those involved in the complement system.

We have sequenced, assembled and annotated a transcriptome for the brown trout, providing a useful resource for further research in this species. Using this information, we investigated the molecular mechanisms of tolerance to metals in a brown trout population exposed to significantly elevated concentrations of multiple metals in the River Hayle, U.K. and anchored our data to metal accumulation in tissues and river water concentrations. Tissue metal accumulation patterns indicate the gill represents the major route of metal uptake in Hayle fish, while the accumulation in the kidney and liver reflects their important role in metal storage and detoxification. The considerable tissue metal accumulation observed in the absence of overt toxicity confirms the metal tolerance of Hayle brown trout. Global gene expression profiling revealed that the two broad strategies likely to contribute to the metal tolerance of this population are the regulation of metal homeostasis pathways and ion homeostasis pathways, with up-regulation of the antioxidant system playing a relatively minor role. Within these, several mechanisms appear of particular importance. These include increased synthesis of metallothionein, the prominence of the kidney in regulating ion balance and the putative role of iron-handling pathways in wider metal homeostasis. Although our data set highlighted some potential mechanisms of metal toxicity, particularly inhibition of the immune system, there is little to suggest that the brown trout inhabiting the River Hayle are incurring adverse health effects as a result of the presence of toxic concentrations of metals in their environment. This contrasts with more extensive evidence of metal toxicity from studies on yellow perch, potentially causing adverse impact at both the individual and population levels. A possible explanation for this is that metal contamination in the River Hayle has been present for a greater length of time, perhaps leading to a greater degree of metal tolerance in this population. Whether this is a result of an inherent genetic plasticity, allowing individual acclimation, or local population adaptation, leading to inherited metal-tolerance, is unclear and would require further research to be elucidated.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental experimental section, metal concentrations in the river Hayle and river Teign, description of cDNA libraries sequenced, fold changes between the Hayle and Tein fish of potential control genes for RT-QPCR analysis, target genes, primers and assay details for RT-QPCR analysis, custer diagrams of tissue metal concentration, summary statistics of sequence read processing and QC filtering, transcript abundance level in each tissue, results of RT-QPCR analysis, heatmaps of expression values of differentially expressed genes and gene ontology terms over-represented in the lists of differentially expressed genes between Hayle and Teign fish for each tissue. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*T.M.U.W.: E-mail tu202@exeter.ac.uk, phone +44 (0)1392 724677, fax +44 (0)1392 263434. E.M.S.: E-mail E.Santos@exeter.ac.uk, phone +44 (0)1392 264607, fax +44 (0)1392 263434.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Environment Agency for facilitating the fish sampling and for provision of historical chemistry data for the rivers studied in this project. We also thank J. Shears and P. Shears for support with the fish sampling, P. Kille for encouraging us to conduct this project, A. Moorhouse, C. Sambles and K. Paszkiewicz for facilitating the sequencing experiments and K. Uren for providing the image of the River Hayle. We acknowledge funding from the Salmon & Trout Association and from the Natural Environment Research Council, grant number NE/1528326/1.

■ REFERENCES

(1) Pierron, F.; Bourret, V.; St-Cyr, J.; Campbell, P. G. C.; Bernatchez, L.; Couture, P. Transcriptional responses to environmental metal exposure in wild yellow perch (*Perca flavescens*) collected in lakes with differing environmental metal concentrations (Cd, Cu, Ni). *Ecotoxicology* **2009**, *18* (5), 620−631.

(2) Pierron, F.; Normandeau, E.; Defo, M.; Campbell, P.; Bernatchez, L.; Couture, P. Effects of chronic metal exposure on wild fish populations revealed by high-throughput cDNA sequencing. *Ecotoxicology* **2011**, *20* (6), 1388−1399.

(3) Couture, P.; Rajender Kumar, P. Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (*Perca flavescens*). *Aquat. Toxicol.* **2003**, *64* (1), 107−120.

(4) Rajotte, J. W.; Couture, P. Effects of environmental metal contamination on the condition, swimming performance and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* **2002**, *59* (8), 1296−1304.

(5) Levesque, H. M.; Moon, T. W.; Campbell, P. G. C.; Hontela, A. Seasonal variation in carbohydrate and lipid metabolism of yellow perch (*Perca flavescens*) chronically exposed to metals in the field. *Aquat. Toxicol.* **2002**, *60*, 257−267.

(6) Levesque, H. M.; Dorval, J.; Hontela, A.; Van Der Kraak, G. J.; Campbell, P. G. C. Hormonal, morphological and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures. *J. Toxicol. Environ. Health, Part A* **2003**, *66* (7), 657−676.

(7) Giguare, A.; Campbell, P. G. C.; Hare, L.; Couture, P. Subcellular partitioning of cadmium, copper, nickel and zinc in indigenous yellow perch (*Perca flavescens*) sampled along a polymetallic gradient. *Aquat. Toxicol.* **2006**, *77* (2), 178−189.

(8) Bourret, V.; Couture, P.; Campbell, P. G. C.; Bernatchez, L. Evolutionary ecotoxicology of wild yellow perch (*Perca flavescens*) populations chronically exposed to a polymetallic gradient. *Aquat. Toxicol.* **2008**, *86* (1), 76−90.

(9) Brown, B. E. Effects of mine drainage on the river hayle, cornwall a) factors affecting concentrations of copper, zinc and iron in water, sediments and dominant invertebrate fauna. *Hydrobiologia* **1977**, *52* (2), 221−233.
(10) Durrant, C. J.; Stevens, J. R.; Hogstrand, C.; Bury, N. R. The effect of metal pollution on the population genetic structure of brown trout (*Salmo trutta L.*) residing in the River Hayle, Cornwall, UK. *Environ. Pollut.* **2011**, *159* (12), 3595−3603.
(11) Hansen, B. H.; Romma, S.; Softeland, L. I. R.; Olsvik, P. A.; Andersen, R. A. Induction and activity of oxidative stress-related proteins during waterborne Cu-exposure in brown trout (*Salmo trutta*). *Chemosphere* **2006**, *65* (10), 1707−1714.
(12) Beaumont, M.; Butler, P.; Taylor, E. Exposure of brown trout *Salmo trutta* to a sublethal concentration of copper in soft acidic water: effects upon gas exchange and ammonia accumulation. *J. Exp. Biol.* **2003**, *206* (1), 153−162.
(13) Everall, N.; Macfarlane, N.; Sedgwick, R. The interactions of water hardness and pH with the acute toxicity of zinc to the brown trout, *Salmo trutta L. J. Fish Biol.* **2006**, *35* (1), 27−36.
(14) Hansen, B. H.; Romma, S.; Garmo, Ã. A.; Olsvik, P. A.; Andersen, R. A. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2006**, *143* (3), 263−274.
(15) Farag, A. d. M.; Stansbury, M. A.; Bergman, H. L.; Hogstrand, C.; MacConnell, E. The physiological impairment of free-ranging brown trout exposed to metals in the Clark Fork River, Montana. *Can. J. Fish. Aquat. Sci.* **1995**, *52* (9), 2038−2050.
(16) Kolde, R. *Pretty Heatmaps*, R package version 0.7.4, 2012.
(17) Zerbino, D. R.; Birney, E. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* **2008**, *18* (5), 821−829.
(18) Schulz, M. H.; Zerbino, D. R.; Vingron, M.; Birney, E. Oases: Robust de novo RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics* **2012**, *28* (8), 1086−1092.
(19) Li, B.; Dewey, C. N. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinf.* **2011**, *28*, 1086−1092.
(20) Grabherr, M. G.; Haas, B. J.; Yassour, M.; Levin, J. Z.; Thompson, D. A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **2011**, *29* (7), 644−652.
(21) Robinson, M. D.; McCarthy, D. J.; Smyth, G. K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010**, *26* (1), 139−140.
(22) Chen, H.; Boutros, P. C. VennDiagram: A package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinf.* **2011**, *24*, 801−803.
(23) Field, D.; Tiwari, B.; Booth, T.; Houten, S.; Swan, D.; Bertrand, N.; Thurston, M. Open software for biologists: from famine to feast. *Nat. Biotechnol.* **2006**, *24* (7), 801−804.
(24) Huang, D. W.; Sherman, B. T.; Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **2008**, *4* (1), 44−57.
(25) Uren-Webster, T. M.; Lewis, C.; Filby, A. L.; Paull, G. C.; Santos, E. M. Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. *Aquat. Toxicol.* **2010**, *99*, 360−369.
(26) Filby, A. L.; Tyler, C. R. Molecular characterization of estrogen receptors 1, 2a and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biol. Reprod.* **2005**, *73* (4), 648.
(27) Bury, N. R.; Walker, P. A.; Glover, C. N. Nutritive metal uptake in teleost fish. *J. Exp. Biol.* **2003**, *206* (1), 11−23.
(28) Wood, C. M., An introduction to metals in fish physiology and toxicology: Basic principles. In *Homeostasis and Toxicology of Essential Metals*; Wood, C. M., Farrel, A. P., Brauner, C. J., Eds.; Academic Press: London, 2012; pp 1−53.
(29) Olsvik, P. l. A.; Gundersen, P. l.; Andersen, R. A.; Zachariassen, K. E. Metal accumulation and metallothionein in brown trout, *Salmo trutta*, from two Norwegian rivers differently contaminated with Cd, Cu and Zn. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2001**, *128* (2), 189−201.
(30) Eastwood, S.; Couture, P. Seasonal variations in condition and liver metal concentrations of yellow perch (*Perca flavescens*) from a metal-contaminated environment. *Aquat. Toxicol.* **2002**, *58*, 43−56.
(31) Goto, D.; Wallace, W. G. Metal intracellular partitioning as a detoxification mechanism for mummichogs (*Fundulus heteroclitus*) living in metal-polluted salt marshes. *Mar. Environ. Res.* **2010**, *69* (3), 163−171.
(32) Chowdhury, M. J.; Baldisserotto, B.; Wood, C. M. Tissue-specific cadmium and metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary cadmium. *Arch. Environ. Contam. Toxicol.* **2005**, *48* (3), 381−390.
(33) Hogstrand, C.; Haux, C. Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **1991**, *100* (12), 137−141.
(34) Dang, F.; Zhong, H.; Wang, W.-X. Copper uptake kinetics and regulation in a marine fish after waterborne copper acclimation. *Aquat. Toxicol.* **2009**, *94* (3), 238−244.
(35) Craig, P. M.; Galus, M.; Wood, C. M.; McClelland, G. B. Dietary iron alters waterborne copper-induced gene expression in soft water acclimated zebrafish (*Danio rerio*). *Am. J. Physiol.: Regul., Integr. Comp. Physiol.* **2009**, *296* (2), 362−373.
(36) Minghetti, M.; Leaver, M. J.; Carpena, E.; George, S. G. Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2008**, *147* (4), 450−459.
(37) Minghetti, M.; Leaver, M. J.; George, S. G. Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*. *Aquat. Toxicol.* **2011**, *97* (1), 23−33.
(38) Zheng, D.; Feeney, G. P.; Kille, P.; Hogstrand, C. Regulation of ZIP and ZnT zinc transporters in zebrafish gill: zinc repression of ZIP10 transcription by an intronic MRE cluster. *Physiol. Genom.* **2008**, *34* (2), 205−214.
(39) Xie, L.; Klerks, P. L. Changes in cadmium accumulation as a mechanism for cadmium resistance in the least killifish Heterandria formosa. *Aquat. Toxicol.* **2004**, *66* (1), 73−81.
(40) Gale, S. A.; Smith, S. V.; Lim, R. P.; Jeffree, R. A.; Petocz, P. Insights into the mechanisms of copper tolerance of a population of black-banded rainbowfish (Melanotaenia nigrans) (Richardson) exposed to mine leachate, using 64/67Cu. *Aquat. Toxicol.* **2003**, *62* (2), 135−153.
(41) Craig, P. M.; Hogstrand, C.; Wood, C. M.; McClelland, G. B. Gene expression endpoints following chronic waterborne copper exposure in a genomic model organism, the zebrafish, *Danio rerio*. *Physiol. Genom.* **2009**, *40* (1), 23−33.
(42) Kwong, R. W. M.; Andres, J. A.; Niyogi, S. Molecular evidence and physiological characterization of iron absorption in isolated enterocytes of rainbow trout (*Oncorhynchus mykiss)*: Implications for dietary cadmium and lead absorption. *Aquat. Toxicol.* **2010**, *99* (3), 343−350.
(43) Kwong, R. W. M.; Andres, J. A.; Niyogi, S. Effects of dietary cadmium exposure on tissue-specific cadmium accumulation, iron status and expression of iron-handling and stress-inducible genes in rainbow trout: Influence of elevated dietary iron. *Aquat. Toxicol.* **2011**, *102* (12), 1−9.
(44) Hu, Y.-h.; Zheng, W.-j.; Sun, L. Identification and molecular analysis of a ferritin subunit from red drum (*Sciaenops ocellatus*). *Fish Shellfish Immunol.* **2010**, *28* (4), 678−686.
(45) Zhang, X.; Cornelis, R.; De Kimpe, J.; Mees, L.; Lameire, N. Study of arsenicprotein binding in serum of patients on continuous ambulatory peritoneal dialysis. *Clin. Chem.* **1998**, *44* (1), 141−147.

(46) Grosell, M., Copper. In *Homeostasis and Toxicology of Essential Metals*; Wood, C. M., Farrel, A. P., Brauner, C. J., Eds.; Academic Press: London, 2012; pp 54−135.
(47) Hogstrand, C., Zinc. In *Homeostasis and Toxicology of Essential Metals*; Wood, C. M., Farrel, A. P., Brauner, C. J., Eds.; Academic Press: London, 2012; pp 136−201.
(48) Mager, A. M., Lead. In *Homeostasis and Toxicology of Non-Essential Metals*; Wood, C. M., Farrel, A. P., Brauner, C. J., Eds.; Academic Press: London, 2012; pp 186−237.
(49) McGeer, J. C.; Niyogi, S.; Smith, D. S., Cadmium. In *Homeostasis and Toxicology of Non-Essential Metals*; Wood, C. M., Farrel, A. P., Brauner, C. J., Eds.; Academic Press: London, 2012; pp 126−185.
(50) Lauren, D. J.; McDonald, D. G. Acclimation to Copper by Rainbow Trout, *Salmo gairdneri*: Biochemistry. *Can. J. Fish. Aquat. Sci.* **1987**, *44* (1), 105−111.
(51) Lauren, D. J.; McDonald, D. G. Acclimation to Copper by Rainbow Trout, *Salmo gairdneri*: Physiology. *Can. J. Fish. Aquat. Sci.* **1987**, *44* (1), 99−104.
(52) Pelgrom, S. M. G. J.; Lock, R. A. C.; Balm, P. H. M.; Bonga, S. E. W. Integrated physiological response of tilapia, *Oreochromis mossambicus*, to sublethal copper exposure. *Aquat. Toxicol.* **1995**, *32* (4), 303−320.
(53) Valko, M.; Morris, H.; Cronin, M. T. D. Metals, toxicity and oxidative stress. *Curr. Med. Chem.* **2005**, *12* (10), 1161−1208.
(54) De Smet, H.; Blust, R. Stress responses and changes in protein metabolism in carp *Cyprinus carpio* during cadmium exposure. *Ecotoxicol. Environ. Saf.* **2001**, *48* (3), 255−262.
(55) Cain, D. J.; Luoma, S. N.; Wallace, W. G. Linking metal bioaccumulation of aquatic insects to their distribution patterns in a mining-impacted river. *Environ. Toxicol. Chem.* **2004**, *23* (6), 1463−1473.
(56) Audet, D.; Couture, P. Seasonal variations in tissue metabolic capacities of yellow perch (*Perca flavescens*) from clean and metal-contaminated environments. *Can. J. Fish. Aquat. Sci.* **2003**, *60* (3), 269−278.
(57) Zelikoff, J. T.; Bowser, D.; Squibb, K. S.; Frenkel, K. Immunotoxicity of low level cadmium exposure in fish: An alternative animal model for immunotoxicological studies. *J. Toxicol. Environ. Health* **1995**, *45* (3), 235−248.
(58) Nayak, A. S.; Lage, C. R.; Kim, C. H. Effects of low concentrations of arsenic on the innate immune system of the zebrafish (*Danio rerio*). *Toxicol. Sci.* **2007**, *98* (1), 118−124.
(59) Esteve, C.; Alcaide, E.; Urena, R. The effect of metals on condition and pathologies of European eel (*Anguilla anguilla*): In situ and laboratory experiments. *Aquat. Toxicol.* **2012**, *109* (0), 176−184.
(60) Reynders, H.; van der Ven, K.; Moens, L. N.; van Remortel, P.; De Coen, W. M.; Blust, R. Patterns of gene expression in carp liver after exposure to a mixture of waterborne and dietary cadmium using a custom-made microarray. *Aquat. Toxicol.* **2006**, *80* (2), 180−193.

Supporting Information

Global transcriptome profiling reveals molecular mechanisms of metal tolerance in a chronically exposed wild population of brown trout.

T. M. Uren Webster[1]*, N. Bury[2], R. van Aerle[1] and E. M. Santos[1]*

1. Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD
2. Diabetes & Nutritional Sciences Division, 3.83 Franklin-Wilkins Building, King's College London, London, SE1 9NH

* Corresponding authors

This Supporting Information contains:

Supplemental Experimental Section

Sample collection

Eggs and sperm were stripped from five female and two male brown trout obtained from a trout farm and mixed to facilitate fertilisation. Fertilised eggs were incubated at 8±1 °C on gravel beds in flow-through de-chlorinated tap water. Embryos were collected at 10 developmental stages identified according to [16], as follows: unfertilised eggs (0 days post fertilisation (dpf)), blastula (2 dpf), gastrula (6 dpf), early somitogenesis (10 dpf), late somitogenesis (14 dpf), early organogenesis (21 dpf), mid organogenesis (31 dpf), late organogenesis (41dpf), hatched alevins (51 dpf) and swim-up fry just prior to commencement of feeding (70 dpf). All embryos were snap frozen in liquid nitrogen then stored at -80 °C prior to RNA extraction.

For collection of adult tissues, five brown trout from the River Hayle at Relubbus in Cornwall (N 50° 8.476774' , W 5° 24.661446') and 10 brown trout from the control site, the relatively un-impacted River Teign at Gidleigh Park in Devon (N 50° 40.568816' , W 3° 52.407188') were caught by electric fishing on the 19th September 2010 and 11th October 2010 respectively. The fish were humanely killed with a lethal dose of benzocaine (0.5 g L^{-1}; Sigma-Aldrich) and individual tissues (gill, liver, heart, spleen, stomach, intestine, gonad, head kidney, trunk kidney, eye, brain, pituitary, muscle, skin and caudal fin) were dissected and transported on dry ice to the University of Exeter where they were stored at -80 °C prior to RNA extraction or analysis of metal content.

RNA extraction, cDNA Library preparation and sequencing

Total RNA was extracted from all individual wild fish tissues and from individual embryos using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. The isopropanol precipitation step was modified by addition of a high salt solution (0.8 M sodium citrate, 1.2 M NaCl) to remove proteoglycon and polysaccharide contamination [1] during the embryo extractions. The concentration and purity of the resulting RNA was assessed using absorbance measurements at 260 nm and by monitoring the 230/260 and 260/280 nm absorbance ratios, using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). The integrity of the RNA was further assessed by gel electrophoresis (1% agarose). Equal amounts of total RNA from five embryos were pooled for each developmental stage, before these were combined into a single embryonic sample for sequencing. For the adult fish, equal amounts of total RNA from individual fish tissues were pooled into 12 samples for sequencing to form the

following pools: gill, trunk kidney, liver and gut (consisting of stomach and intestine) from both Hayle and Teign fish; ovary and testis from Teign fish (from mature and maturing fish only); and mixed remaining tissues from the Hayle and from the Teign trout (Table S2). This strategy was adopted to allow for comparisons of transcript abundance between the Hayle and Teign fish for tissues hypothesised to be involved in metal tolerance (gill, gut, kidney and liver), and to maximise the likelihood of sequencing genes specific for each tissue. All RNA samples were treated with DNase and cleaned up on Qiagen RNeasy MinElute columns, then quality and concentration were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). All RNA input to library construction was of high quality with a RIN > 8. cDNA libraries were prepared from each RNA sample using the Illumina TruSeq RNA Sample Preparation kit, and according to the manufacturer's instructions. The single embryonic cDNA library was sequenced in one lane of the Illumina GAIIx Genome Analyzer generating 100 bp paired-end reads. All cDNA libraries constructed from the wild fish were multiplexed 12x and sequenced in another single lane, generating 76 bp paired-end reads. The average insert size of the multiplexed libraries was 153 bp, and of the embryonic library was 142 bp.

Bioinformatics

The FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) was used to clip remaining Illumina adapter sequences from the sequence reads and to trim the first 12 bp at the 5' end to remove bias caused by random hexamer priming [2]. Quality trimming of the 3' end of the reads using a sliding window at the first base with a quality Phred score of < 20 was performed (http://wiki.bioinformatics.ucdavis.edu/index.php/Trim.slidingWindow.pl) and reads shorter than 30 bp were discarded from the dataset. Paired reads were separated from orphan reads for each of the adult tissue and embryonic libraries, using the script from https://github.com/lexnederbragt/denovo-assembly-tutorial/blob/master/scripts/pair_up_reads.py. All 'forward' reads (read 1) and 'reverse' reads (read 2) of the adult tissue libraries were pooled into 2 separate fastq files and interleaved using the shuffleSequences_fastq.pl script provided by the Velvet package in preparation for assembly. Similarly, interleaved fastq files were created for the embryonic tissue library.

The interleaved paired and orphan sequences for adult tissues and embryos were assembled *de novo* using Velvet (version 1.2.08; [3]) and Oases (version 0.2.08; [4]). An

initial assembly was created using a k-mer of 73 and using the following parameters for Oases: ins_length 50 -ins_length_sd 200. Subsequently, assemblies were created using k-mers ranging from 65 to 41 (with steps of 8), such that the transcripts generated by the previous assembly were used as a –long input for the next assembly. The resulting transcripts of the final assembly (the brown trout transcriptome) were then annotated using Blast and all available Ensembl cDNA sequences for zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), nile tilapia (*Oreochromis niloticus*), stickleback (*Gasterosteus aculeatus*), human and mouse (Release 69; October 2012), (non-human) vertebrate RefSeq RNA and protein sequences and EST sequences (Database of 2012-11-09). In addition, transcripts were also annotated using the Blast service at the Bioportal, University of Oslo, using the non-redundant nucleotides (nt) and proteins (nr) databases [5]. The resulting blast outputs were parsed using the blast2table.pl script from ftp://ftp.genome.ou.edu/pub/programs/Blast2table keeping only the top hits with an e-value cut off < $1e^{-15}$. Annotations were assigned in the following preferential order: zebrafish, medaka, nile tilapia, stickleback, human, mouse (Ensembl cDNA), RefSeq vertebrates RNA, nt, RefSeq vertebrates proteins, and nr. When no annotation could be found, the transcript ID was given.

Gene expression was determined in the gill, gut, kidney and liver of fish inhabiting the metal-contaminated river Hayle and the reference river Teign using RSEM [6]. To reduce the redundancy of the dataset, accession numbers of the various annotations were used as gene ID and the transcript names generated by Oases were used as transcript IDs. Reads were mapped against the brown trout reference transcriptome (generated using the --no_polyA parameter) and using default settings. Subsequent analyses in RSEM were conducted using a selection of scripts provided as part of the Trinity assembly package (version r2012-10-05; [7], following the differential expression analysis pipeline described on http://trinityrnaseq.sourceforge.net/analysis/diff_expression_analysis.html. Statistical differences in gene expression levels between tissues of the 2 rivers were calculated using edgeR [8]. Genes were considered differentially expressed when FDR < 0.1 (Benjamini-Hochberg correction). Hierarchical clustering was performed on all differentially expressed genes (> 2-fold and FDR < 0.1) between Teign and Hayle brown trout using the analyze_diff_expr.pl script provided by Trinity [6]. Hierarchical trees were generated using the Euclidean distance metric and complete linkage clustering. A 4-way Venn diagram showing overlapping differentially-expressed genes was produced using VennDiagram [9] in R/Bioconductor.

All analyses were carried out on a local server running under the NEBC Bio-Linux 7 environment [10] unless stated otherwise.

RT-QPCR validation of gene expression profiles in Hayle and Teign fish

Validation of the quantification of gene expression in Teign and Hayle fish was conducted using real time quantitative PCR (RT-QPCR) for five transcripts (*mtb, gpx1b, cat, slc40a1* and *arpc3*), in gill, gut, kidney and liver samples from all individual fish. Primers for each target gene were designed with Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) using the transcript sequences assembled during this project. Specificity of primer sets throughout the range of detection was confirmed by the observation of single amplification products of the expected size and Tm, and optimised by performing a standard curve for each primer pair as described by Filby and Tyler [11]. Over the detection range, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.943- 2.134. The sequences, PCR product sizes, annealing temperatures and PCR efficiencies for each primer pair are shown in Table S3. cDNA was synthesised according to manufacturer's instructions from 2 µg of total RNA treated with RQ1 DNase (Promega, Southampton, UK), using random hexamers (MWG-Biotech) and M-MLV reverse transcriptase (Promega). cDNA was diluted (1:2) then RT-QPCR was performed using an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) with SYBR Green chemistry as described by Filby and Tyler [11]. A template-minus negative control was run in triplicate on each plate to verify the absence of cDNA contamination. Efficiency-corrected relative expression levels were determined by normalizing to the control gene, Actin-related protein 2/3 complex 3 (*arpc3*). To select an appropriate control gene, we examined our assembly to find transcripts where expression levels were high and consistent between Hayle and Teign fish for all tissues. A comparison between the expression ratios (Hayle/Teign) in the gill, gut, kidney and liver for *arpc3* and other candidate control genes is presented in Table S3a.

References

1. Chomczynski, P.; Mackey, K., Modification of the TRI Reagent procedure for isolation of RNA from polysaccharide -and proteoglycan - rich sources. *Biotechniques* **1995,** *19*, 942-945.
2. Hansen, K.D., Brenner, S.E., and Dudoit S. Biases in Illumina transcriptome sequencing caused by random hexamer priming. *Nucleic Acids Research* **2010**, *38* (12), e131.
3. Zerbino and Birney. Velvet: Algorithms for *de novo* short read assembly using de Bruijn graphs. *Genome Research* **2008**, *18*, 821-829.
4. Schulz, M.H., Zerbino, D.R., Vingron, M., and Birney, E. Oases: robust de novo RNA-seq assembly across the dynamic range of expression. *Bioinformatics* **2012**, *28*, 1086.
5. Kumar, S., Skjæveland, Å., Orr, RJS, Enger, P., Ruden, T., Mevik, B-H., Burki, F., Botnen, A., and Shalchian-Tabrizi, K. AIR: A batch-oriented web program package for construction of supermatrices ready for phylogenomic analyses. *BMC Bioinformatics* **2009**, *10*, 357.
6. Li, B. and Dewey, C.N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **2011**, *12*, 323.
7. Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **2011,** *29*, 644-652.
8. Robinson, M.D., McCarthy, D.J. and Smyth, G.K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010**, *26*, (1), 139–140.
9. Chen, H. and Boutros, P.C. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinformatics* **2011**, *12*, 35.
10. Field, D., Tiwari, B., Booth, T., Houten, S., Swan, D., Bertrand, N., Thurston, M. Open software for biologists: from famine to feast. *Nature Biotechnology* **2006**, *24*, 801-803.
11. Filby, A. L.; Tyler, C. R., Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). *Biology of reproduction* **2005,** *73*, (4), 648.
12. Huang, D. W.; Sherman, B. T.; Lempicki, R. A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **2008,** *4*, (1), 44-57.

Table S1 - Metal concentrations in the river Hayle and river Teign. Mean values, and range of values, are presented from data collected at monthly intervals by the Environment Agency monitoring programme throughout the year between 1990-1996 and 2010-2012 for the river Hayle, and from 1991-2012 (zinc only) and 1991-1995 (all other metals) for the river Teign. The data contained in this table was kindly provided by the UK Environment Agency.

	River Hayle (Relubbus)		River Teign (Gidleigh Park)	
	Total metal (µg/L)	Filtered metal (µg/L)	Total metal (µg/L)	Filtered metal (µg/L)
Zinc	638.9 (404-858)	599.2 (390-780)	10.4 (2-204)	4.7 (2-12)
Copper	42.3 (24-193)	34.9 (17-86.5)	<1 (<1)	-
Iron	199.2 (60-2690)	80.5 (34-210)	172.0 (50-760)	128.9 (60-280)
Arsenic	8.4 (1.1-101)	4.5 (0.4-8)	1.2 (0.6-5.4)	1.0 (0.5-2.4)
Cadmium	1.4 (0.9-4)	1.3 (0.9-1.6)	<0.2 (<0.2)	<0.2 (<0.2)
Nickel	27.1 (18-39)	25.8 (17.6-37.4)	1.0 (1)	1.3 (1-2)
Lead	<2 (<1-12)	<2 (<2)	<1 (<1)	<1 (<1)

Table S2 - Description of cDNA libraries sequenced. Mixed tissue samples from the Hayle and the Teign contained equal amounts of RNA from the heart, spleen, head kidney, eye, brain, pituitary, muscle, skin and caudal fin.

Sample no.	**Sample description**	**Lane no.**	**Read characteristics**
1	Embryonic (10 developmental stages pooled)	1	100 bp paired end
2	Teign Mixed Tissue	2	76 bp paired end (multiplexed)
3	Hayle Mixed Tissue	2	76 bp paired end (multiplexed)
4	Hayle Gill	2	76 bp paired end (multiplexed)
5	Hayle Gut	2	76 bp paired end (multiplexed)
6	Hayle Kidney	2	76 bp paired end (multiplexed)
7	Hayle Liver	2	76 bp paired end (multiplexed)
8	Teign Gill	2	76 bp paired end (multiplexed)
9	Teign Gut	2	76 bp paired end (multiplexed)
10	Teign Kidney	2	76 bp paired end (multiplexed)
11	Teign Liver	2	76 bp paired end (multiplexed)
12	Teign Ovary	2	76 bp paired end (multiplexed)
13	Teign Testis	2	76 bp paired end (multiplexed)

Table S3a – Fold changes in expression level quantified by RNA-seq of potential control genes for RT-QPCR analysis.

Gene		Fold change (Hayle/Teign) in expression			
		Gill	Gut	Kidney	Liver
Actin-related protein 2/3 complex 3	*arpc3*	1.25	1.17	0.97	1.10
Ribosomal protein L8	*rpl8*	0.92	0.61	0.49	0.77
Ribosomal protein L7	*rpl7*	1.25	1.27	0.75	2.04
Beta Actin	*bactin*	1.25	1.18	0.72	1.41
Glucose-6-phosphate dehydrogenase	*g6pdh*	1.67	1.53	0.75	1.49
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	0.58	0.95	1.75	0.61

Table S3b – Target genes, primers and assay details for RT-QPCR analysis.

Target Gene		Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR efficiency
Actin-related protein 2/3 complex 3	*arpc3*	CCAGCAACAAGCAGGAAGAC	ACGGTCACACAGCCTCAG	83	58.5	96.2 %
Ferroportin	*slc401a*	GGCACATAGAGCACAGGTTC	GACAGGACAGCAGCAAGC	162	58.5	113.4 %
Metallothionein b	*mtb*	ACCAGTTGTGAAAGCAAG	GTCAGTCATAGGGAATGG	155	55.0	109.9 %
Glutathione peroxidise 1b	*gpx1b*	GCCAAGCACATTTCCCAAAG	GAGAGCCATTCAAGCGTTATG	200	55.0	94.3 %
Catalase	*cat*	CGGCTCTCACACCTTCAAG	GTCTCGGATGGCGTAGTC	148	57.0	102.9 %

Figure S1: Cluster diagrams displaying the concentration of each metal in individual fish from the river Hayle (h1-h5) and river Teign (t1-t10), illustrating the similarity of distribution profiles of metals in each tissue. Values given are log transformed metal concentrations (µg/g).

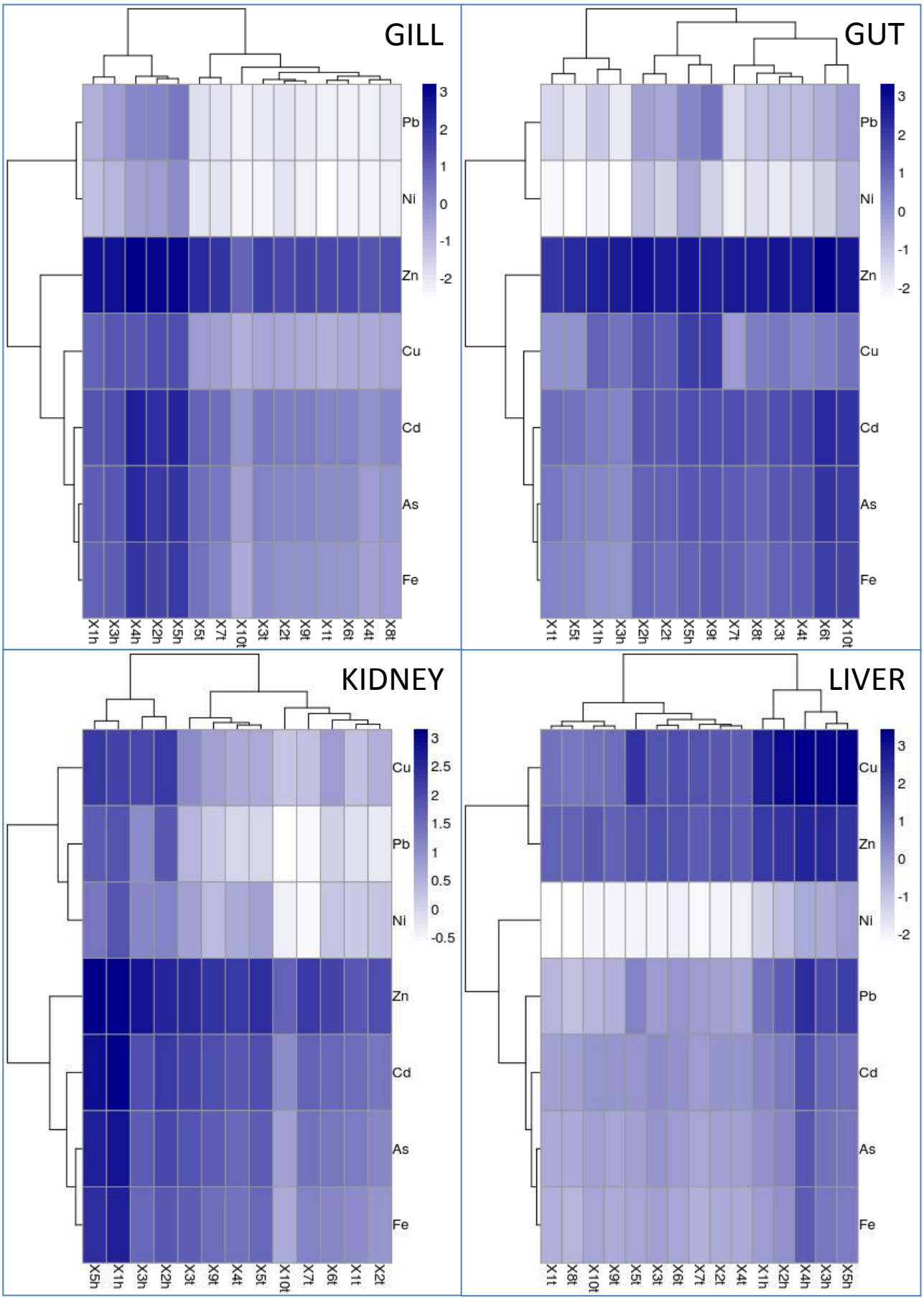


Table S4 - Summary statistics of raw sequencing reads, numbers of reads retained after adaptor removal and quality filtering and retained for input into transcriptome assembly as either paired reads or orphans. 1 and 2 refer to the forward and reverse reads in each paired-end sequence read.

	No. raw reads		Adaptors removed		Quality Filtered		No. Paired reads		No. orphans	
Sample	**Read 1**	**Read 2**	**Read 1**	**Read 2**	**Read 1**	**Read 2**	**Read 1**	**Read 2**	**Read 1**	**Read 2**
Hayle Gill	2,936,116	2,936,116	2,680,643	2,683,739	2,455,767	2,520,215	2,156,170	2,156,170	299,597	364,045
Hayle Gut	3,198,880	3,198,880	2,912,539	2,916,540	2,659,209	2,724,901	2,321,001	2,321,001	338,208	403,900
Hayle Kidney	3,587,826	3,587,826	3,272,885	3,276,952	2,996,962	3,090,315	2,637,910	2,637,910	359,052	452,405
Hayle Liver	3,648,593	3,648,593	3,329,308	3,334,596	3,043,407	3,124,718	2,669,069	2,669,069	374,338	455,649
Hayle Mixed Tissue	2,541,870	2,541,870	2,303,275	2,305,787	2,099,076	2,156,734	1,825,364	1,825,364	273,712	331,370
Teign Gill	3,489,607	3,489,607	3,186,023	3,189,248	2,912,245	2,993,566	2,555,857	2,555,857	356,388	437,709
Teign Gut	3,418,757	3,418,757	3,115,427	3,118,565	2,850,298	2,930,476	2,500,343	2,500,343	349,955	430,133
Teign Kidney	3,147,358	3,147,358	2,875,181	2,877,401	2,637,264	2,710,844	2,321,788	2,321,788	315,476	389,056
Teign Liver	3,855,726	3,855,726	3,523,064	3,527,418	3,215,926	3,300,338	2,818,972	2,818,972	396,954	481,366
Teign Ovary	2,591,346	2,591,346	2,364,740	2,368,163	2,166,788	2,198,275	1,886,219	1,886,219	280,569	312,056
Teign Testis	3,540,738	3,540,738	3,228,934	3,235,082	2,942,162	3,028,973	2,577,349	2,577,349	364,813	451,624
Teign Mixed Tissue	3,415,701	3,415,701	3,099,156	3,106,756	2,820,832	2,907,199	2,460,718	2,460,718	360,114	446,481
Total Adult Tissues	**39,372,518**	**39,372,518**	**35,891,175**	**35,940,247**	**32,799,936**	**33,686,554**	**28,730,760**	**28,730,760**	**4,069,176**	**4,955,794**
Embryonic	34,411,228	34,411,228	30,970,124	29,448,455	30,829,882	29,311,104	27,162,593	27,162,593	3,667,289	2,148,511
TOTAL	**73,783,746**	**73,783,746**	**66,861,299**	**65,388,702**	**63,629,818**	**62,997,658**	**55,893,353**	**55,893,353**	**7,736,465**	**7,104,305**

Table S5 - Number of transcripts in the final transcriptome assembly and relative expression levels for each tissue. Expression is presented as Fragments Per Kilobase of Exon Per Million Fragments Mapped (FPKM).

	No. transcripts							
Expression level (FPKM)	**H Gill**	**T Gill**	**H Gut**	**T Gut**	**H Kidney**	**T Kidney**	**H Liver**	**T Liver**
≤ 1	1194	1472	2825	2626	1874	1314	5348	4893
1-10	30608	33783	27904	29047	34121	28273	24208	23809
10-100	13587	13758	8790	8992	12834	13911	4431	4306
100-1000	1094	1068	953	978	1039	1122	603	656
> 1000	117	116	86	110	96	155	102	130
Total transcripts expressed	**46600**	**50197**	**40558**	**41753**	**49964**	**44775**	**34693**	**33794**
Transcripts not expressed	**50083**	**46486**	**56125**	**54930**	**46719**	**51908**	**61991**	**62889**

Table S6 - Comparison between the fold differences in expression levels for selected transcripts generated based on the global analysis (RNA-Seq) and on the individual gene quantification (RT-QPCR). Values presented are mean expression of transcripts for the Hayle population relative to Teign (Hayle; n=5, Teign; n=10). Fold differences in expression measured using RNA-Seq are in blue, and those obtained by RT-QPCR are in red. Asterisks indicate a significant difference in expression between the two populations.

Gene	Gill	Gut	Kidney	Liver
slc40a1	1.2 0.7	1.7 0.8	4.3 0.5	1.2 1.3
mtb	8.2 * 9.8 *	7.7 * 5.7 *	2.2 7.1	5.6 * 14.7 *
gpx1b	2.5 0.8	1.3 9.1	0.48 0.8	1.6 1.5
cat	1.1 0.2	1.1 1.2	0.4 1.2	0.9 0.2

Figure S2- Heatmaps illustrating changes in gene expression for all differentially expressed genes (> 2-fold and FDR <0.1) between Teign and Hayle brown trout, in the four separate tissues. Hierarchical trees were generated using the Euclidean distance metric and complete linkage clustering.

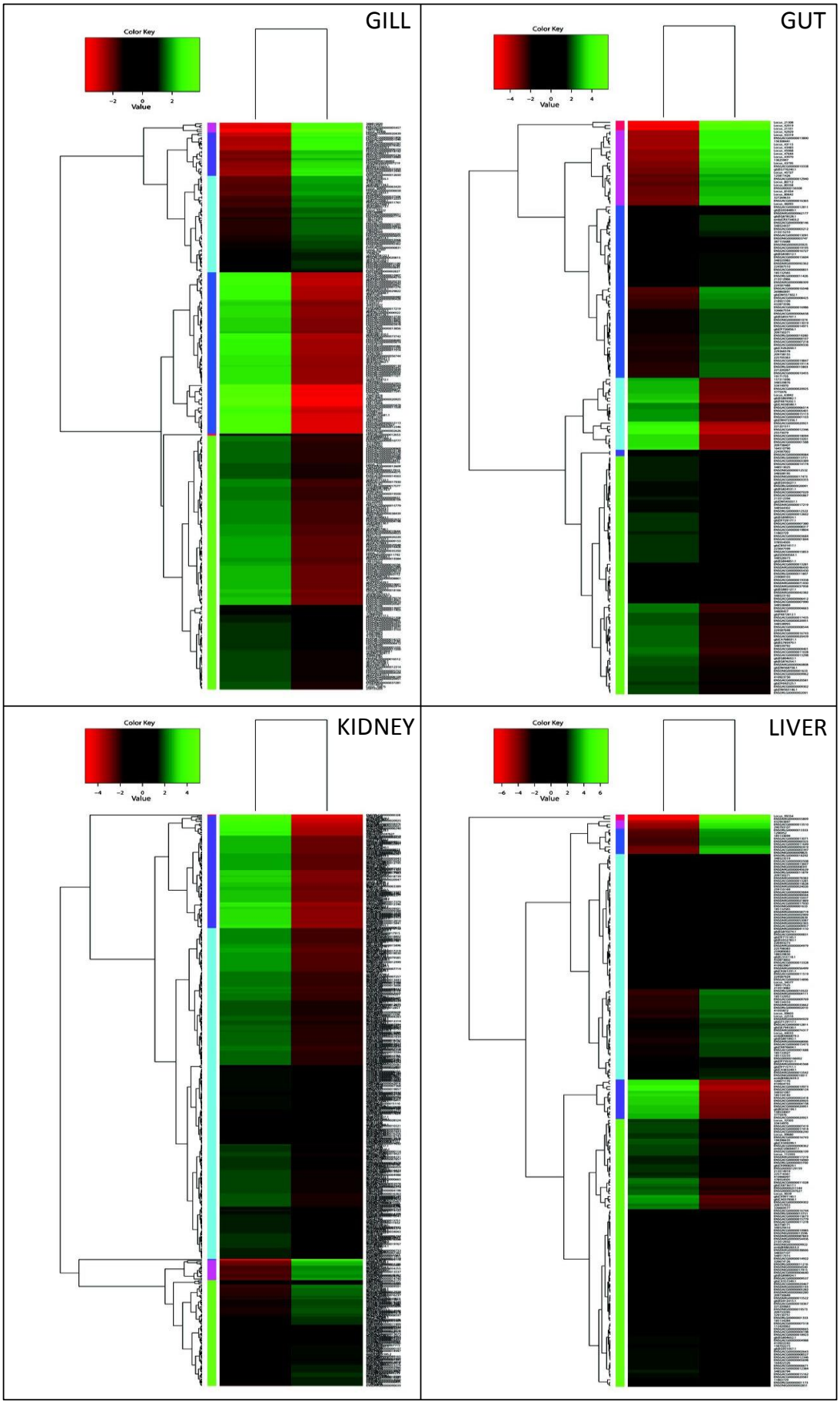


Table S7- Gene Ontology Terms over-represented in the lists of differentially expressed genes between Hayle and Teign fish for each tissue. Values presented are the number and percentage of genes in the tissue-specific gene lists associated with each term and the P-values associated with this over-representation. This analysis was conducted using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.7; [12]), using our whole brown trout transcriptome assembly as a background.

GUT								
Category	Term	Count	%	P-Value	Bonferroni	Benjamini	FDR	Fisher Exact
GOTERM_BP_FAT	Proteolysis	11	7.10	8.90E-06	7.50E-04	7.50E-04	9.60E-03	1.60E-06
GOTERM_BP_FAT	platelet activation	2	1.30	2.00E-02	8.10E-01	5.70E-01	1.90E+01	1.60E-04
GOTERM_BP_FAT	cell activation	2	1.3	5.80E-02	9.90E-01	8.10E-01	4.70E+01	1.70E-03
GOTERM_BP_FAT	Coagulation	2	1.3	6.50E-02	1.00E+00	7.60E-01	5.20E+01	2.10E-03
GOTERM_BP_FAT	Hemostasis	2	1.3	6.50E-02	1.00E+00	7.60E-01	5.20E+01	2.10E-03
GOTERM_BP_FAT	regulation of body fluid levels	2	1.3	6.50E-02	1.00E+00	7.60E-01	5.20E+01	2.10E-03
GOTERM_BP_FAT	blood coagulation	2	1.3	6.50E-02	1.00E+00	7.60E-01	5.20E+01	2.10E-03
GOTERM_BP_FAT	protein polymerization	2	1.3	9.50E-02	1.00E+00	8.10E-01	6.60E+01	4.60E-03
GOTERM_CC_FAT	extracellular space	5	3.2	1.30E-05	3.70E-04	3.70E-04	1.10E-02	3.80E-07
GOTERM_CC_FAT	extracellular region	8	5.2	1.20E-04	3.50E-03	1.70E-03	1.00E-01	2.00E-05
GOTERM_CC_FAT	extracellular region part	5	3.2	1.10E-03	3.00E-02	1.00E-02	8.90E-01	1.00E-04
GOTERM_CC_FAT	fibrinogen complex	2	1.3	8.20E-03	2.10E-01	5.80E-02	6.70E+00	1.80E-05
GOTERM_MF_FAT	serine hydrolase activity	8	5.2	4.30E-07	3.30E-05	3.30E-05	4.60E-04	2.50E-08
GOTERM_MF_FAT	serine-type peptidase activity	8	5.2	4.30E-07	3.30E-05	3.30E-05	4.60E-04	2.50E-08
GOTERM_MF_FAT	serine-type endopeptidase activity	7	4.5	1.90E-06	1.50E-04	7.40E-05	2.00E-03	1.00E-07
GOTERM_MF_FAT	peptidase activity, acting on L-amino acid peptides	11	7.1	8.10E-06	6.30E-04	2.10E-04	8.60E-03	1.30E-06
GOTERM_MF_FAT	peptidase activity	11	7.1	1.80E-05	1.40E-03	3.40E-04	1.90E-02	3.10E-06
GOTERM_MF_FAT	endopeptidase activity	8	5.2	1.10E-04	8.50E-03	1.70E-03	1.20E-01	1.50E-05
GOTERM_MF_FAT	carboxypeptidase activity	3	1.9	5.00E-03	3.20E-01	6.20E-02	5.20E+00	1.70E-04
GOTERM_MF_FAT	protein binding, bridging	2	1.3	2.40E-02	8.50E-01	2.40E-01	2.30E+01	2.40E-04
GOTERM_MF_FAT	exopeptidase activity	3	1.9	3.80E-02	9.50E-01	3.10E-01	3.40E+01	3.90E-03
GOTERM_MF_FAT	metallocarboxypeptidase activity	2	1.3	5.20E-02	9.80E-01	3.70E-01	4.30E+01	1.30E-03

KIDNEY								
Category	Term	Count	%	P-Value	Bonferroni	Benjamini	FDR	Fisher Exact
GOTERM_BP_ALL	response to organic substance	4	3.3	3.50E-04	8.20E-02	8.20E-02	4.50E-01	1.20E-05
GOTERM_BP_ALL	response to estradiol stimulus	2	1.6	1.10E-02	9.30E-01	7.40E-01	1.30E+01	4.20E-05
GOTERM_BP_ALL	homeostatic process	4	3.3	1.70E-02	9.90E-01	7.60E-01	2.00E+01	2.30E-03
GOTERM_BP_ALL	response to estrogen stimulus	2	1.6	1.80E-02	9.90E-01	6.80E-01	2.10E+01	1.40E-04
GOTERM_BP_ALL	chemical homeostasis	3	2.4	2.20E-02	1.00E+00	6.60E-01	2.50E+01	1.60E-03
GOTERM_BP_ALL	response to hexose	2	1.6	2.20E-02	1.00E+00	5.90E-01	2.50E+01	2.10E-04

	stimulus							
GOTERM_BP_ALL	response to carbohydrate stimulus	2	1.6	2.20E-02	1.00E+00	5.90E-01	2.50E+01	2.10E-04
GOTERM_BP_ALL	response to steroid hormone stimulus	2	1.6	2.20E-02	1.00E+00	5.90E-01	2.50E+01	2.10E-04
GOTERM_BP_ALL	response to glucose stimulus	2	1.6	2.20E-02	1.00E+00	5.90E-01	2.50E+01	2.10E-04
GOTERM_BP_ALL	response to monosaccharide stimulus	2	1.6	2.20E-02	1.00E+00	5.90E-01	2.50E+01	2.10E-04
GOTERM_BP_ALL	regulation of transcription, DNA-dependent	10	8.1	2.60E-02	1.00E+00	6.00E-01	2.90E+01	1.10E-02
GOTERM_BP_ALL	regulation of RNA metabolic process	10	8.1	2.70E-02	1.00E+00	5.60E-01	3.00E+01	1.20E-02
GOTERM_BP_ALL	regulation of macromolecule biosynthetic process	11	8.9	3.80E-02	1.00E+00	6.50E-01	3.90E+01	1.90E-02
GOTERM_BP_ALL	regulation of biosynthetic process	11	8.9	3.90E-02	1.00E+00	6.20E-01	4.00E+01	1.90E-02
GOTERM_BP_ALL	regulation of cellular biosynthetic process	11	8.9	3.90E-02	1.00E+00	6.20E-01	4.00E+01	1.90E-02
GOTERM_BP_ALL	response to chemical stimulus	4	3.3	3.90E-02	1.00E+00	5.80E-01	4.00E+01	7.10E-03
GOTERM_BP_ALL	response to hormone stimulus	2	1.6	4.00E-02	1.00E+00	5.60E-01	4.10E+01	7.50E-04
GOTERM_BP_ALL	glucose metabolic process	3	2.4	4.20E-02	1.00E+00	5.50E-01	4.30E+01	4.50E-03
GOTERM_BP_ALL	carboxylic acid metabolic process	5	4.1	4.20E-02	1.00E+00	5.30E-01	4.30E+01	1.10E-02
GOTERM_BP_ALL	oxoacid metabolic process	5	4.1	4.20E-02	1.00E+00	5.30E-01	4.30E+01	1.10E-02
GOTERM_BP_ALL	organic acid metabolic process	5	4.1	4.40E-02	1.00E+00	5.20E-01	4.40E+01	1.20E-02
GOTERM_BP_ALL	cellular ketone metabolic process	5	4.1	4.50E-02	1.00E+00	5.10E-01	4.50E+01	1.20E-02
GOTERM_BP_ALL	regulation of cellular metabolic process	11	8.9	4.60E-02	1.00E+00	5.00E-01	4.60E+01	2.40E-02
GOTERM_BP_ALL	response to endogenous stimulus	2	1.6	4.70E-02	1.00E+00	4.80E-01	4.60E+01	1.10E-03
GOTERM_MF_FAT	sequence-specific DNA binding	10	8.1	3.50E-04	3.10E-02	3.10E-02	3.80E-01	8.20E-05
GOTERM_MF_FAT	transcription factor activity	10	8.1	7.10E-03	4.70E-01	2.80E-01	7.50E+00	2.50E-03
GOTERM_MF_FAT	hexokinase activity	2	1.6	1.90E-02	8.20E-01	4.30E-01	1.90E+01	1.50E-04
GOTERM_MF_FAT	transcription regulator activity	10	8.1	3.60E-02	9.60E-01	5.60E-01	3.30E+01	1.60E-02

LIVER								
Category	Term	Count	%	P-Value	Bonferroni	Benjamini	FDR	Fisher Exact
GOTERM_BP_FAT	response to organic substance	4	3.3	4.80E-04	7.70E-02	7.70E-02	5.80E-01	1.80E-05
GOTERM_BP_FAT	response to estradiol stimulus	2	1.6	1.20E-02	8.70E-01	6.40E-01	1.40E+01	5.20E-05
GOTERM_BP_FAT	response to estrogen stimulus	2	1.6	2.00E-02	9.70E-01	6.80E-01	2.20E+01	1.70E-04
GOTERM_BP_FAT	homeostatic process	4	3.3	2.30E-02	9.80E-01	6.20E-01	2.50E+01	3.50E-03
GOTERM_BP_FAT	response to hexose stimulus	2	1.6	2.40E-02	9.80E-01	5.60E-01	2.60E+01	2.60E-04
GOTERM_BP_FAT	response to steroid hormone stimulus	2	1.6	2.40E-02	9.80E-01	5.60E-01	2.60E+01	2.60E-04
GOTERM_BP_FAT	response to glucose stimulus	2	1.6	2.40E-02	9.80E-01	5.60E-01	2.60E+01	2.60E-04
GOTERM_BP_FAT	response to monosaccharide stimulus	2	1.6	2.40E-02	9.80E-01	5.60E-01	2.60E+01	2.60E-04
GOTERM_BP_FAT	response to carbohydrate stimulus	2	1.6	2.40E-02	9.80E-01	5.60E-01	2.60E+01	2.60E-04

GOTERM_BP_FAT	chemical homeostasis	3	2.4	2.60E-02	9.90E-01	5.30E-01	2.80E+01	2.20E-03
GOTERM_BP_FAT	response to hormone stimulus	2	1.6	4.40E-02	1.00E+00	6.60E-01	4.20E+01	9.40E-04
GOTERM_BP_FAT	regulation of transcription, DNA-dependent	10	8.1	4.80E-02	1.00E+00	6.40E-01	4.50E+01	2.30E-02
GOTERM_BP_FAT	regulation of RNA metabolic process	10	8.1	4.90E-02	1.00E+00	6.10E-01	4.60E+01	2.40E-02
GOTERM_BP_FAT	glucose metabolic process	3	2.4	5.10E-02	1.00E+00	5.80E-01	4.70E+01	6.10E-03
GOTERM_BP_FAT	response to endogenous stimulus	2	1.6	5.20E-02	1.00E+00	5.60E-01	4.80E+01	1.30E-03
GOTERM_BP_FAT	carboxylic acid biosynthetic process	3	2.4	7.20E-02	1.00E+00	6.50E-01	6.00E+01	1.00E-02
GOTERM_BP_FAT	organic acid biosynthetic process	3	2.4	7.30E-02	1.00E+00	6.20E-01	6.00E+01	1.10E-02
GOTERM_BP_FAT	hexose metabolic process	3	2.4	7.90E-02	1.00E+00	6.30E-01	6.30E+01	1.20E-02
GOTERM_BP_FAT	monosaccharide metabolic process	3	2.4	9.50E-02	1.00E+00	6.70E-01	7.00E+01	1.60E-02
GOTERM_MF_FAT	sequence-specific DNA binding	10	8.1	3.50E-04	3.10E-02	3.10E-02	3.80E-01	8.20E-05
GOTERM_MF_FAT	transcription factor activity	10	8.1	7.10E-03	4.70E-01	2.80E-01	7.50E+00	2.50E-03
GOTERM_MF_FAT	hexokinase activity	2	1.6	1.90E-02	8.20E-01	4.30E-01	1.90E+01	1.50E-04
GOTERM_MF_FAT	transcription regulator activity	10	8.1	3.60E-02	9.60E-01	5.60E-01	3.30E+01	1.60E-02
GOTERM_MF_FAT	carbohydrate kinase activity	2	1.6	7.30E-02	1.00E+00	7.50E-01	5.60E+01	2.70E-03
GOTERM_MF_FAT	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	2	1.6	9.40E-02	1.00E+00	7.70E-01	6.60E+01	4.60E-03
GOTERM_MF_FAT	NADH dehydrogenase (quinone) activity	2	1.6	9.40E-02	1.00E+00	7.70E-01	6.60E+01	4.60E-03
GOTERM_MF_FAT	NADH dehydrogenase (ubiquinone) activity	2	1.6	9.40E-02	1.00E+00	7.70E-01	6.60E+01	4.60E-03

Table S8- List of differentially expressed genes generated using EdgeR including FDR values <0.1 and fold changes (FC) for each tissue. Significantly up-regulated genes in the Hayle fish compared to the Teign fish are highlighted in red and significantly down-regulated genes are highlighted in green. Where no expression was calculated for a transcript in one sample, direction of change is indicated by 'up' or 'down'.

			GILL		GUT		KIDNEY		LIVER	
GeneID	Symbol/ Accession	Database	FDR	FC	FDR	FC	FDR	FC	FDR	FC
ENSGACG00000003077	*abhd12b (2 of 2)*	Ensembl_stickleback	-	-1.4	-	1.5	4.41E-02	6.4	-	-1.1
ENSGACG00000016406	*abi1 (1 of 2)*	Ensembl_stickleback	-	0.0	4.41E-02	down	-	-1.4	-	down
ENSGACG00000020048	*ascl1*	Ensembl_stickleback	-	-1.7	1.77E-03	down	-	down	-	down
ENSGACG00000007368	*arpc5*	Ensembl_stickleback	-	1.1	-	-1.8	8.67E-02	-6.9	-	down
ENSGACG00000012675	*actr10*	Ensembl_stickleback	-	up	-	down	5.65E-02	down	-	down
ENSGACG00000014650	*acot11 (1 of 2)*	Ensembl_stickleback	-	-1.7	-	up	3.02E-02	up	-	-1.4
ENSGACG00000006135	*adam10*	Ensembl_stickleback	-	4.0	-	-1.5	2.72E-02	-7.7	-	1.0
ENSGACG00000015076	*adamts1*	Ensembl_stickleback	-	-1.5	-	-5.2	4.76E-02	9.9	-	2.2
ENSORLG00000006708	*ak1*	Ensembl_medaka	-	1.8	-	-8.3	3.11E-02	-24.3	-	up
ENSDARG00000042382	*arf4 (2 of 3)*	Ensembl_zebrafish	4.69E-02	down	-	down	-	-1.6	-	up
5453305	AF141606.1	nt	-	up	-	2.9	7.14E-03	-11.2	-	2.8
14581944	AF256852.1	nt	-	-1.1	-	-1.2	3.02E-02	18.1	-	-1.1
13625997	AAK35224.1	nr	1.17E-06	up	-	0.0	-	0.0	-	0.0
ENSORLG00000017577	*aqps*	Ensembl_medaka	-	-2.2	1.27E-03	down	-	-2.4	-	up
ENSGACG00000013328	*alyref*	Ensembl_stickleback	-	1.4	-	1.7	-	1.6	1.59E-02	10.5
AM402664	emb|AM402664.1	EST_others	-	1.0	3.54E-02	-6.9	-	0.0	-	-1.4
ENSGACG00000000493	*npepl1*	Ensembl_stickleback	-	1.7	-	2.3	5.65E-02	down	-	0.0
ENSDARG00000013856	*amy2a*	Ensembl_zebrafish	-	0.0	2.58E-06	-24.0	-	0.0	-	-2.8
ENSGACG00000013115	*aplp2*	Ensembl_stickleback	-	down	-	down	6.99E-02	down	-	down
ENSGACG00000018367	*anxa6*	Ensembl_stickleback	-	0.0	-	0.0	2.51E-04	down	1.57E-03	down
ENSDARG00000070480	*agr2*	Ensembl_zebrafish	-	3.3	-	-1.1	9.43E-02	5.5	-	2.8
ENSDARG00000053279	*apln*	Ensembl_zebrafish	-	-2.5	-	0.0	8.78E-02	17.3	-	1.1
ENSORLG00000012653	*apob (6 of 6)*	Ensembl_medaka	-	-1.3	8.77E-02	-12.3	-	-5.7	-	-1.7
ENSGACG00000012729	*apoo (2 of 2)*	Ensembl_stickleback	-	1.1	-	-1.4	3.22E-03	-8.7	-	1.8
ENSORLG00000017624	*aqp1*	Ensembl_medaka	-	-1.8	-	down	8.79E-02	-5.0	-	-1.1
ENSDARG00000007086	*aqp10a*	Ensembl_zebrafish	-	up	3.50E-05	-24.9	-	2.2	-	1.2
ENSGACG00000012346	*arg2*	Ensembl_stickleback	7.49E-12	down	6.21E-08	down	3.52E-10	-145.8	1.48E-02	down
388815820	JQ764761.1	nt	-	0.0	4.98E-05	up	-	0.0	-	0.0
188529830	EU541926.1	nt	-	up	3.77E-12	307.9	-	up	5.87E-02	24.0
ENSGACG00000002986	*armc1 (1 of 2)*	Ensembl_stickleback	-	down	-	4.8	3.02E-02	up	-	1.9
ENSGACG00000019091	*asna1*	Ensembl_stickleback	-	-145.0	-	1.4	4.67E-03	down	-	3.8
ENSGACG00000007981	*aspg*	Ensembl_stickleback	-	down	-	-3.0	6.99E-02	down	-	down
ENSGACG00000007419	*abcc2*	Ensembl_stickleback	-	0.0	-	down	-	down	5.91E-02	down
66471773	AJ971743.1	nt	-	down	5.76E-04	-13.8	-	-1.4	-	0.0
ENSGACG00000015604	*bcl7a*	Ensembl_stickleback	3.75E-02	up	-	-2.1	-	-1.5	-	-1.1
ENSGACG00000004283	*bcl2l1 (1 of 2)*	Ensembl_stickleback	-	up	-	down	1.22E-03	down	-	1.2
ENSGACG00000001688	*bmper*	Ensembl_stickleback	-	up	-	-2.1	-	down	1.57E-03	up
ENSGACG00000012929	*bre*	Ensembl_stickleback	-	0.0	-	0.0	5.65E-02	up	-	0.0
ENSDARG00000045568	*bcat1*	Ensembl_zebrafish	-	1.6	-	up	-	1.5	1.46E-04	17.6
ENSORLG00000015803	*brox*	Ensembl_medaka	3.35E-03	up	-	-1.2	-	-2.9	-	2.2
ENSGACG00000004158	*baz1a*	Ensembl_stickleback	-	1.2	-	-1.2	-	2.4	3.37E-15	-318.8
ENSGACG00000005734	*baz2b (4 of 4)*	Ensembl_stickleback	-	down	-	0.0	6.04E-02	-13.7	-	0.0
ENSGACG00000010106	*brd4*	Ensembl_stickleback	-	-4.3	-	-3.4	1.09E-03	-24.1	-	-2.8
213514654	NP_001134905.1	Refseq_proteins	-	1.5	-	1.4	9.82E-02	5.2	-	-1.7
ENSGACG00000005753	*celsr3*	Ensembl_stickleback	-	-2.2	9.39E-03	down	6.99E-02	up	-	2.5
ENSGACG00000012602	*cabp1 (2 of 2)*	Ensembl_stickleback	7.32E-02	down	-	1.7	-	-1.5	-	-1.6
ENSDARG00000019990	*cabp1a*	Ensembl_zebrafish	-	2.4	-	1.1	8.79E-02	up	-	2.1
ENSORLG00000010644	*cald1 (1 of 2)*	Ensembl_medaka	-	-4.2	1.77E-03	-14.4	-	2.9	-	0.0
ENSGACG00000012384	*camta1 (1 of 2)*	Ensembl_stickleback	-	1.0	-	-2.0	-	-2.4	2.28E-03	-46.3
ENSGACG00000016957	*calml4*	Ensembl_stickleback	-	down	-	1.5	5.64E-02	-8.0	-	0.0
ENSGACG00000013281	*capn5 (2 of 2)*	Ensembl_stickleback	9.28E-02	down	-	up	-	0.0	1.48E-02	up
ENSGACG00000006412	*cnn1*	Ensembl_stickleback	6.54E-03	down	7.64E-03	down	1.42E-03	down	-	0.0
ENSDARG00000093937	*cnn1b*	Ensembl_zebrafish	-	-1.7	5.21E-02	-5.3	-	-1.3	-	1.4
ENSGACG00000014122	*casq1 (2 of 2)*	Ensembl_stickleback	-	-1.1	-	1.3	5.65E-02	down	-	up
ENSDARG00000054456	*clip3*	Ensembl_zebrafish	-	down	-	2.1	-	-1.3	7.77E-02	-6.4
ENSDARG00000056499	*ca6*	Ensembl_zebrafish	-	1.5	-	-2.1	-	-1.1	4.66E-03	37.9
ENSORLG00000014439	*cel (2 of 3)*	Ensembl_medaka	-	0.0	4.56E-06	-33.0	-	0.0	-	0.0
ENSDARG00000029822	*cel.2*	Ensembl_zebrafish	-	-2.5	4.50E-08	-49.2	-	-1.7	-	6.6
ENSDARG00000021339	*cpa5*	Ensembl_zebrafish	-	1.5	1.11E-07	-35.6	-	1.6	-	down
ENSDARG00000045442	*cpb1*	Ensembl_zebrafish	-	0.0	1.39E-07	-34.7	-	0.0	-	-1.1
ENSGACG00000015162	*cpb2*	Ensembl_stickleback	-	-2.7	-	1.6	-	1.8	2.26E-03	-16.4
ENSORLG00000010823	*ctsc*	Ensembl_medaka	-	up	-	-1.3	5.66E-03	up	-	-1.9
CX349299	gb|CX349299.1	EST_others	-	0.0	-	0.0	-	0.0	3.22E-03	down
ENSDARG00000013628	*cd164*	Ensembl_zebrafish	-	1.0	-	1.8	4.20E-02	6.2	7.20E-02	6.5
ENSGACG00000017930	*cisd2*	Ensembl_stickleback	-	2.9	2.45E-03	down	-	2.5	3.74E-02	up
ENSGACG00000004663	*cdca4*	Ensembl_stickleback	9.28E-02	down	-	2.1	-	1.6	-	1.0
ENSGACG00000006495	*cdca7l*	Ensembl_stickleback	-	15.5	-	up	1.59E-02	up	-	0.0
ENSGACG00000009388	*cep290*	Ensembl_stickleback	-	0.0	-	0.0	1.96E-02	down	-	0.0
ENSDARG00000002365	*cers5*	Ensembl_zebrafish	-	1.3	-	1.3	-	-1.2	9.95E-02	up
ENSGACG00000002643	*cct5*	Ensembl_stickleback	-	1.1	-	0.0	-	0.0	8.58E-03	down
ENSGACG00000019847	*cct7*	Ensembl_stickleback	3.85E-03	up	-	down	-	-1.0	-	up
ENSGACG00000018739	*cxcl12 (1 of 2)*	Ensembl_stickleback	-	1.3	-	down	1.43E-09	-65.8	-	2.2
ENSDARG00000055100	*cxcl12b*	Ensembl_zebrafish	-	-1.2	-	1.4	8.09E-06	-20.4	-	1.1
ENSGACG00000008146	*chn2*	Ensembl_stickleback	5.86E-02	up	-	up	-	up	-	up
ENSDARG00000093193	*chia.6*	Ensembl_zebrafish	-	-1.1	-	-2.1	-	-2.8	4.66E-03	-13.6
ENSGACG00000017379	*clic2*	Ensembl_stickleback	-	0.0	-	-1.8	4.05E-05	down	-	0.0
ENSGACG00000007280	*clic4*	Ensembl_stickleback	-	-4.0	-	3.7	1.96E-02	down	-	down
ENSGACG00000004855	*cbx7 (1 of 2)*	Ensembl_stickleback	-	1.2	9.01E-02	7.5	-	up	-	2.2
ENSG00000166002	*c11orf75*	Ensembl_human	-	down	5.52E-06	up	-	-1.3	1.95E-04	up
ENSORLG00000001844	*c17orf67*	Ensembl_medaka	-	-3.6	-	0.0	2.11E-02	-7.4	-	2.1
ENSORLG00000014223	*c4orf33*	Ensembl_medaka	-	1.2	3.54E-02	-5.8	-	-1.3	-	1.3
ENSONIG00000002851	*c9orf16*	Ensembl_nile_tilapia	-	3.0	-	-1.0	-	-1.3	7.68E-02	down
ENSORLG00000004586	*ctrc (2 of 2)*	Ensembl_medaka	-	0.0	1.10E-07	-37.4	-	up	-	-3.3

ENSDARG00000068680	*ctrl*	Ensembl_zebrafish	-	0.0	1.39E-07	-41.8	-	0.0	-	1.8
ENSONIG00000003112	*cela3a*	Ensembl_nile_tilapia	-	0.0	1.82E-03	-19.3	-	0.0	-	0.0
ENSORLG00000006922	*cela1 (2 of 2)*	Ensembl_medaka	-	1.2	1.86E-06	-27.4	-	0.0	-	down
ENSDARG00000017314	*cela1 (1 of 7)*	Ensembl_zebrafish	-	1.2	2.12E-06	-39.5	-	0.0	-	0.0
ENSONIG00000006852	*cela1 (2 of 2)*	Ensembl_nile_tilapia	-	down	1.77E-03	down	-	0.0	-	0.0
ENSDARG00000090428	*ctrb1*	Ensembl_zebrafish	-	0.0	1.35E-07	-46.7	-	0.0	-	down
ENSGACG00000000480	*cotl1*	Ensembl_stickleback	-	down	3.04E-04	down	5.65E-02	up	-	down
ENSORLG00000013399	*f5*	Ensembl_medaka	-	down	-	0.0	6.99E-02	up	-	-1.3
ENSGACG00000014046	*cc2d2a (1 of 2)*	Ensembl_stickleback	-	-1.3	-	-2.9	5.66E-03	up	-	down
ENSDARG00000028524	*col5a3b*	Ensembl_zebrafish	-	-14.4	-	up	5.65E-02	down	-	0.0
213515210	NP_001134256.1	Refseq_proteins	4.90E-02	19.0	-	1.5	1.47E-02	-17.8	-	-1.0
ENSONIG00000016437	*c3 (4 of 4)*	Ensembl_nile_tilapia	-	down	1.27E-05	down	-	-1.4	-	-1.8
ENSGACG00000011303	*coro7*	Ensembl_stickleback	-	-1.9	-	1.4	3.24E-03	down	-	up
CR371595	emb|CR371595.2	EST_others	-	up	-	3.5	4.49E-02	up	-	up
CR373403	emb|CR373403.2	EST_others	1.36E-02	up	-	-7.2	-	-2.3	-	down
ENSONIG00000020925	*crebzf*	Ensembl_nile_tilapia	9.89E-02	10.6	-	1.3	-	1.5	-	4.1
113671701	NP_001038787.1	Refseq_proteins	-	3.7	-	-2.2	1.96E-02	up	-	-4.6
ENSGACG00000006281	*ctdspl2 (1 of 2)*	Ensembl_stickleback	-	-1.4	-	-1.2	4.49E-02	down	-	up
CU069447	emb|CU069447.1	EST_others	-	0.0	-	0.0	-	down	4.78E-02	down
ENSGACG00000008425	*cuedc2*	Ensembl_stickleback	1.11E-07	200.3	-	0.0	-	down	-	7.0
ENSONIG00000003295	*cdk18*	Ensembl_nile_tilapia	-	-2.1	-	0.0	6.99E-02	down	-	0.0
375196821	JN739111.1	nt	-	-1.8	6.22E-03	down	-	-1.8	-	down
ENSGACG00000009382	*cth*	Ensembl_stickleback	-	-5.9	-	1.1	2.57E-04	-19.4	-	1.0
ENSONIG00000001633	*cox4i2*	Ensembl_nile_tilapia	9.61E-03	down	-	-2.1	-	1.8	4.61E-02	6.3
ENSONIG00000001074	*cyp46a1*	Ensembl_nile_tilapia	4.68E-03	up	-	2.8	5.78E-03	10.2	-	-1.2
ENSGACG00000017414	*cyp8b1*	Ensembl_stickleback	-	-3.1	-	0.0	-	up	8.31E-05	-36.4
ENSDARG00000053068	*cyp8b1*	Ensembl_zebrafish	-	0.0	1.87E-02	12.8	-	1.1	-	-1.2
ENSGACG00000011632	*cyth3 (1 of 2)*	Ensembl_stickleback	-	-4.0	-	-4.4	6.86E-02	-6.2	-	-3.1
ENSGACG00000008861	*dao*	Ensembl_stickleback	-	0.0	9.01E-02	down	-	down	-	down
41055872	NM_200993.1	Refseq_genes	-	-3.2	-	-1.4	-	1.5	2.11E-07	up
ENSGACG00000016060	*ddx21*	Ensembl_stickleback	-	-16.6	-	-3.8	-	-1.9	1.37E-03	down
ENSGACG00000010201	*dapk2 (2 of 2)*	Ensembl_stickleback	3.13E-08	down	-	0.0	-	0.0	-	down
ENSGACG00000001844	*daxx*	Ensembl_stickleback	2.42E-03	down	-	down	-	2.1	-	-1.8
ENSGACG00000018923	*dock4*	Ensembl_stickleback	-	1.4	-	1.3	-	1.1	6.43E-04	-11.7
ENSGACG00000002377	*dhrs7c (2 of 2)*	Ensembl_stickleback	-	down	-	down	1.04E-02	down	-	0.0
ENSGACG00000008597	*degs2*	Ensembl_stickleback	-	1.2	-	5.1	7.29E-04	11.5	-	2.9
ENSGACG00000019427	*dtx4 (1 of 2)*	Ensembl_stickleback	-	-1.8	-	1.1	2.43E-02	down	-	down
ENSGACG00000005878	*dnase1*	Ensembl_stickleback	-	0.00	1.26E-07	down	-	0.0	-	down
ENSGACG00000015594	*dda1 (2 of 2)*	Ensembl_stickleback	-	down	-	-1.8	1.28E-02	down	-	-4.4
ENSGACG00000018166	*dpysl2 (1 of 2)*	Ensembl_stickleback	-	-6.8	6.40E-02	-24.7	4.39E-02	-30.3	-	-4.3
ENSGACG00000006240	*dip2a*	Ensembl_stickleback	-	-5.3	-	-3.2	1.22E-03	down	5.14E-04	-28.6
ENSGACG00000000671	*dip2b (2 of 2)*	Ensembl_stickleback	-	-2.2	-	-1.6	-	-3.1	2.80E-03	-16.8
19171735	AF382036.1	nt	8.88E-02	27.2	-	up	-	1.4	-	-1.1
ENSDARG00000062177	*dcbld2*	Ensembl_zebrafish	4.69E-02	up	-	up	4.49E-02	up	-	down
ENSORLG00000011807	*dcbld2*	Ensembl_medaka	6.54E-03	down	-	-1.2	3.65E-02	down	-	1.0
ENSDARG00000041110	*dnajc3*	Ensembl_zebrafish	-	2.7	-	2.6	-	1.5	1.13E-02	9.1
ENSGACG00000016454	*dusp22 (1 of 2)*	Ensembl_stickleback	-	0.00	-	down	2.28E-03	down	-	down
ENSGACG00000003355	*dynlt3 (1 of 2)*	Ensembl_stickleback	9.28E-02	down	-	down	-	1.6	-	up
ENSORLG00000002091	*elf2*	Ensembl_medaka	5.65E-03	down	-	-2.7	-	-3.1	-	1.8
ENSGACG00000003978	*egfl6*	Ensembl_stickleback	-	down	2.87E-03	down	4.39E-02	-28.0	-	0.0
ENSGACG00000015896	*egfl8*	Ensembl_stickleback	-	down	-	-2.8	5.65E-02	down	-	0.0
ENSDARG00000056744	*ela2*	Ensembl_zebrafish	-	0.00	3.21E-07	-30.7	-	0.0	-	down
ENSDARG00000007276	*ela3l*	Ensembl_zebrafish	-	0.00	1.10E-07	-46.4	-	0.0	-	down
ENSDARG00000045639	*elavl4*	Ensembl_zebrafish	-	up	-	0.0	-	up	9.95E-02	up
ENSGACG00000014563	*eaf2*	Ensembl_stickleback	-	0.00	9.01E-02	down	-	0.0	-	0.0
ENSDARG00000004979	*elovl5*	Ensembl_zebrafish	-	1.5	-	1.4	-	-1.3	3.47E-03	8.4
ENSORLG00000020058	*endou*	Ensembl_medaka	-	0.00	1.52E-06	-39.3	-	0.0	-	3.7
ENSGACG00000005596	*erlec1*	Ensembl_stickleback	-	-1.1	-	3.7	3.18E-04	up	-	1.3
269860691	XM_002650019.1	nt	7.32E-02	up	-	0.0	-	0.0	-	0.0
ENSGACG00000009051	*ephb3 (1 of 2)*	Ensembl_stickleback	-	4.0	4.13E-02	12.6	-	4.0	-	1.5
238817522	FJ443041.1	nt	-	0.00	1.08E-03	up	-	0.0	-	0.0
ENSGACG00000002632	*epcam*	Ensembl_stickleback	-	-1.2	3.54E-02	down	8.30E-03	down	-	up
ENSONIG00000019196	*ect2*	Ensembl_nile_tilapia	-	0.00	-	up	3.02E-02	down	-	1.6
225715605	BT079225.1	nt	-	0.00	2.53E-03	-18.3	-	up	-	down
225716561	BT079703.1	nt	-	0.00	-	1.2	-	0.0	2.72E-05	-25.6
225716185	BT079515.1	nt	-	up	-	0.0	9.52E-06	down	-	0.0
ENSDARG00000004111	*esr1*	Ensembl_zebrafish	-	0.00	-	up	-	2.9	1.19E-10	105.2
ENSG00000156508	*eef1a1*	Ensembl_human	8.70E-06	up	-	0.0	-	0.0	-	0.0
ENSGACG00000008569	*eif2s1*	Ensembl_stickleback	-	0.00	1.32E-10	-454.3	-	2.2	-	-1.1
ENSGACG00000013800	*eif2b3*	Ensembl_stickleback	2.14E-08	up	-	down	-	down	-	0.0
ENSGACG00000004504	*eif4h*	Ensembl_stickleback	-	0.00	1.84E-02	down	-	-1.7	-	up
ENSGACG00000009227	*esyt1 (2 of 2)*	Ensembl_stickleback	-	-1.9	-	-4.7	6.46E-05	-19.0	-	-1.2
ENSGACG00000018094	*fam129b*	Ensembl_stickleback	8.82E-07	-132.5	-	up	-	up	-	down
ENSDARG00000074317	*fam20c (2 of 2)*	Ensembl_zebrafish	-	1.6	-	1.5	-	0.0	1.36E-04	40.3
ENSGACG00000003212	*fam204a*	Ensembl_stickleback	4.90E-02	16.0	-	up	-	0.0	-	0.0
ENSGACG00000019804	*fam3c*	Ensembl_stickleback	3.75E-02	down	-	-1.1	-	2.1	-	1.2
ENSDARG00000010437	*fam46c*	Ensembl_zebrafish	-	-4.2	-	1.2	-	-1.9	2.05E-02	11.1
ENSGACG00000004704	*fam65b*	Ensembl_stickleback	-	-2.0	-	down	2.47E-02	-6.6	-	up
ENSGACG00000007029	*fancc*	Ensembl_stickleback	5.86E-02	down	-	down	2.85E-09	down	-	down
ENSDARG00000038439	*fabp10a*	Ensembl_zebrafish	-	-1.7	5.44E-02	-25.7	-	-1.4	-	-1.8
ENSGACG00000010321	*fbn3*	Ensembl_stickleback	-	-1.4	-	-1.2	8.06E-02	-5.2	-	-4.3
ENSDARG00000008969	*fgb*	Ensembl_zebrafish	-	4.7	2.66E-02	-30.1	-	2.9	-	-2.0
ENSDARG00000037281	*fgg*	Ensembl_zebrafish	-	-1.3	1.70E-02	-19.4	-	down	-	-1.7
ENSORLG00000001333	*fscb*	Ensembl_medaka	-	-2.0	7.81E-02	-6.9	5.97E-02	-8.5	1.96E-02	-15.5
ENSGACG00000013103	*flna (2 of 2)*	Ensembl_stickleback	-	1.3	7.08E-02	-5.6	-	-3.9	-	2.0
301069358	NP_571346.2	Refseq_proteins	-	down	2.45E-03	up	-	2.0	-	0.0
ENSGACG00000017029	*foxo4*	Ensembl_stickleback	-	-1.0	-	-2.0	1.02E-04	-27.6	-	down
ENSGACG00000007994	*fhod3 (1 of 2)*	Ensembl_stickleback	-	0.00	-	down	3.67E-05	down	-	0.0
ENSGACG00000014208	*fmnl1 (2 of 2)*	Ensembl_stickleback	-	-1.1	-	-1.3	3.17E-02	-8.2	-	down
ENSGACG00000010242	*fhl5*	Ensembl_stickleback	-	down	-	down	3.90E-03	down	-	0.0
ENSGACG00000018742	*fmr1*	Ensembl_stickleback	-	up	2.45E-03	up	-	up	-	0.0
ENSDARG00000089165	*fut9 (14 of 16)*	Ensembl_zebrafish	-	-1.2	-	1.9	8.81E-02	14.8	-	up
ENSGACG00000011403	*fh*	Ensembl_stickleback	-	-1.5	9.01E-02	down	-	-1.7	-	down
ENSORLG00000013751	*fundc1*	Ensembl_medaka	4.69E-02	down	-	1.4	6.99E-02	down	5.91E-02	down
ENSGACG00000009962	*gabpb2*	Ensembl_stickleback	9.28E-02	down	-	-1.8	-	-2.1	-	-1.2
112419938	BT026755.1	nt	-	-1.0	-	-6.3	6.60E-02	-8.2	-	up
112420062	BT026879.1	nt	-	0.00	-	0.0	-	0.0	1.46E-03	-11.1
112420848	BT027665.1	nt	-	-1.1	-	-4.1	2.28E-03	up	-	up
ENSORLG00000000978	*gatsl3*	Ensembl_medaka	-	down	-	1.3	8.81E-02	11.9	-	1.0
ENSORLG00000020655	*gle1*	Ensembl_medaka	-	2.0	-	down	8.79E-02	down	-	-2.1
ENSGACG00000005766	*gltscr2*	Ensembl_stickleback	-	-1.7	-	0.0	6.99E-02	down	-	down
ENSORLG00000002010	*gck*	Ensembl_medaka	-	0.00	-	0.0	-	0.0	1.87E-02	up

ENSDARG00000068006	*gck*	Ensembl_zebrafish	-	0.00	-	down	-	0.0	4.94E-08	42.9
ENSGACG00000011182	*gclm*	Ensembl_stickleback	-	1.2	-	-2.0	3.02E-02	down	-	up
ENSGACG00000006959	*gpct*	Ensembl_stickleback	-	down	-	4.9	6.71E-03	-11.0	-	-1.7
ENSGACG00000005698	*gnmt*	Ensembl_stickleback	-	-3.3	-	-5.3	-	-1.2	5.17E-05	-23.2
ENSDARG00000036239	*gatm*	Ensembl_zebrafish	-	0.00	-	1.1	3.24E-03	down	-	0.0
ENSGACG00000009501	*grhl3*	Ensembl_stickleback	-	up	-	up	2.75E-04	up	-	up
ENSGACG00000006103	*grem1*	Ensembl_stickleback	-	down	1.84E-02	up	-	up	-	-1.1
ENSGACG00000020233	*hspb1*	Ensembl_stickleback	-	1.2	5.59E-02	down	-	-2.2	-	2.2
ENSDARG00000092362	*hsp70.2*	Ensembl_zebrafish	5.91E-03	8.9	1.93E-02	7.3	-	1.6	-	-1.3
ENSGACG00000011278	*hectd4 (1 of 2)*	Ensembl_stickleback	-	-1.4	-	-1.0	-	-1.8	3.09E-02	-6.3
ENSGACG00000003389	*hhatl (2 of 2)*	Ensembl_stickleback	4.69E-02	down	-	0.0	2.51E-07	down	-	0.0
ENSGACG00000012795	*hhatl (1 of 2)*	Ensembl_stickleback	-	0.00	-	0.0	2.43E-02	down	-	0.0
ENSONIG00000012532	*helq*	Ensembl_nile_tilapia	9.28E-02	down	-	0.0	-	-1.1	-	down
ENSGACG00000010755	*hltf*	Ensembl_stickleback	-	-2.0	-	-2.8	2.75E-04	down	-	-2.9
ENSDARG00000012609	*hpx*	Ensembl_zebrafish	-	-2.3	3.33E-02	-12.0	-	-1.5	-	-1.5
ENSONIG00000017473	*hlcs*	Ensembl_nile_tilapia	1.61E-02	down	-	0.0	-	-1.6	-	-1.2
ENSGACG00000009401	*hoxc8*	Ensembl_stickleback	9.37E-03	-35.8	-	-1.5	3.65E-02	-31.3	-	down
ENSGACG00000010338	*htra1 (1 of 2)*	Ensembl_stickleback	1.45E-07	127.2	-	down	-	down	-	0.0
ENSORLG00000013140	*habp2*	Ensembl_medaka	-	down	4.41E-02	down	-	0.0	-	-1.8
ENSGACG00000013673	*hsd3b7*	Ensembl_stickleback	-	-2.1	-	1.0	9.50E-02	4.9	2.33E-02	-7.8
260783280	XP_002586704.1	Refseq_proteins	-	-1.5	-	up	9.04E-04	down	-	0.0
156308441	XP_001617664.1	nr	1.25E-07	up	-	0.0	-	0.0	-	0.0
ENSDARG00000096430	*ighv9-1*	Ensembl_zebrafish	9.28E-02	down	-	0.0	-	2.6	-	down
ENSONIG00000008551	*ino80d*	Ensembl_nile_tilapia	-	-2.9	-	-3.4	3.24E-02	-6.7	-	-1.3
ENSDARG00000011909	*itpr2*	Ensembl_zebrafish	-	-1.1	-	1.8	8.78E-02	17.4	-	-2.7
ENSGACG00000000831	*ip6k2 (1 of 2)*	Ensembl_stickleback	7.33E-03	8.7	1.51E-02	6.7	-	2.2	1.15E-02	7.4
ENSGACG00000015412	*ipmk*	Ensembl_stickleback	-	down	-	12.9	1.70E-04	down	-	1.2
ENSDARG00000014947	*igfbp1a*	Ensembl_zebrafish	-	-1.3	-	-1.3	5.65E-02	up	-	-2.2
ENSDARG00000038666	*igfbp1b*	Ensembl_zebrafish	-	0.00	-	down	-	down	5.91E-02	-5.4
ENSGACG00000002508	*igfbp5 (1 of 2)*	Ensembl_stickleback	-	1.1	-	0.0	-	-1.1	2.94E-02	up
ENSDARG00000034043	*irx5a*	Ensembl_zebrafish	-	0.00	-	0.0	8.79E-02	down	-	0.0
ENSGACG00000011519	*jph2*	Ensembl_stickleback	-	up	-	1.0	-	-2.8	4.60E-03	14.4
ENSGACG00000009872	*kat7 (1 of 2)*	Ensembl_stickleback	-	-1.6	-	2.0	3.65E-02	down	-	0.0
ENSORLG00000011216	*kansl3*	Ensembl_medaka	-	down	-	down	-	-3.1	5.91E-02	down
ENSDARG00000015815	*kdrl*	Ensembl_zebrafish	-	1.4	-	1.5	4.39E-02	21.0	-	-1.4
ENSGACG00000011649	*lace1 (1 of 2)*	Ensembl_stickleback	-	0.00	-	0.0	-	0.0	1.70E-04	up
ENSONIG00000014788	*lrig2*	Ensembl_nile_tilapia	-	1.4	1.49E-02	up	1.50E-04	up	-	-1.1
ENSGACG00000000675	*lta4h*	Ensembl_stickleback	-	0.00	-	up	3.67E-05	down	-	up
ENSGACG00000008716	*limk2*	Ensembl_stickleback	-	-2.3	-	1.8	3.11E-02	22.4	-	2.6
ENSGACG00000005614	*lmf1*	Ensembl_stickleback	-	1.6	4.56E-06	down	-	1.2	-	-1.6
ENSGACG00000019500	*ldlr (2 of 2)*	Ensembl_stickleback	-	up	4.41E-02	down	-	-4.8	-	down
ENSGACG00000011854	*kdm2b (2 of 2)*	Ensembl_stickleback	-	-2.4	-	-2.2	1.34E-04	down	-	down
ENSDARG00000013542	*lpgat1*	Ensembl_zebrafish	-	-3.4	-	1.5	-	up	5.61E-03	up
ENSGACG00000018078	*man1b1 (1 of 2)*	Ensembl_stickleback	-	-1.5	-	-2.4	6.99E-02	down	-	1.3
ENSGACG00000013607	*man1c1*	Ensembl_stickleback	-	1.1	9.01E-02	down	-	1.5	7.68E-02	up
ENSDARG00000042816	*mmp9*	Ensembl_zebrafish	-	-1.7	-	-1.5	1.41E-02	-6.6	-	-2.2
ENSDARG00000060808	*mecom*	Ensembl_zebrafish	3.75E-02	-27.0	-	1.0	-	up	-	-1.1
ENSGACG00000012999	*mep1b (1 of 2)*	Ensembl_stickleback	-	down	-	down	9.04E-04	down	-	down
ENSGACG00000015955	*mthfsd*	Ensembl_stickleback	-	-1.5	-	2.9	8.79E-02	down	-	up
ENSDARG00000090044	*mthfd2*	Ensembl_zebrafish	-	2.1	-	1.4	-	1.4	1.19E-02	up
ENSDARG00000053087	*mthfr*	Ensembl_zebrafish	-	1.2	-	-1.7	-	1.2	2.69E-02	28.0
ENSGACG00000016984	*msmo1*	Ensembl_stickleback	-	0.00	3.54E-02	up	-	down	-	up
ENSGACG00000005096	*mettl5*	Ensembl_stickleback	-	-1.1	-	-2.2	8.04E-02	-4.8	-	-1.1
ENSGACG00000006537	*mcph1*	Ensembl_stickleback	-	1.5	-	down	1.28E-02	down	-	-2.6
ENSONIG00000009665	*mcph1*	Ensembl_nile_tilapia	-	0.00	9.01E-02	down	-	-6.6	-	0.0
ENSONIG00000000210	*mfap4 (8 of 9)*	Ensembl_nile_tilapia	-	0.00	1.49E-02	-9.0	-	0.0	-	0.0
ENSGACG00000010541	*mrpl12*	Ensembl_stickleback	-	5.4	-	1.4	6.99E-02	down	-	-2.2
ENSGACG00000002919	*mrpl30*	Ensembl_stickleback	-	up	-	-3.0	1.07E-03	down	-	down
ENSGACG00000013019	*mapk8ip3*	Ensembl_stickleback	7.43E-04	65.8	-	2.5	-	-2.5	-	4.6
159490	M28397.1	nt	-	0.00	2.00E-05	up	-	up	-	0.0
ENSGACG00000009336	*mre11a*	Ensembl_stickleback	7.32E-02	up	-	2.3	6.84E-03	up	-	1.1
ENSG00000247627	*mtnd4p12*	Ensembl_human	-	down	7.76E-04	down	1.03E-04	down	2.27E-04	-67.2
ENSG00000251544	*mtnd5p12*	Ensembl_human	-	down	-	down	-	down	9.95E-02	down
ENSGACG00000001806	*megf8*	Ensembl_stickleback	-	1.0	9.02E-05	up	-	-2.0	-	1.6
ENSGACG00000007818	*mras*	Ensembl_stickleback	-	1.0	-	-3.9	3.92E-03	40.7	-	-7.8
ENSGACG00000008124	*myof (1 of 2)*	Ensembl_stickleback	-	up	-	up	-	-1.4	1.33E-14	down
ENSGACG00000000114	*myo18b (1 of 2)*	Ensembl_stickleback	-	0.00	-	down	3.24E-03	down	-	0.0
ENSGACG00000010907	*myoz1 (1 of 2)*	Ensembl_stickleback	-	0.00	6.88E-05	down	-	0.0	-	up
ENSDARG00000023369	*mxd*	Ensembl_zebrafish	-	-2.5	2.29E-07	31.6	-	-1.4	-	0.0
ENSORLG00000020815	*nans*	Ensembl_medaka	-	0.00	9.39E-03	up	-	-5.1	-	down
ENSGACG00000002420	*ndufb8*	Ensembl_stickleback	-	down	-	0.0	2.21E-03	-16.4	-	-1.6
ENSONIG00000015110	*ndufaf2*	Ensembl_nile_tilapia	-	0.00	-	down	3.65E-02	down	-	0.0
ENSGACG00000020925	*nd1*	Ensembl_stickleback	2.42E-03	down	1.43E-06	down	8.60E-08	down	1.55E-09	down
ENSGACG00000020947	*nd4*	Ensembl_stickleback	-	down	-	down	8.79E-02	down	-	down
ENSGACG00000020951	*nd5*	Ensembl_stickleback	6.09E-02	-24.5	8.40E-02	-6.8	1.77E-03	-55.2	5.64E-06	down
ENSGACG00000014896	*nckap5*	Ensembl_stickleback	-	1.3	-	-1.9	-	-3.2	8.58E-03	up
ENSONIG00000017915	*ndfip1 (2 of 2)*	Ensembl_nile_tilapia	-	-1.5	5.59E-02	down	5.83E-05	down	1.19E-02	down
ENSGACG00000013006	*napa*	Ensembl_stickleback	-	down	-	0.0	2.26E-02	-34.4	-	0.0
ENSGACG00000006514	*nsf (1 of 2)*	Ensembl_stickleback	4.19E-04	down	5.07E-03	-40.2	8.79E-02	down	-	0.0
ENSGACG00000014174	*nbeal1*	Ensembl_stickleback	1.24E-02	-10.5	-	1.3	-	-1.1	-	-3.4
ENSGACG00000020827	*ncf1*	Ensembl_stickleback	-	-1.4	-	1.9	3.04E-02	-6.8	-	down
ENSGACG00000004210	*nid1 (1 of 2)*	Ensembl_stickleback	-	0.00	1.13E-04	down	-	up	-	-1.6
ENSGACG00000001588	*ninj1*	Ensembl_stickleback	3.75E-08	-199.3	-	-3.1	-	1.5	-	-1.1
ENSDARG00000037958	*nosip*	Ensembl_zebrafish	3.75E-02	down	-	-2.1	-	-3.8	-	2.3
ENSDARG00000044075	*nkx6.2*	Ensembl_zebrafish	-	0.00	1.45E-02	-9.0	-	up	-	0.0
ENSGACG00000016365	*nfatc3*	Ensembl_stickleback	1.34E-08	97.1	-	1.0	-	2.2	-	0.0
ENSDARG00000057741	*nr1h4*	Ensembl_zebrafish	-	0.00	-	1.8	6.04E-02	19.5	-	-1.1
ENSGACG00000012768	*nucb1*	Ensembl_stickleback	-	-1.3	-	-1.5	3.65E-02	down	-	-2.3
ENSGACG00000015626	*nucb2 (1 of 2)*	Ensembl_stickleback	-	1.6	-	-1.4	6.60E-02	-6.9	-	up
ENSORLG00000011426	NOLC1 (1 of 2)	Ensembl_medaka	4.69E-02	up	-	-4.2	-	down	-	-5.5
ENSGACG00000019459	*nap1l1*	Ensembl_stickleback	-	-4.3	-	up	3.02E-02	down	-	down
ENSDARG00000016256	*nudt3a*	Ensembl_zebrafish	-	1.0	2.24E-04	down	3.27E-02	-7.4	-	1.2
1296952	X92804.1	nt	-	0.00	-	0.0	-	0.0	9.87E-04	up
ENSGACG00000016390	*odz3 (2 of 2)*	Ensembl_stickleback	-	2.4	-	-2.1	5.07E-02	-9.7	-	down
ES555559	gb|ES555559.1	EST_others	-	down	-	-3.6	4.94E-04	-27.4	-	-1.1
2258079	AF009794.1	nt	-	down	-	down	3.90E-03	down	-	0.0
164422326	EU325858.1	nt	-	0.00	-	0.0	-	0.0	5.91E-02	-16.2
185133427	NM_001124346.1	Refseq_genes	-	1.5	-	-1.8	-	5.0	3.31E-05	up
225705221	BT074033.1	nt	-	up	2.29E-02	up	-	1.2	-	up
225705363	BT074104.1	nt	3.75E-02	up	-	up	-	up	-	0.0
225704491	BT073668.1	nt	-	1.7	-	3.1	6.04E-02	8.1	-	4.6
225705745	BT074295.1	nt	-	1.6	4.41E-02	up	-	1.4	-	3.3

185135625	NM_001124385.1	Refseq_genes	-	down	4.41E-02	down	-	up	-	-2.0
350537414	NM_001246346.1	Refseq_genes	-	-1.0	-	2.1	6.04E-02	-5.0	-	-2.0
185134284	NM_001124556.1	Refseq_genes	-	down	-	-8.3	-	-1.4	9.49E-03	-7.2
261245070	NM_001160506.1	Refseq_genes	-	-1.3	-	-1.5	7.58E-02	-4.9	-	-1.2
185132277	NM_001124400.1	Refseq_genes	-	2.0	-	1.5	6.85E-02	6.6	-	0.0
185132952	NM_001124249.1	Refseq_genes	-	2.4	-	down	-	-1.4	1.28E-04	67.1
259089083	NM_001165108.1	Refseq_genes	-	1.5	-	1.3	-	up	4.52E-03	10.2
350537622	NM_001246355.1	Refseq_genes	-	2.3	-	0.0	2.27E-04	-30.9	-	-1.2
185135498	NM_001124308.1	Refseq_genes	-	2.5	-	-1.7	9.34E-06	-119.0	-	0.0
34809457	AY386796.1	nt	3.85E-03	down	-	-2.5	4.02E-02	-9.8	-	-4.3
185135324	NM_001124376.1	Refseq_genes	-	1.4	-	6.9	6.99E-02	up	-	1.4
185135579	NM_001124309.1	Refseq_genes	-	down	-	down	1.95E-02	-17.9	-	down
259089112	NM_001165121.1	Refseq_genes	-	1.5	3.04E-04	down	-	2.3	-	-2.3
238231538	NM_001160480.1	Refseq_genes	-	0.00	-	-2.7	2.82E-04	-13.2	-	down
33414970	AY278452.1	nt	2.78E-03	down	2.21E-04	-39.1	7.24E-05	down	1.87E-02	down
14389032	AF375014.1	nt	-	3.4	-	up	1.42E-03	up	-	0.0
40794777	AY518339.1	nt	-	-3.0	-	-1.9	8.46E-02	-6.9	-	-1.1
387155688	FN824527.1	nt	8.69E-02	9.6	-	0.0	-	-1.6	-	3.7
11863729	AJ303076.1	nt	7.42E-04	down	-	-1.7	8.79E-02	-6.0	2.94E-02	down
259089457	NM_001165057.1	Refseq_genes	-	-2.3	-	1.8	1.28E-02	up	-	0.0
185134543	NM_001124290.1	Refseq_genes	-	0.00	-	0.0	4.31E-10	down	-	0.0
318065039	HM190266.1	nt	-	2.7	4.41E-02	down	-	3.2	-	-1.1
194018416	NM_001129986.1	Refseq_genes	-	down	6.22E-03	down	-	0.0	-	0.0
238231355	NM_001160640.1	Refseq_genes	-	down	-	0.0	6.99E-02	down	-	down
185134302	NM_001124285.1	Refseq_genes	-	-1.6	-	-6.2	6.04E-02	-13.8	-	up
185134310	NM_001124274.1	Refseq_genes	-	down	-	down	-	0.0	1.49E-09	49.1
259089103	NM_001165118.1	Refseq_genes	3.75E-02	down	-	-1.5	-	-2.1	-	down
185132233	NM_001124600.1	Refseq_genes	-	0.00	-	0.0	-	0.0	4.15E-04	up
71381926	DQ025596.1	nt	-	down	-	down	5.70E-06	down	-	-3.1
24637708	AF527060.1	nt	-	-1.3	-	-2.1	6.27E-02	-5.4	-	1.1
157311696	NM_001105103.1	Refseq_genes	8.93E-05	-72.6	2.66E-02	-30.7	1.68E-03	down	-	down
225708383	BT075614.1	nt	-	1.3	-	1.3	-	2.2	1.00E-02	11.2
225706089	BT074467.1	nt	-	0.00	2.85E-02	down	-	0.0	-	0.0
ENSGACG00000004331	*pank4*	Ensembl_stickleback	-	down	-	2.4	6.03E-03	-11.3	-	1.4
ENSGACG00000016727	*papd5*	Ensembl_stickleback	8.13E-02	8.9	-	1.1	-	1.2	-	5.6
ENSGACG00000007828	*pon1*	Ensembl_stickleback	-	0.00	-	down	4.67E-03	down	-	19.0
ENSGACG00000007536	*ptms*	Ensembl_stickleback	-	2.2	-	2.5	5.27E-02	5.5	-	up
ENSDARG00000031777	*pparaa*	Ensembl_zebrafish	-	-1.6	-	1.3	8.53E-02	6.0	-	1.7
ENSGACG00000009302	*pes1*	Ensembl_stickleback	5.86E-02	-15.6	-	0.0	-	0.0	1.70E-04	down
ENSORLG00000006231	*pten*	Ensembl_medaka	-	0.00	-	1.1	5.65E-02	down	-	0.0
ENSGACG00000011516	*ppap2c (1 of 2)*	Ensembl_stickleback	-	-1.0	-	down	5.65E-02	down	-	-1.6
ENSGACG00000015853	*pigb*	Ensembl_stickleback	2.00E-02	down	-	0.0	-	0.0	-	down
ENSGACG00000005401	*ptdss1 (2 of 2)*	Ensembl_stickleback	2.19E-04	-64.1	-	0.0	-	up	-	up
ENSGACG00000002045	*pde6d*	Ensembl_stickleback	-	down	-	0.0	1.70E-04	down	-	down
ENSDARG00000060280	*pde9a (1 of 2)*	Ensembl_zebrafish	-	-2.1	-	-2.8	-	2.8	2.31E-02	down
ENSDARG00000013522	*pck1*	Ensembl_zebrafish	-	0.00	-	up	-	3.7	4.39E-03	-11.1
ENSDARG00000009153	*pla2g1b*	Ensembl_zebrafish	-	-1.7	6.16E-05	-19.8	-	-1.0	-	2.2
ENSGACG00000012490	*plcb1 (1 of 2)*	Ensembl_stickleback	-	1.2	-	1.3	3.68E-02	12.5	-	-1.1
ENSDARG00000037506	*prps1b*	Ensembl_zebrafish	-	1.8	9.89E-05	up	-	1.7	-	1.0
ENSGACG00000019001	*pim3*	Ensembl_stickleback	-	1.1	-	1.0	7.50E-02	5.4	-	up
ENSGACG00000010777	*pls1*	Ensembl_stickleback	-	down	1.08E-03	-9.7	-	0.0	-	1.4
ENSGACG00000016743	*psd4*	Ensembl_stickleback	5.74E-06	down	-	0.0	3.65E-02	down	6.61E-08	down
ENSGACG00000005254	*plekhm2*	Ensembl_stickleback	-	-7.2	-	1.0	8.87E-02	-7.4	-	-1.5
ENSGACG00000007218	*plk2*	Ensembl_stickleback	3.04E-05	19.9	-	1.9	-	-1.6	-	-1.9
ENSDARG00000017219	*pabpc1a*	Ensembl_zebrafish	1.61E-02	-8.0	1.19E-06	-32.8	2.23E-07	-40.1	2.49E-05	-20.5
ENSGACG00000019366	*polr2a*	Ensembl_stickleback	-	-1.0	-	-1.4	1.81E-03	-22.3	-	1.0
ENSG00000129159	*kcnc1*	Ensembl_human	-	down	-	1.0	-	-4.1	5.46E-03	-41.3
ENSGACG00000004944	*pglc2*	Ensembl_stickleback	-	-3.3	-	-3.1	4.57E-02	-6.7	-	-1.7
327274185	XM_003221811.1	Refseq_genes	-	down	7.30E-02	-7.0	-	0.0	-	2.8
326678863	XP_001922687.3	Refseq_proteins	-	1.1	5.49E-02	-15.5	-	-2.7	-	-2.3
410923967	XP_003975453.1	Refseq_proteins	-	0.00	1.43E-06	-39.9	-	0.0	1.35E-02	10.4
410900828	XP_003963898.1	Refseq_proteins	-	-1.0	-	-1.9	7.67E-02	12.2	-	-1.8
432943847	XP_004083297.1	Refseq_proteins	-	up	-	up	-	-1.4	1.01E-20	up
432882457	XP_004074040.1	Refseq_proteins	-	down	-	1.1	1.59E-02	down	-	0.0
326671170	XP_003199376.1	Refseq_proteins	-	0.00	-	0.0	-	0.0	3.38E-15	down
348501087	XP_003438102.1	Refseq_proteins	-	0.00	-	0.0	-	0.0	8.65E-13	down
348517015	XP_003446031.1	Refseq_proteins	-	0.00	-	0.0	-	0.0	8.24E-02	-7.4
326669577	XP_001923321.2	Refseq_proteins	-	0.00	5.59E-02	down	-	0.0	4.84E-11	down
348538469	XP_003456713.1	Refseq_proteins	7.32E-02	down	-	0.0	-	down	-	0.0
326667554	XP_002667068.2	Refseq_proteins	2.78E-03	15.2	-	0.0	-	-1.0	-	down
410932293	XP_003979528.1	Refseq_proteins	-	0.00	2.45E-03	up	-	1.8	-	-1.5
348521084	XP_003448056.1	Refseq_proteins	-	-1.1	-	6.3	4.49E-02	up	-	down
410910654	XP_003968805.1	Refseq_proteins	-	2.4	8.77E-02	-14.4	-	0.0	-	0.0
348525982	XP_003450500.1	Refseq_proteins	1.36E-02	8.8	5.59E-02	up	-	up	-	0.0
338718465	XM_001498233.3	nt	-	0.00	1.60E-12	-243.0	-	0.0	-	up
327269633	XP_003219598.1	Refseq_proteins	2.08E-03	up	-	0.0	-	0.0	-	0.0
363738171	XP_001231970.2	Refseq_proteins	-	-1.7	-	-9.3	-	2.2	3.74E-02	down
348525178	XP_003450099.1	Refseq_proteins	-	-1.1	-	-1.0	1.96E-04	down	-	0.0
348539876	XP_003457415.1	Refseq_proteins	3.56E-06	down	-	0.0	-	0.0	-	0.0
125807411	XP_001343562.1	Refseq_proteins	-	2.5	2.02E-04	-13.5	9.69E-02	5.6	-	down
189517525	XP_001923568.1	Refseq_proteins	-	down	-	down	-	0.0	5.60E-04	11.4
327281562	XP_003225516.1	Refseq_proteins	-	0.00	-	0.0	6.99E-02	down	-	up
348526073	XP_003450545.1	Refseq_proteins	3.46E-03	-13.1	6.88E-05	down	5.66E-03	down	-	up
348526794	XP_003450904.1	Refseq_proteins	-	0.00	-	1.3	-	0.0	2.07E-04	-19.6
348513025	XP_003444043.1	Refseq_proteins	3.15E-02	down	-	0.0	3.65E-02	up	-	up
348539792	XP_003457373.1	Refseq_proteins	1.65E-03	-47.6	1.27E-03	-49.6	-	0.0	-	0.0
348545569	XP_003460252.1	Refseq_proteins	-	down	-	down	4.49E-02	down	-	down
348529410	XP_003452206.1	Refseq_proteins	-	0.00	-	down	-	0.0	4.20E-02	-6.5
68444937	XP_706427.1	Refseq_proteins	-	4.4	2.87E-03	up	-	up	-	0.0
125811426	XP_001335256.1	Refseq_proteins	2.19E-08	75.4	8.58E-10	-166.1	-	down	-	down
410906097	XP_003966528.1	Refseq_proteins	-	-2.5	-	down	-	-2.8	2.94E-02	down
326674126	XP_003200076.1	Refseq_proteins	-	-1.8	-	1.2	-	-1.5	6.95E-03	down
326665123	XP_691524.5	Refseq_proteins	-	-1.1	-	1.5	7.71E-05	-88.2	-	up
189514417	XP_001345882.2	Refseq_proteins	-	-7.9	3.54E-02	9.3	-	1.9	-	-7.0
326670954	XP_001336175.4	Refseq_proteins	-	-4.0	2.12E-06	52.4	-	-3.2	-	2.9
432873596	XP_004072295.1	Refseq_proteins	1.51E-06	28.5	-	3.6	-	down	-	0.0
348532506	XP_003453747.1	Refseq_proteins	-	down	7.27E-02	down	3.59E-04	down	-	2.3
348507107	XR_134778.1	Refseq_genes	-	-1.7	-	1.2	-	1.5	1.00E-02	down
348500648	XM_003437837.1	Refseq_genes	-	down	-	0.0	3.02E-02	down	-	up
348523192	XM_003449060.1	Refseq_genes	1.36E-02	down	-	-2.0	-	-2.6	-	0.0
348544302	XM_003459573.1	Refseq_genes	3.15E-02	down	9.39E-03	down	1.97E-05	down	-	down
348526849	XM_003450884.1	Refseq_genes	-	0.00	-	down	4.86E-03	7.8	-	0.0

348524437	XM_003449682.1	Refseq_genes	3.15E-02	up	-	up	4.67E-03	up	-	down
348517992	XP_003446516.1	Refseq_proteins	-	0.00	3.74E-03	-13.5	-	-1.8	-	1.3
348523519	XP_003449271.1	Refseq_proteins	-	-2.4	-	up	-	0.0	9.95E-02	up
348538993	XP_003456974.1	Refseq_proteins	7.32E-02	down	-	-4.3	-	0.0	-	down
432873602	XP_004072298.1	Refseq_proteins	-	0.00	-	down	-	down	1.21E-02	13.4
301624357	XP_002941477.1	Refseq_proteins	-	0.00	7.90E-02	-11.7	-	0.0	-	0.0
348516493	XP_003445773.1	Refseq_proteins	-	3.2	-	1.0	8.79E-02	down	-	3.7
326663987	XP_003197704.1	Refseq_proteins	-	0.00	7.34E-02	-6.8	-	0.0	-	down
348506200	XP_003440648.1	Refseq_proteins	-	4.8	4.41E-02	down	-	2.2	-	up
410924755	XM_003975798.1	Refseq_genes	-	0.00	-	0.0	-	up	2.29E-18	down
410923730	XM_003975286.1	Refseq_genes	1.61E-02	down	-	-1.2	-	-3.5	-	-1.1
410919522	XM_003973185.1	Refseq_genes	-	0.00	5.95E-04	-34.0	-	0.0	-	down
410922242	XM_003974543.1	Refseq_genes	-	1.2	9.79E-02	-6.9	-	0.0	8.31E-04	down
410926977	XP_003976944.1	Refseq_proteins	-	up	3.12E-04	-20.6	-	0.0	-	-2.7
348508195	XP_003441640.1	Refseq_proteins	1.36E-02	down	-	1.0	-	1.3	-	-1.5
432926066	XP_004080813.1	Refseq_proteins	-	0.00	-	down	9.62E-02	8.5	-	1.3
410905289	XP_003966124.1	Refseq_proteins	-	-3.3	-	-1.8	4.63E-10	-110.8	-	down
410929355	XP_003978065.1	Refseq_proteins	-	-3.4	2.85E-02	down	-	0.0	-	4.7
ENSDARG00000035350	*ins*	Ensembl_zebrafish	-	0.00	3.65E-03	-17.3	-	0.0	-	0.0
ENSGACG00000009139	*pfn2 (1 of 2)*	Ensembl_stickleback	-	down	3.51E-04	down	4.49E-02	down	-	down
ENSONIG00000007715	*prlr (1 of 2)*	Ensembl_nile_tilapia	-	-1.5	-	-2.1	3.23E-02	8.9	-	0.0
ENSONIG00000009922	*psapl1*	Ensembl_nile_tilapia	-	down	2.07E-03	down	8.21E-08	down	3.74E-02	down
ENSDARG00000017213	*prss35*	Ensembl_zebrafish	-	1.0	-	1.9	7.05E-02	6.5	-	2.7
ENSGACG00000000107	*psmb9 (5 of 5)*	Ensembl_stickleback	6.83E-05	up	2.61E-02	9.5	1.04E-02	up	-	0.0
ENSGACG00000019857	*pacsin2*	Ensembl_stickleback	-	-1.4	-	-1.3	3.02E-02	-8.3	-	9.6
ENSORLG00000015712	*prkch*	Ensembl_medaka	-	1.7	-	-2.7	2.77E-03	down	-	2.2
ENSDARG00000079585	*pkn3*	Ensembl_zebrafish	-	-2.3	-	3.7	2.99E-03	-50.1	-	up
ENSDARG00000089608	*ppp1cbl*	Ensembl_zebrafish	-	1.9	-	3.2	1.03E-04	down	-	up
ENSGACG00000005491	*ppp1r21*	Ensembl_stickleback	-	1.7	-	1.1	1.08E-03	-29.9	-	up
ENSGACG00000007843	*ptprc*	Ensembl_stickleback	-	-1.9	-	-3.2	6.99E-02	-5.8	-	-1.8
ENSGACG00000012481	*ralgapa1*	Ensembl_stickleback	-	-6.2	-	-2.7	5.07E-02	-29.1	-	-4.1
ENSDARG00000005989	*rgl1*	Ensembl_zebrafish	-	-1.5	-	1.7	3.40E-02	12.6	-	2.8
223646095	NP_001138713.1	Refseq_proteins	-	0.00	5.59E-02	up	-	0.0	-	0.0
ENSGACG00000002543	*rgcd1*	Ensembl_stickleback	-	-11.6	9.39E-03	down	1.73E-05	-44.1	-	0.0
ENSDARG00000021869	*rcan2*	Ensembl_zebrafish	-	-1.7	-	-1.3	-	-1.7	8.21E-02	6.1
ENSDARG00000090039	*reck*	Ensembl_zebrafish	-	-1.4	-	1.3	6.99E-02	up	-	-1.1
ENSGACG00000013298	*arhgdia (1 of 2)*	Ensembl_stickleback	3.85E-03	down	-	83.1	-	1.0	-	up
ENSGACG00000019358	*arhgef15*	Ensembl_stickleback	3.85E-03	down	-	1.2	-	-2.3	-	up
222137251	FJ002822.1	nt	-	0.00	1.52E-06	123.1	-	0.0	-	0.0
ENSONIG00000002839	*rpp21*	Ensembl_nile_tilapia	-	-1.3	-	-1.2	-	1.2	2.30E-02	7.4
ENSGACG00000012811	*rps6ka3*	Ensembl_stickleback	9.69E-02	5.6	-	2.2	-	1.1	2.74E-06	52.0
ENSORLG00000011879	*rrbp1*	Ensembl_medaka	-	-1.1	-	1.1	-	1.2	8.95E-02	5.5
ENSONIG00000010011	*rrbp1*	Ensembl_nile_tilapia	-	0.00	-	1.2	-	-1.6	1.00E-02	up
ENSMUSG00000026955	2010317E24Rik	Ensembl_mouse	-	0.00	4.56E-06	down	-	0.0	-	0.0
ENSGACG00000016138	*rnf165 (1 of 2)*	Ensembl_stickleback	-	0.00	4.41E-02	down	9.33E-05	down	-	down
ENSGACG00000005671	*rbms1 (1 of 2)*	Ensembl_stickleback	-	down	-	down	3.02E-02	down	-	up
ENSDARG00000045930	*rbpms2 (1 of 2)*	Ensembl_zebrafish	-	1.0	7.78E-02	-6.1	-	1.8	-	up
ENSONIG00000008341	*rbfox2 (1 of 2)*	Ensembl_nile_tilapia	-	down	-	up	-	down	1.87E-02	up
ENSGACG00000020220	*ruvbl2*	Ensembl_stickleback	-	0.00	5.75E-05	-15.9	-	0.0	-	0.0
213513492	NM_001140687.1	Refseq_genes	-	1.3	5.59E-02	up	-	1.8	-	down
213513418	NM_001139890.1	Refseq_genes	-	-2.5	-	down	4.41E-02	-9.6	-	-1.6
259155117	NM_001165329.1	Refseq_genes	-	1.1	-	-5.2	8.79E-02	-5.3	-	2.2
291190403	NM_001173641.1	Refseq_genes	-	2.6	-	1.9	8.78E-02	-25.6	-	0.0
213513162	NM_001141362.1	Refseq_genes	-	-1.0	5.68E-03	-7.8	-	3.6	-	-1.7
291190593	NM_001173915.1	Refseq_genes	-	0.00	-	1.9	6.99E-02	up	-	0.0
259155215	NM_001165377.1	Refseq_genes	-	-1.9	-	-2.1	6.99E-02	-6.9	-	1.9
213511949	NM_001140737.1	Refseq_genes	-	0.00	-	1.6	2.18E-02	7.2	-	up
185134143	NM_001123580.1	Refseq_genes	-	down	-	up	-	0.0	9.06E-15	down
213512283	NM_001140113.1	Refseq_genes	-	0.00	1.19E-08	down	3.24E-07	down	-	0.0
213514195	NM_001140522.1	Refseq_genes	-	0.00	-	0.0	3.02E-02	down	-	0.0
213514355	NM_001140779.1	Refseq_genes	-	-2.5	-	1.5	1.34E-03	10.6	-	-1.0
226443385	NM_001146421.1	Refseq_genes	-	down	-	down	6.99E-02	down	-	down
291190368	NM_001173776.1	Refseq_genes	-	0.00	9.39E-03	down	-	0.0	-	0.0
10505157	AF273013.1	nt	-	1.1	-	down	1.79E-02	-6.9	-	up
221221511	BT057545.1	nt	3.43E-12	down	-	0.0	-	0.0	-	0.0
209737133	BT049635.1	nt	-	-1.1	9.01E-02	down	-	-4.1	-	-1.5
209733395	BT047766.1	nt	-	down	4.50E-08	down	2.85E-09	down	7.68E-02	down
221220955	BT057267.1	nt	-	-4.9	1.27E-03	down	2.73E-11	-195.2	-	-4.1
209733843	BT047990.1	nt	-	-2.2	-	-4.0	1.96E-02	-6.4	-	-3.9
221220457	BT057018.1	nt	-	0.00	-	down	1.13E-09	down	-	down
209738407	BT050272.1	nt	7.51E-06	down	-	0.0	-	0.0	-	0.0
221219843	BT056711.1	nt	-	down	-	down	5.65E-02	down	-	down
221219477	BT056528.1	nt	-	0.00	2.87E-03	down	4.67E-03	down	-	down
209737953	BT050045.1	nt	-	down	6.22E-03	down	-	down	2.26E-03	down
221220267	BT056923.1	nt	3.75E-02	up	-	up	-	up	-	0.0
221221335	BT057457.1	nt	-	-2.0	-	1.9	1.62E-03	-14.2	-	0.0
209731161	BT046649.1	nt	-	1.9	9.01E-02	down	-	-1.8	-	3.1
221221243	BT057411.1	nt	-	down	-	down	1.18E-04	down	-	down
221220085	BT056832.1	nt	-	-2.8	-	-4.4	8.30E-03	-21.0	-	-1.3
303663498	BT125368.1	nt	-	down	-	-4.9	6.99E-02	down	-	0.0
209734397	BT048267.1	nt	-	down	3.07E-10	-273.9	-	up	-	up
221220663	BT057121.1	nt	-	0.00	-	-1.2	-	4.1	1.18E-02	-10.4
304376917	BT050094.2	nt	-	-1.4	2.87E-03	down	5.66E-03	down	-	-3.2
209730649	BT046393.1	nt	-	0.00	-	2.0	-	5.2	1.07E-02	-8.3
209738155	BT050146.1	nt	8.88E-02	27.2	-	0.0	-	0.0	-	0.0
209735875	BT049006.1	nt	-	0.00	9.10E-07	-49.4	-	0.0	-	0.0
209730271	BT046204.1	nt	3.14E-04	up	-	0.0	-	-5.4	5.91E-02	up
224587002	BT071859.1	nt	8.00E-03	down	-	-1.5	8.30E-03	-22.0	-	up
209148888	BT044698.1	nt	-	0.00	4.41E-02	up	-	down	-	0.0
224587036	BT071893.1	nt	-	-1.1	-	-1.7	4.44E-02	-5.7	-	-1.3
224587090	BT071953.1	nt	-	down	-	down	6.38E-08	down	-	0.0
223647599	BT058845.1	nt	3.75E-02	down	-	up	-	-2.9	-	0.0
209154129	BT045035.1	nt	-	down	-	-5.4	3.65E-02	down	-	down
223647861	BT058976.1	nt	-	-1.9	-	down	8.02E-14	-312.0	-	4.7
223647865	BT058978.1	nt	-	-1.3	-	-1.3	3.02E-02	-11.7	-	down
224587384	BT072238.1	nt	-	-1.6	6.21E-08	-121.6	9.52E-03	-8.0	-	-5.4
224587388	BT072243.1	nt	-	0.00	-	0.0	1.91E-06	down	-	-1.2
224587400	BT072256.1	nt	-	down	-	down	8.79E-02	down	-	0.0
224587424	BT072278.1	nt	-	1.2	-	3.7	8.09E-06	up	8.31E-04	up
209154931	BT045436.1	nt	-	0.00	1.19E-02	down	-	0.0	-	up
224587488	BT072334.1	nt	7.32E-02	up	-	-6.3	-	1.4	-	1.1
223649215	BT059653.1	nt	-	-2.0	-	-2.2	4.73E-02	-7.3	-	-5.9

224587510	BT072359.1	nt	3.75E-02	up	-	-1.8	-	-1.7	-	0.0
209155099	BT045520.1	nt	-	0.00	-	down	2.77E-03	down	-	0.0
209155209	BT045575.1	nt	-	4.1	-	3.7	3.02E-02	down	-	-1.8
224613399	BT072448.1	nt	-	1.1	-	1.1	5.65E-02	-5.1	-	1.1
224613403	BT072459.1	nt	-	down	-	down	9.71E-10	down	-	down
224587622	BT072484.1	nt	-	down	-	down	3.24E-03	down	-	down
224587648	BT072508.1	nt	1.58E-03	-29.2	-	-5.3	4.41E-02	-7.8	-	-2.2
223649117	BT059604.1	nt	-	-2.5	-	-19.7	7.77E-04	-31.8	-	down
223648461	BT059276.1	nt	-	0.00	-	down	7.67E-02	-14.6	-	down
209156037	BT045989.1	nt	-	0.00	-	0.0	4.16E-06	down	-	down
224587895	BT072767.1	nt	-	1.4	-	-2.0	5.91E-03	-9.9	-	0.0
224587922	BT072796.1	nt	-	-1.2	-	1.4	8.79E-02	up	-	0.0
223672306	BT059975.1	nt	-	down	-	up	8.79E-02	-5.4	-	0.0
223672718	BT060181.1	nt	-	down	-	down	1.50E-04	down	-	-1.1
291190370	NM_001173637.1	Refseq_genes	-	down	-	down	4.05E-05	down	-	down
213513351	NM_001141001.1	Refseq_genes	-	down	-	down	1.07E-03	down	-	down
291190431	NM_001173900.1	Refseq_genes	-	-5.1	-	-4.1	6.97E-02	-4.9	-	-8.7
226442587	NM_001146430.1	Refseq_genes	-	-1.1	-	2.5	4.44E-02	8.0	-	-1.4
226443114	NM_001146569.1	Refseq_genes	-	3.4	-	-1.7	6.04E-02	-18.4	-	-1.2
213512792	NM_001140055.1	Refseq_genes	-	down	8.39E-02	-6.7	-	1.1	-	-1.9
185133694	NM_001123697.1	Refseq_genes	-	0.00	-	0.0	-	0.0	7.25E-10	131.5
213512966	NM_001139774.1	Refseq_genes	1.24E-02	8.6	-	-1.4	-	up	-	0.0
194396635	EU643669.1	nt	-	down	9.01E-02	down	3.65E-02	down	7.28E-04	-56.2
213512932	NM_001140849.1	Refseq_genes	-	down	-	-2.0	-	-14.6	6.72E-02	-5.2
158702273	EU025707.1	nt	-	1.0	-	-1.1	-	-1.5	6.25E-04	-11.5
213513791	NM_001140121.1	Refseq_genes	-	-1.0	-	1.1	6.49E-05	up	-	1.4
259155205	NM_001165372.1	Refseq_genes	-	up	3.54E-02	down	-	-1.8	-	up
356640272	NM_001252361.1	Refseq_genes	-	down	-	-1.6	9.04E-04	-472.8	-	down
213514485	NM_001140576.1	Refseq_genes	-	up	-	-2.3	8.30E-03	down	-	0.0
218931109	NM_001140986.1	Refseq_genes	2.16E-05	29.6	-	-2.7	-	down	-	down
213512998	NM_001140457.1	Refseq_genes	-	1.2	-	down	4.41E-02	-12.4	-	-2.2
185132565	NM_001123669.1	Refseq_genes	9.37E-03	8.2	6.22E-03	7.7	-	2.7	4.46E-02	5.6
25573079	AF504023.1	nt	2.19E-08	down	7.25E-07	-142.8	7.44E-06	down	-	down
363548533	JN897012.1	nt	-	down	1.84E-02	down	-	down	-	down
378554505	JQ390056.1	nt	5.37E-03	-11.4	2.37E-05	-22.9	7.44E-06	-23.7	2.60E-08	-57.1
3775976	*u12143.1*	nt	1.51E-06	down	1.38E-08	down	3.07E-11	down	4.47E-12	down
259155233	NM_001165386.1	Refseq_genes	-	-1.9	-	down	7.89E-04	down	-	down
259155169	NM_001165355.1	Refseq_genes	-	-1.2	-	1.2	-	1.2	3.97E-02	7.0
226443008	NM_001146539.1	Refseq_genes	-	0.00	-	down	1.04E-02	down	-	0.0
329130751	HM133629.1	nt	-	0.00	-	0.0	-	0.0	4.61E-02	-8.4
329130737	HM133622.1	nt	-	-7.6	-	-3.4	6.47E-04	-16.4	-	-5.8
213514523	NM_001140090.1	Refseq_genes	-	0.00	7.73E-07	down	2.75E-04	down	-	0.0
304555577	NM_001195198.1	Refseq_genes	-	down	6.16E-05	down	6.99E-02	down	-	down
213513391	NM_001140288.1	Refseq_genes	-	-4.3	3.54E-02	down	8.25E-07	down	-	down
226443419	NM_001146429.1	Refseq_genes	-	down	-	up	1.96E-02	down	-	0.0
213513082	NM_001141336.1	Refseq_genes	-	1.4	-	3.0	-	-1.6	1.31E-03	13.0
226443273	NM_001146626.1	Refseq_genes	-	1.2	-	0.0	-	2.2	9.95E-02	9.0
213512635	NM_001140741.1	Refseq_genes	-	2.0	2.06E-02	6.9	-	up	-	0.0
213514719	NM_001141345.1	Refseq_genes	-	-3.0	2.29E-02	down	-	up	-	0.0
213515517	NM_001140898.1	Refseq_genes	-	-13.5	-	1.5	4.39E-02	-15.2	-	-1.1
213512394	NM_001141358.1	Refseq_genes	9.61E-03	down	-	-1.1	-	-1.1	-	0.0
291190832	NM_001173937.1	Refseq_genes	-	1.7	-	-2.1	5.28E-02	-6.4	-	3.6
185133566	NM_001123692.1	Refseq_genes	-	1.0	1.03E-03	-13.4	-	-1.6	-	-1.5
185133997	NM_001123569.1	Refseq_genes	-	up	-	-1.1	1.98E-02	6.7	-	up
213514919	NM_001141637.1	Refseq_genes	-	-4.2	8.47E-02	-7.3	3.98E-02	-6.6	2.27E-02	-25.6
213513389	NM_001140261.1	Refseq_genes	-	-9.4	-	-1.1	8.45E-06	down	-	1.2
291190285	NM_001173882.1	Refseq_genes	-	0.00	-	0.0	6.84E-03	down	-	up
185135858	NM_001123711.1	Refseq_genes	-	0.00	2.24E-04	down	-	0.0	-	0.0
213514129	NM_001139633.1	Refseq_genes	-	down	-	1.0	3.02E-02	down	-	down
185135317	NM_001123588.1	Refseq_genes	-	up	1.95E-04	-17.0	-	4.6	-	2.0
7769634	AF228581.1	nt	-	3.0	9.01E-02	up	-	-1.4	-	1.7
164510790	AM262766.1	nt	6.67E-04	down	2.07E-03	down	-	0.0	-	down
4102912	AF017232.1	nt	-	-1.6	7.27E-02	down	-	-1.4	-	1.1
ENSGACG00000001474	*sar1b*	Ensembl_stickleback	-	-4.8	-	0.0	1.60E-02	-37.0	-	-3.6
ENSDARG00000069983	CU929159.1	Ensembl_zebrafish	-	-1.1	-	-1.8	1.31E-05	down	-	2.0
ENSGACG00000016757	*sec24b*	Ensembl_stickleback	-	-1.1	-	down	9.04E-04	down	-	-1.4
ENSGACG00000000830	*sec61a1 (2 of 2)*	Ensembl_stickleback	-	1.5	-	-1.2	6.84E-03	down	-	1.2
ENSDARG00000090286	*serpina1*	Ensembl_zebrafish	-	2.4	6.39E-03	-15.7	-	-1.4	-	-1.7
ENSDARG00000021208	*serpind1*	Ensembl_zebrafish	-	2.3	7.81E-02	-23.2	-	-3.2	-	-1.4
ENSGACG00000007719	*serinc2 (1 of 2)*	Ensembl_stickleback	-	down	-	down	1.28E-02	down	-	down
ENSGACG00000007834	*srsf5*	Ensembl_stickleback	-	-1.9	-	-3.0	1.77E-03	-27.3	-	-2.2
ENSORLG00000016512	LOC100144362	Ensembl_medaka	-	-1.1	1.67E-02	-8.5	1.81E-03	-11.6	-	1.3
ENSGACG00000010973	*sgk1*	Ensembl_stickleback	-	2.2	-	-4.1	-	0.0	4.42E-20	-862.7
ENSONIG00000011932	*sbf1*	Ensembl_nile_tilapia	-	down	-	down	5.07E-02	-28.3	-	-2.1
ENSGACG00000014971	*sdr42e1*	Ensembl_stickleback	2.42E-03	up	-	1.3	-	0.0	-	0.0
ENSDARG00000095304	si:ch211-207c6.2	Ensembl_zebrafish	-	-3.8	-	0.0	4.41E-02	-12.4	-	up
ENSDARG00000073742	si:ch73-103b2.3	Ensembl_zebrafish	-	0.00	4.50E-08	-38.7	-	up	-	-2.8
ENSDARG00000093374	si:ch73-18b11.1	Ensembl_zebrafish	-	down	-	2.8	3.65E-02	down	-	-1.0
ENSDARG00000058719	si:dkey-119f1.1	Ensembl_zebrafish	-	-3.6	-	1.0	-	-2.8	1.00E-02	16.4
ENSDARG00000060325	si:dkey-179j5.2	Ensembl_zebrafish	-	0.00	-	0.0	-	up	1.18E-10	up
ENSDARG00000094929	si:dkey-7f3.15	Ensembl_zebrafish	-	down	-	1.6	-	up	4.66E-06	42.9
ENSDARG00000068515	si:zfos-1762d12.1	Ensembl_zebrafish	-	-1.5	-	1.5	4.52E-02	8.3	-	1.6
ENSGACG00000002914	*neu1*	Ensembl_stickleback	-	up	1.58E-02	-32.8	-	up	-	up
ENSGACG00000016749	*srp72*	Ensembl_stickleback	-	1.6	-	up	3.65E-02	up	-	up
ENSORLG00000006430	*srprb*	Ensembl_medaka	-	1.0	9.01E-02	up	-	0.0	-	1.3
ENSONIG00000012851	*sin3a (1 of 2)*	Ensembl_nile_tilapia	-	0.00	-	down	4.67E-03	down	-	up
ENSGACG00000008432	*six1*	Ensembl_stickleback	-	3.6	-	-1.1	8.79E-02	-97.0	-	3.2
ENSDARG00000052578	*c6ast4*	Ensembl_zebrafish	-	0.00	2.12E-06	-23.4	-	0.0	-	down
BQ036199	gb|BQ036199.1	EST_others	-	down	-	0.0	-	0.0	2.57E-15	down
ENSGACG00000016411	*slit3*	Ensembl_stickleback	-	0.00	-	down	5.51E-02	-8.9	-	up
ENSDARG00000071430	*smyhc1*	Ensembl_zebrafish	2.44E-03	-27.0	-	1.6	-	-1.5	-	0.0
ENSGACG00000015581	*sumo1*	Ensembl_stickleback	-	-1.1	-	-3.6	2.24E-02	-14.4	-	1.2
DW592332	gb|DW592332.1	EST_others	-	4.1	-	-1.3	3.65E-02	up	-	up
ENSONIG00000004201	*slc12a9*	Ensembl_nile_tilapia	-	1.6	-	13.6	3.67E-05	down	-	-1.3
ENSDARG00000013855	*slc12a3*	Ensembl_zebrafish	-	1.3	-	1.3	7.27E-02	4.8	-	up
ENSGACG00000007935	*slc16a4*	Ensembl_stickleback	-	0.00	1.49E-02	down	1.42E-03	down	-	down
ENSGACG00000010985	*slc2a2*	Ensembl_stickleback	-	up	-	-1.5	-	1.6	8.09E-02	-7.9
ENSGACG00000019384	*slc2a4*	Ensembl_stickleback	-	down	5.07E-03	-39.2	8.60E-08	down	-	down
ENSONIG00000013596	*slc24a6*	Ensembl_nile_tilapia	-	1.9	-	-1.3	1.68E-03	down	5.91E-02	down
ENSGACG00000015473	*slc25a36 (2 of 2)*	Ensembl_stickleback	-	up	-	up	-	-28.1	5.61E-03	up
ENSGACG00000005439	*slc35e2b*	Ensembl_stickleback	-	-1.1	-	12.6	1.96E-03	-19.2	-	down
ENSGACG00000016873	*slc39a2*	Ensembl_stickleback	-	-1.6	-	down	8.30E-03	-18.4	-	0.0

ENSGACG00000007949	*slc48a1*	Ensembl_stickleback	-	-1.8	-	-4.6	1.08E-03	-29.6	-	3.7
ENSGACG00000004673	*snx5*	Ensembl_stickleback	-	up	-	0.0	5.84E-02	-7.9	-	down
ENSGACG00000006733	*spc24*	Ensembl_stickleback	-	0.00	-	0.0	4.50E-02	-10.5	-	up
ENSGACG00000020699	*spag7*	Ensembl_stickleback	-	1.1	-	-1.9	8.67E-02	-7.6	-	1.1
ENSGACG00000013537	*s1pr1*	Ensembl_stickleback	-	2.1	-	-1.2	1.85E-07	up	-	-1.2
ENSGACG00000008317	*skap2*	Ensembl_stickleback	3.75E-02	down	-	1.9	-	-1.1	-	down
ENSGACG00000011185	*sox9 (2 of 2)*	Ensembl_stickleback	-	0.00	-	4.1	1.96E-02	up	-	-3.3
ENSGACG00000016008	*st6galnac6*	Ensembl_stickleback	-	-1.4	-	1.3	5.07E-02	-9.0	-	-1.1
ENSORLG00000018292	*stab2*	Ensembl_medaka	-	-1.1	-	3.7	-	-1.2	9.95E-02	12.2
ENSORLG00000000328	*stc1 (2 of 2)*	Ensembl_medaka	-	down	-	up	2.36E-18	down	-	0.0
ENSDARG00000058476	*stc1l*	Ensembl_zebrafish	-	down	-	up	2.30E-12	down	-	0.0
ENSDARG00000033662	*scd*	Ensembl_zebrafish	-	1.2	-	3.5	-	1.0	8.55E-10	54.2
ENSDARG00000024026	*sdf2*	Ensembl_zebrafish	-	-1.2	-	1.3	-	1.8	5.55E-02	5.5
ENSGACG00000020581	*sod1*	Ensembl_stickleback	1.15E-02	down	6.61E-04	down	2.28E-03	down	1.89E-03	down
ENSGACG00000013091	*st14 (1 of 3)*	Ensembl_stickleback	6.09E-02	29.6	-	-1.3	1.96E-02	30.7	-	0.0
ENSGACG00000006093	*st7l*	Ensembl_stickleback	-	0.00	2.62E-05	-33.1	-	0.0	-	up
ENSGACG00000016739	*sapcd2*	Ensembl_stickleback	-	-1.9	4.41E-02	25.0	-	-1.3	-	-1.1
ENSGACG00000007257	*sufu*	Ensembl_stickleback	-	down	-	down	3.65E-02	down	-	up
ENSGACG00000013526	*suv420h2*	Ensembl_stickleback	-	up	-	down	6.99E-02	down	-	up
ENSGACG00000012650	*smarcc1 (1 of 2)*	Ensembl_stickleback	-	0.00	2.29E-02	up	-	down	-	2.1
ENSGACG00000003136	*syt11*	Ensembl_stickleback	-	-1.0	8.90E-08	down	-	-2.8	-	-1.3
288548571	GU569096.1	nt	-	0.00	6.59E-03	8.6	-	0.0	-	0.0
ENSDARG00000054255	*tctex1d2*	Ensembl_zebrafish	-	1.3	-	1.3	2.28E-03	up	-	1.9
ENSONIG00000012488	*tctex1d2*	Ensembl_nile_tilapia	-	0.00	-	0.0	6.99E-02	down	-	0.0
56326278	CR650765.2	nt	-	1.2	9.01E-02	up	-	1.4	-	2.0
ENSGACG00000005236	*tspan2 (2 of 2)*	Ensembl_stickleback	-	up	5.20E-03	up	-	down	-	-1.7
ENSDARG00000008407	*tspan7b*	Ensembl_zebrafish	-	-3.0	-	-2.5	5.07E-02	-14.4	-	up
ENSONIG00000003747	*ttc14*	Ensembl_nile_tilapia	6.54E-03	up	-	down	6.84E-03	up	-	1.0
290793107	GU217573.1	nt	-	0.00	-	0.0	-	up	1.57E-20	up
ENSGACG00000016986	*trip4*	Ensembl_stickleback	1.61E-02	up	-	up	-	-1.0	-	up
ENSDARG00000002909	*tjp3*	Ensembl_zebrafish	-	1.2	-	1.1	1.59E-02	11.6	2.14E-02	12.3
ENSGACG00000003028	*timp2 (2 of 2)*	Ensembl_stickleback	-	0.00	-	down	2.75E-04	down	-	0.0
ENSORLG00000012522	*tsta3*	Ensembl_medaka	2.00E-02	down	3.88E-05	down	-	0.0	-	0.0
ENSDARG00000063420	*tox4 (2 of 2)*	Ensembl_zebrafish	-	-15.1	6.40E-02	23.9	-	4.7	-	down
ENSGACG00000017200	*taldo1*	Ensembl_stickleback	-	down	2.29E-02	down	-	-1.6	-	0.0
ENSONIG00000009845	*tf*	Ensembl_nile_tilapia	-	down	9.01E-02	up	8.78E-08	down	-	-1.1
ENSGACG00000019195	*tmc7*	Ensembl_stickleback	5.86E-02	up	-	-1.3	-	down	-	up
ENSGACG00000015584	*tmed2*	Ensembl_stickleback	-	0.00	-	0.0	8.79E-02	down	-	0.0
ENSGACG00000016363	*tmed9*	Ensembl_stickleback	-	1.8	-	-2.7	6.04E-03	-22.0	-	up
ENSGACG00000004023	*tmem106c*	Ensembl_stickleback	-	0.00	1.71E-02	-15.7	-	0.0	-	down
ENSGACG00000009084	*tmem136 (2 of 2)*	Ensembl_stickleback	2.50E-02	down	-	up	8.79E-02	up	-	0.0
ENSGACG00000013071	*tmem161a*	Ensembl_stickleback	-	up	5.69E-02	-5.4	-	0.0	3.31E-05	up
ENSGACG00000016548	*tmem38b*	Ensembl_stickleback	4.11E-07	43.7	-	1.1	-	-1.3	-	5.6
ENSGACG00000003118	*tmem42*	Ensembl_stickleback	-	-4.4	-	0.0	2.01E-17	up	-	down
ENSGACG00000004640	*tmem64*	Ensembl_stickleback	-	-1.8	-	-4.3	-	-5.3	2.31E-02	down
ENSGACG00000015841	*tnpo1*	Ensembl_stickleback	-	down	-	down	3.24E-03	down	-	-1.1
ENSGACG00000007382	*trim36*	Ensembl_stickleback	-	-7.5	-	-4.0	8.81E-02	-10.2	-	-7.6
ENSDARG00000028027	*trim63*	Ensembl_zebrafish	-	-1.7	-	1.0	2.69E-02	-16.5	-	down
ENSGACG00000004198	*tnnc2 (1 of 2)*	Ensembl_stickleback	-	-6.2	1.17E-03	-12.4	3.31E-04	-12.8	1.38E-03	-11.1
ENSDARG00000042993	*try*	Ensembl_zebrafish	-	1.2	1.73E-07	-32.3	-	-1.1	-	down
229366578	*acq58269.1*	nr	3.04E-05	19.4	-	0.0	-	0.0	-	0.0
ENSONIG00000003233	*tdrd3*	Ensembl_nile_tilapia	-	-1.5	-	1.6	5.65E-02	up	-	-3.3
ENSGACG00000009537	*tpcn1*	Ensembl_stickleback	-	1.4	-	-4.7	-	2.2	9.97E-03	-9.0
ENSDARG00000036832	*cyt1l*	Ensembl_zebrafish	-	1.1	3.95E-03	9.4	-	2.6	-	1.0
ENSGACG00000005854	*uchl5*	Ensembl_stickleback	-	down	-	0.0	4.49E-02	up	-	up
ENSDARG00000087495	*usp2 (2 of 3)*	Ensembl_zebrafish	-	2.4	-	2.2	3.36E-02	6.6	-	1.7
ENSGACG00000011028	*usp36*	Ensembl_stickleback	1.08E-02	-34.3	2.07E-03	down	1.42E-03	down	2.53E-05	-90.0
ENSDARG00000007714	*ube2q1*	Ensembl_zebrafish	-	-3.7	3.54E-02	up	-	1.4	-	0.0
ENSDARG00000011537	*ugt2a5*	Ensembl_zebrafish	-	down	-	down	1.91E-07	down	-	-1.6
ENSDARG00000039501	*ugt2a6*	Ensembl_zebrafish	-	0.00	-	down	4.36E-09	down	-	0.0
ENSORLG00000007327	*galntl4*	Ensembl_medaka	-	down	-	0.0	1.96E-02	down	-	0.0
ENSGACG00000009985	*unc45b*	Ensembl_stickleback	-	1.8	-	up	6.59E-02	-5.5	-	-6.7
ENSDARG00000078382	CABZ01075938.1	Ensembl_zebrafish	-	-1.6	6.21E-08	up	9.52E-06	up	5.91E-02	up
ENSDARG00000087843	CABZ01092722.1	Ensembl_zebrafish	-	-1.1	-	3.0	-	1.3	2.50E-02	-6.9
ENSDARG00000088309	*ct573337.1*	Ensembl_zebrafish	3.75E-02	8.5	-	up	-	up	-	0.0
116517246	NP_001070844.1	Refseq_proteins	-	1.2	-	1.0	2.26E-02	24.6	-	2.8
158534007	NP_001103579.1	Refseq_proteins	-	0.00	-	down	-	0.0	8.84E-12	-20469
ENSGACG00000017435	*upf3b*	Ensembl_stickleback	2.50E-02	down	-	0.0	1.50E-04	down	-	0.0
ENSGACG00000014740	*utp18*	Ensembl_stickleback	-	up	-	0.0	1.28E-02	up	-	0.0
ENSGACG00000005430	*crk (1 of 2)*	Ensembl_stickleback	4.69E-02	down	-	-1.6	-	1.4	-	down
ENSGACG00000005867	*vsx2*	Ensembl_stickleback	7.32E-02	down	-	1.0	-	1.8	-	-1.2
ENSORLG00000016336	*vmo1*	Ensembl_medaka	-	0.00	9.89E-05	-17.4	-	up	-	-2.2
ENSDARG00000055809	*vtg2*	Ensembl_zebrafish	-	-1.2	-	2.7	-	-1.5	9.15E-33	20268.3
ENSDARG00000092419	*vtg7*	Ensembl_zebrafish	-	-1.5	-	-1.6	-	-1.4	2.57E-15	317.5
ENSGACG00000007380	*wdr61*	Ensembl_stickleback	1.36E-02	down	3.54E-02	down	-	0.0	-	down
ENSORLG00000005229	*wls*	Ensembl_medaka	-	2.8	-	-1.2	9.04E-04	down	-	down
ENSGACG00000019114	*xrcc6bp1*	Ensembl_stickleback	7.95E-05	23.6	-	up	-	-1.0	-	down
ENSGACG00000020724	*yipf5*	Ensembl_stickleback	-	down	-	down	3.65E-02	down	-	up
238943009	CU695216.16	nt	-	1.3	1.76E-04	up	-	5.1	-	1.2
156764000	CU464084.9	nt	-	1.6	2.85E-02	-63.5	-	-2.6	-	-1.4
ENSDARG00000093844	zgc:136461	Ensembl_zebrafish	-	down	2.78E-07	-34.5	-	0.0	-	down
ENSDARG00000079274	zgc:66382	Ensembl_zebrafish	-	down	4.52E-06	-20.9	-	1.4	-	down
ENSGACG00000015779	*zfhx3*	Ensembl_stickleback	-	1.0	4.30E-03	down	4.65E-02	-7.9	3.74E-02	down
ENSGACG00000002418	*zfhx4*	Ensembl_stickleback	-	-3.9	-	-6.0	-	-1.7	6.34E-11	-403.4
ENSGACG00000002626	*znf423*	Ensembl_stickleback	-	-16.1	1.49E-02	down	1.98E-03	-53.9	-	down
ENSGACG00000016481	*znf710 (1 of 2)*	Ensembl_stickleback	-	up	-	up	3.02E-02	up	-	3.2
ENSGACG00000010661	*znf750*	Ensembl_stickleback	-	-1.1	9.89E-05	down	-	up	-	down
ENSGACG00000005282	*zcchc11*	Ensembl_stickleback	-	1.3	-	-1.4	-	2.2	5.72E-03	-9.7
ENSGACG00000009457	*zranb2*	Ensembl_stickleback	-	1.0	-	down	-	-2.1	5.91E-02	up
ENSGACG00000005043	*zwilch*	Ensembl_stickleback	-	down	-	down	1.28E-02	down	-	down
CX256055	gb|CX256055.1	EST_others	-	0.00	1.29E-03	-23.7	-	0.0	-	1.4
CX261231	gb|CX261231.1	EST_others	-	0.00	-	0.0	-	0.0	7.12E-03	11.2
CX262650	gb|CX262650.1	EST_others	1.36E-02	up	-	1.0	1.59E-02	up	-	0.0
CX035424	gb|CX035424.1	EST_others	-	up	-	1.0	3.02E-02	down	-	0.0
CX036514	gb|CX036514.1	EST_others	-	1.4	2.85E-02	up	-	1.4	-	up
CX039308	gb|CX039308.1	EST_others	-	1.3	4.12E-02	-11.4	-	-2.0	-	1.4
ENSGACG00000012314	*nt5dc2*	Ensembl_stickleback	-	down	7.27E-02	down	9.04E-04	down	-	down
CA350320	gb|CA350320.1	EST_others	-	0.00	-	1.3	4.49E-02	down	-	up
CA367945	gb|CA367945.1	EST_others	-	1.4	-	0.0	1.59E-02	up	-	0.0
CA376054	gb|CA376054.1	EST_others	-	-3.6	9.01E-02	down	-	3.0	-	down
CA383249	gb|CA383249.1	EST_others	-	-1.1	-	1.5	-	up	6.86E-02	23.1
BX079066	emb|BX079066.3	EST_others	-	0.00	-	2.3	3.65E-02	-31.7	-	0.0

BX081886	emb|BX081886.2	EST_others	-	-1.4	2.87E-03	down	-	-1.3	-	1.9
BX085529	emb|BX085529.3	EST_others	-	-1.5	-	-1.9	4.39E-02	-6.4	-	1.0
BX860267	emb|BX860267.3	EST_others	-	0.00	6.34E-04	-22.7	-	-1.4	-	0.0
BX862653	emb|BX862653.3	EST_others	-	0.00	-	0.0	7.19E-06	down	4.78E-02	down
BX862659	emb|BX862659.3	EST_others	-	2.4	-	1.7	-	1.7	4.71E-06	up
BX863438	emb|BX863438.3	EST_others	-	-1.5	-	0.0	1.22E-03	down	-	down
BX866879	emb|BX866879.3	EST_others	-	-1.7	-	up	-	up	1.19E-02	up
BX871489	emb|BX871489.2	EST_others	-	-1.1	5.82E-03	-10.2	-	up	-	down
ENSGACG00000000665	ENSGACG00000000665	Ensembl_stickleback	-	down	-	down	3.11E-02	-32.4	3.24E-02	-29.2
ENSGACG00000001046	ENSGACG00000001046	Ensembl_stickleback	-	0.00	5.20E-03	up	-	up	-	0.0
ENSGACG00000001103	ENSGACG00000001103	Ensembl_stickleback	8.33E-04	-53.1	-	1.1	-	-1.3	-	2.3
ENSGACG00000002397	ENSGACG00000002397	Ensembl_stickleback	-	-1.2	-	down	-	-17.9	5.56E-10	up
ENSGACG00000003684	ENSGACG00000003684	Ensembl_stickleback	2.50E-02	down	-	1.1	-	1.4	9.95E-02	up
ENSGACG00000003787	ENSGACG00000003787	Ensembl_stickleback	-	1.0	4.56E-06	up	-	0.0	-	up
ENSGACG00000004988	ENSGACG00000004988	Ensembl_stickleback	-	down	-	0.0	1.95E-02	-34.6	1.00E-02	down
ENSGACG00000005457	ENSGACG00000005457	Ensembl_stickleback	-	up	1.20E-06	128.1	-	up	-	down
ENSGACG00000005610	ENSGACG00000005610	Ensembl_stickleback	-	-1.4	7.31E-06	-20.2	-	-1.1	-	1.2
ENSGACG00000006023	ENSGACG00000006023	Ensembl_stickleback	-	1.3	-	3.7	2.43E-02	up	-	0.0
ENSGACG00000006109	ENSGACG00000006109	Ensembl_stickleback	-	-2.6	7.81E-02	-6.9	3.76E-02	-6.3	9.93E-06	-36.5
ENSGACG00000006376	ENSGACG00000006376	Ensembl_stickleback	-	-3.3	2.28E-02	-9.6	-	-1.4	-	-2.1
ENSGACG00000006658	ENSGACG00000006658	Ensembl_stickleback	7.32E-02	up	2.85E-02	up	-	-1.1	-	0.0
ENSGACG00000007518	ENSGACG00000007518	Ensembl_stickleback	-	2.6	-	up	8.30E-03	down	5.91E-02	down
ENSGACG00000007857	ENSGACG00000007857	Ensembl_stickleback	-	0.00	-	0.0	1.07E-03	down	-	0.0
ENSGACG00000007990	ENSGACG00000007990	Ensembl_stickleback	2.78E-03	down	-	1.3	3.90E-03	down	-	down
ENSGACG00000008104	ENSGACG00000008104	Ensembl_stickleback	-	-1.4	1.36E-03	-11.0	-	1.7	-	-1.8
ENSGACG00000008362	ENSGACG00000008362	Ensembl_stickleback	-	0.00	-	down	-	up	3.31E-05	down
ENSGACG00000008527	ENSGACG00000008527	Ensembl_stickleback	-	up	-	0.0	-	0.0	4.66E-03	down
ENSGACG00000008544	ENSGACG00000008544	Ensembl_stickleback	1.61E-02	down	-	1.6	-	-1.6	-	-1.2
ENSGACG00000008811	ENSGACG00000008811	Ensembl_stickleback	-	0.00	-	0.0	8.30E-03	down	-	0.0
ENSGACG00000009769	ENSGACG00000009769	Ensembl_stickleback	-	down	-	up	-	down	3.46E-09	990.0
ENSGACG00000010278	ENSGACG00000010278	Ensembl_stickleback	-	-1.1	-	down	4.99E-02	-8.7	-	-3.1
ENSGACG00000010455	ENSGACG00000010455	Ensembl_stickleback	1.27E-03	28.4	-	0.0	-	up	-	0.0
ENSGACG00000011411	ENSGACG00000011411	Ensembl_stickleback	-	-6.8	-	-1.7	7.05E-02	-13.1	-	-5.4
ENSGACG00000011761	ENSGACG00000011761	Ensembl_stickleback	-	2.8	1.19E-02	up	-	-8.0	-	up
ENSGACG00000012223	ENSGACG00000012223	Ensembl_stickleback	-	up	9.01E-02	up	-	-1.9	-	-1.4
ENSGACG00000012735	ENSGACG00000012735	Ensembl_stickleback	-	down	6.21E-08	down	-	down	-	up
ENSGACG00000012810	ENSGACG00000012810	Ensembl_stickleback	-	1.1	-	0.0	2.75E-04	down	-	0.0
ENSGACG00000012940	ENSGACG00000012940	Ensembl_stickleback	9.44E-05	up	2.33E-05	51.6	-	-1.3	-	-12.1
ENSGACG00000013362	ENSGACG00000013362	Ensembl_stickleback	-	down	-	down	1.04E-02	down	-	down
ENSGACG00000013443	ENSGACG00000013443	Ensembl_stickleback	-	down	-	0.0	8.79E-02	down	-	2.1
ENSGACG00000013510	ENSGACG00000013510	Ensembl_stickleback	-	-2.5	-	0.0	-	-5.5	9.86E-13	up
ENSGACG00000013819	ENSGACG00000013819	Ensembl_stickleback	-	2.5	-	1.0	3.90E-03	up	-	2.6
ENSGACG00000014561	ENSGACG00000014561	Ensembl_stickleback	-	0.00	4.56E-06	-21.0	-	up	-	-2.2
ENSGACG00000014922	ENSGACG00000014922	Ensembl_stickleback	-	down	-	down	6.81E-04	down	4.78E-02	down
ENSGACG00000015113	ENSGACG00000015113	Ensembl_stickleback	1.53E-03	down	1.20E-06	down	4.79E-06	down	-	-5.7
ENSGACG00000016237	ENSGACG00000016237	Ensembl_stickleback	-	1.1	-	1.2	9.52E-06	up	-	0.0
ENSGACG00000016744	ENSGACG00000016744	Ensembl_stickleback	-	0.00	-	7.5	-	up	5.91E-02	down
ENSGACG00000017541	ENSGACG00000017541	Ensembl_stickleback	-	-1.6	3.51E-09	-180.7	-	1.2	-	up
ENSGACG00000017983	ENSGACG00000017983	Ensembl_stickleback	-	down	-	0.0	8.60E-08	-110.3	-	0.0
ENSGACG00000018030	ENSGACG00000018030	Ensembl_stickleback	-	-5.1	-	-2.6	1.25E-04	-41.5	-	-2.2
ENSGACG00000018802	ENSGACG00000018802	Ensembl_stickleback	-	0.00	9.86E-07	down	1.28E-06	down	-	down
ENSGACG00000019767	ENSGACG00000019767	Ensembl_stickleback	-	0.00	-	up	6.85E-02	-12.0	-	down
ENSGACG00000020439	ENSGACG00000020439	Ensembl_stickleback	1.08E-02	-34.4	1.08E-03	up	-	-5.5	-	down
ENSGACG00000020467	ENSGACG00000020467	Ensembl_stickleback	-	0.00	-	down	3.59E-04	down	7.28E-04	down
ENSGACG00000020921	ENSGACG00000020921	Ensembl_stickleback	2.14E-06	down	7.73E-07	-65.9	3.05E-10	-226.8	7.38E-10	-303.9
ENSONIG00000002827	ENSONIG00000002827	Ensembl_nile_tilapia	-	up	6.22E-03	up	-	down	-	-1.0
ENSONIG00000004540	ENSONIG00000004540	Ensembl_nile_tilapia	-	-1.2	-	2.9	-	-1.7	2.31E-02	down
ENSONIG00000007219	ENSONIG00000007219	Ensembl_nile_tilapia	-	-1.4	2.12E-06	55.2	-	2.8	-	-1.1
ENSONIG00000009825	ENSONIG00000009825	Ensembl_nile_tilapia	-	0.00	-	-2.1	-	5.1	3.37E-15	225.1
ENSONIG00000011792	ENSONIG00000011792	Ensembl_nile_tilapia	-	0.00	2.37E-03	-18.5	-	0.0	-	0.0
ENSONIG00000013985	ENSONIG00000013985	Ensembl_nile_tilapia	-	1.1	-	-1.6	5.07E-02	20.1	-	2.3
ENSONIG00000017323	ENSONIG00000017323	Ensembl_nile_tilapia	-	-1.5	-	-4.0	1.30E-02	-12.0	-	-1.4
ENSONIG00000019573	ENSONIG00000019573	Ensembl_nile_tilapia	-	-1.3	-	-1.4	-	-2.0	7.68E-02	down
ENSORLG00000000460	ENSORLG00000000460	Ensembl_medaka	-	0.00	-	2.1	7.87E-07	143.3	-	-5.8
ENSORLG00000001173	ENSORLG00000001173	Ensembl_medaka	-	1.1	-	3.7	-	1.2	7.68E-02	down
ENSORLG00000001190	ENSORLG00000001190	Ensembl_medaka	-	1.3	6.31E-02	6.1	-	down	-	-2.2
ENSORLG00000003700	ENSORLG00000003700	Ensembl_medaka	-	-2.5	-	down	-	0.0	3.47E-03	-44.5
ENSORLG00000010522	ENSORLG00000010522	Ensembl_medaka	-	0.00	-	0.0	-	0.0	6.43E-04	up
ENSORLG00000011293	ENSORLG00000011293	Ensembl_medaka	-	0.00	4.90E-02	9.7	-	0.0	-	0.0
ENSORLG00000011321	ENSORLG00000011321	Ensembl_medaka	-	0.00	6.16E-05	down	-	2.1	-	-1.9
ENSORLG00000012785	ENSORLG00000012785	Ensembl_medaka	-	-4.9	-	-3.7	1.17E-02	-39.9	-	down
ENSORLG00000013333	ENSORLG00000013333	Ensembl_medaka	-	0.00	-	0.0	-	0.0	1.13E-12	up
ENSORLG00000019280	ENSORLG00000019280	Ensembl_medaka	1.36E-03	21.0	-	up	-	up	-	0.0
ENSORLG00000020091	ENSORLG00000020091	Ensembl_medaka	1.61E-02	down	-	down	-	-1.5	-	down
EV367322	gb|EV367322.1	EST_others	-	down	-	down	1.28E-02	down	-	down
EV368845	gb|EV368845.1	EST_others	-	-1.7	-	up	1.09E-02	-10.4	-	0.0
EV370019	gb|EV370019.1	EST_others	-	1.5	-	-2.4	8.79E-02	-8.6	-	up
EV370268	gb|EV370268.1	EST_others	-	7.7	6.31E-02	14.6	-	4.6	-	6.5
FK872811	gb|FK872811.1	EST_others	-	2.2	-	4.1	2.99E-03	9.4	-	2.8
FK872812	gb|FK872812.1	EST_others	8.88E-02	-22.8	-	0.0	-	-1.4	-	0.0
FK882798	gb|FK882798.1	EST_others	-	2.6	7.27E-02	down	-	-1.4	-	down
FK869364	gb|FK869364.1	EST_others	-	0.00	7.64E-03	down	2.28E-03	down	-	down
FK879202	gb|FK879202.1	EST_others	3.75E-02	down	-	down	-	up	-	0.0
GW640860	gb|GW640860.1	EST_others	-	5.9	-	-1.1	8.30E-03	down	-	1.7
GE839897	gb|GE839897.1	EST_others	-	-1.6	-	-1.3	1.82E-03	-14.9	-	1.5
GE835715	gb|GE835715.1	EST_others	-	4.7	-	-2.6	4.49E-02	up	-	0.0
EL553118	gb|EL553118.1	EST_others	-	-1.7	-	-2.6	-	up	2.48E-03	11.5
EG791873	gb|EG791873.1	EST_others	-	-1.8	-	-1.2	6.16E-02	-10.0	-	1.5
EG793024	gb|EG793024.1	EST_others	-	down	-	0.0	4.49E-02	down	-	0.0
EG795706	gb|EG795706.1	EST_others	-	up	-	0.0	5.85E-04	down	-	down
EG804652	gb|EG804652.1	EST_others	2.08E-03	down	-	-2.0	3.02E-05	-21.9	1.37E-03	down
EG807134	gb|EG807134.1	EST_others	-	3.1	9.89E-05	up	-	1.9	-	-1.2
EG822862	gb|EG822862.1	EST_others	-	up	1.49E-02	down	-	0.0	-	0.0
EG824531	gb|EG824531.1	EST_others	5.86E-02	down	-	1.0	-	-1.9	-	0.0
EG824683	gb|EG824683.1	EST_others	-	-2.4	1.84E-02	down	-	-2.3	-	down
EG806136	gb|EG806136.1	EST_others	-	0.00	5.59E-02	down	2.75E-04	down	-	down
EG785970	gb|EG785970.1	EST_others	1.18E-03	down	2.85E-02	down	4.63E-10	down	-	down
EG776240	gb|EG776240.1	EST_others	4.99E-10	88.9	-	up	-	up	-	0.0
EG801892	gb|EG801892.1	EST_others	-	-1.0	-	1.9	-	down	1.00E-02	up
EG802309	gb|EG802309.1	EST_others	-	4.1	-	9.2	8.79E-02	down	-	up
EG803435	gb|EG803435.1	EST_others	-	1.1	-	up	2.24E-02	-14.3	-	0.0
EG835764	gb|EG835764.1	EST_others	-	-1.3	-	down	2.51E-04	-74.5	-	-2.2

EG838512	gb|EG838512.1	EST_others	2.20E-02	8.1	-	1.0	-	0.0	-	up
EG855707	gb|EG855707.1	EST_others	1.15E-02	up	-	0.0	3.02E-02	down	-	0.0
EG879755	gb|EG879755.1	EST_others	-	down	-	0.0	2.43E-02	down	-	0.0
EG879910	gb|EG879910.1	EST_others	-	3.7	-	-1.3	1.96E-02	up	-	1.4
EG881910	gb|EG881910.1	EST_others	-	down	3.54E-02	down	-	0.0	-	0.0
EG876254	gb|EG876254.1	EST_others	3.75E-02	down	2.85E-02	down	-	-4.5	-	1.8
EG878528	gb|EG878528.1	EST_others	1.27E-03	11.8	-	up	-	1.8	-	0.0
EG880230	gb|EG880230.1	EST_others	-	2.7	-	-1.2	6.99E-02	up	-	-2.9
EG881131	gb|EG881131.1	EST_others	-	down	-	6.7	9.52E-06	down	-	0.0
EG882237	gb|EG882237.1	EST_others	-	21.3	-	up	6.47E-04	33.0	-	4.9
EG882682	gb|EG882682.1	EST_others	-	down	-	down	8.79E-02	down	-	down
EG866124	gb|EG866124.1	EST_others	-	-1.4	-	-1.2	5.77E-02	-5.7	-	down
EG867991	gb|EG867991.1	EST_others	-	1.5	-	-1.2	2.93E-06	up	-	2.0
EG868203	gb|EG868203.1	EST_others	-	4.1	-	2.5	2.99E-03	-24.2	-	up
EG869982	gb|EG869982.1	EST_others	7.42E-04	down	-	down	-	0.0	-	0.0
EG870274	gb|EG870274.1	EST_others	-	4.3	-	down	-	-1.2	1.48E-02	up
EG840326	gb|EG840326.1	EST_others	-	5.6	-	down	8.79E-02	down	-	0.0
EG840695	gb|EG840695.1	EST_others	-	1.2	-	-1.7	7.46E-02	-6.0	-	1.0
EG844851	gb|EG844851.1	EST_others	5.73E-03	-11.1	-	up	-	0.0	-	0.0
EG833455	gb|EG833455.1	EST_others	-	0.00	4.41E-02	up	1.96E-02	down	-	-4.3
EG859641	gb|EG859641.1	EST_others	-	-1.7	-	-3.1	2.33E-02	-10.8	-	-1.1
EG860536	gb|EG860536.1	EST_others	-	down	-	down	2.43E-02	down	-	down
EG849257	gb|EG849257.1	EST_others	-	-2.7	-	down	6.99E-02	down	-	0.0
EG907993	gb|EG907993.1	EST_others	-	0.00	-	0.0	7.05E-02	-26.3	-	0.0
EG908200	gb|EG908200.1	EST_others	-	-2.9	-	-1.4	3.24E-03	down	-	3.5
EG908809	gb|EG908809.1	EST_others	-	down	-	down	4.54E-05	down	-	down
EG911244	gb|EG911244.1	EST_others	-	-2.5	-	-1.8	5.01E-02	-6.8	-	up
EG912415	gb|EG912415.1	EST_others	-	0.00	-	-3.4	-	-1.6	7.68E-02	down
EG913326	gb|EG913326.1	EST_others	-	0.00	-	down	3.65E-02	down	-	down
EG914877	gb|EG914877.1	EST_others	-	2.8	-	1.0	4.22E-03	-13.1	-	1.0
EG942150	gb|EG942150.1	EST_others	-	2.4	-	down	7.56E-02	-6.0	-	down
EG922711	gb|EG922711.1	EST_others	-	down	-	0.0	3.11E-02	-15.9	-	0.0
EG935627	gb|EG935627.1	EST_others	3.15E-02	down	-	-1.1	7.15E-02	-5.1	-	-1.1
EG937327	gb|EG937327.1	EST_others	-	-1.5	2.85E-02	down	-	1.1	-	1.3
EG937416	gb|EG937416.1	EST_others	-	0.00	-	2.9	4.49E-02	up	-	1.9
EG942312	gb|EG942312.1	EST_others	-	-12.9	-	down	2.07E-03	-13.1	-	-5.4
EG899717	gb|EG899717.1	EST_others	-	down	-	-1.4	3.54E-02	7.2	-	down
EG926272	gb|EG926272.1	EST_others	-	0.00	-	down	2.43E-02	down	-	down
EG903128	gb|EG903128.1	EST_others	-	down	5.20E-03	down	2.70E-08	down	-	down
EG928582	gb|EG928582.1	EST_others	-	down	-	down	5.65E-02	down	-	down
EG931057	gb|EG931057.1	EST_others	-	down	-	0.0	-	down	3.00E-03	-15.0
EG932783	gb|EG932783.1	EST_others	-	-1.1	-	1.3	-	-1.7	5.91E-02	up
EG889573	gb|EG889573.1	EST_others	-	1.6	4.41E-02	up	-	3.3	-	up
EG934489	gb|EG934489.1	EST_others	9.28E-02	up	-	up	-	up	-	down
EG898924	gb|EG898924.1	EST_others	1.53E-03	down	-	down	2.73E-05	down	1.18E-03	down
EG901696	gb|EG901696.1	EST_others	-	-2.5	-	1.0	1.60E-02	-18.6	-	down
EG902053	gb|EG902053.1	EST_others	-	1.0	-	-2.1	3.65E-02	down	-	0.0
EG885127	gb|EG885127.1	EST_others	2.00E-02	down	-	-2.5	-	-1.6	-	1.3
EG885488	gb|EG885488.1	EST_others	-	-2.5	-	-3.0	2.28E-03	down	-	-2.7
EG886211	gb|EG886211.1	EST_others	-	up	-	0.0	2.73E-05	-103.6	-	down
EG888853	gb|EG888853.1	EST_others	-	1.5	2.07E-03	down	-	2.2	-	1.7
EG889122	gb|EG889122.1	EST_others	-	0.00	-	-3.7	6.99E-02	down	-	-2.0
EG890543	gb|EG890543.1	EST_others	-	down	-	down	4.20E-04	down	-	0.0
EG893526	gb|EG893526.1	EST_others	-	2.0	-	-1.1	3.11E-02	-7.1	-	down
EG917093	gb|EG917093.1	EST_others	-	down	-	down	2.77E-03	down	-	up
EG917122	gb|EG917122.1	EST_others	-	-3.3	1.50E-03	down	1.61E-05	down	-	1.1
EG918173	gb|EG918173.1	EST_others	-	2.1	-	9.4	8.10E-03	10.2	-	2.5
EG918652	gb|EG918652.1	EST_others	-	down	-	down	8.78E-02	-24.8	-	-2.2
EG920601	gb|EG920601.1	EST_others	-	down	-	-2.1	8.79E-02	down	-	0.0
EG904422	gb|EG904422.1	EST_others	-	0.00	-	0.0	1.59E-02	down	-	0.0
EG907324	gb|EG907324.1	EST_others	-	0.00	-	1.2	6.84E-03	up	-	23.1
DW532716	gb|DW532716.1	EST_others	-	0.00	3.98E-06	-37.4	-	0.0	-	0.0
DW533681	gb|DW533681.1	EST_others	-	0.00	6.22E-03	down	-	0.0	-	0.0
DW533803	gb|DW533803.1	EST_others	-	-1.6	5.76E-04	up	-	up	-	0.0
DY692525	gb|DY692525.1	EST_others	5.86E-02	down	5.59E-02	down	-	down	-	down
DY692639	gb|DY692639.1	EST_others	-	0.00	1.75E-03	-21.5	-	up	-	-4.3
EG755292	gb|EG755292.1	EST_others	-	0.00	5.59E-02	down	-	0.0	-	0.0
DW557302	gb|DW557302.1	EST_others	6.83E-05	up	-	3.8	-	up	-	down
DW557640	gb|DW557640.1	EST_others	-	-1.3	-	-2.1	6.85E-02	-11.0	-	-4.3
DW565146	gb|DW565146.1	EST_others	5.86E-02	down	-	down	3.59E-04	-27.7	-	1.8
DW565771	gb|DW565771.1	EST_others	-	0.00	-	8.7	7.05E-02	18.8	-	0.0
DW566521	gb|DW566521.1	EST_others	-	down	9.39E-03	down	-	down	-	0.0
DW567486	gb|DW567486.1	EST_others	-	3.2	-	6.8	3.92E-03	12.8	-	-1.1
DW568758	gb|DW568758.1	EST_others	6.54E-03	down	7.76E-04	down	8.45E-06	down	-	0.0
DW570084	gb|DW570084.1	EST_others	-	0.00	3.65E-03	-17.2	-	0.0	-	down
DW539469	gb|DW539469.1	EST_others	-	-4.2	-	down	6.99E-02	up	-	up
DW575959	gb|DW575959.1	EST_others	-	-6.9	-	up	4.49E-02	down	-	0.0
DW576807	gb|DW576807.1	EST_others	-	2.4	-	-6.2	1.36E-02	-19.3	-	-1.1
DW577325	gb|DW577325.1	EST_others	-	down	-	down	7.89E-04	down	-	down
DW578341	gb|DW578341.1	EST_others	-	1.2	3.54E-02	down	-	up	-	down
DW581115	gb|DW581115.1	EST_others	-	2.1	-	3.5	1.96E-03	up	-	up
DY697537	gb|DY697537.1	EST_others	-	0.00	-	0.0	3.02E-02	down	-	0.0
DW541038	gb|DW541038.1	EST_others	-	down	-	down	3.65E-02	down	-	down
DY701339	gb|DY701339.1	EST_others	-	down	-	down	7.24E-05	down	-	down
DY705472	gb|DY705472.1	EST_others	-	0.00	4.56E-06	-40.6	-	0.0	-	down
DW542323	gb|DW542323.1	EST_others	-	1.2	4.41E-02	down	-	-2.7	-	-1.1
DY715145	gb|DY715145.1	EST_others	-	down	-	up	-	up	1.57E-03	up
DY715437	gb|DY715437.1	EST_others	-	down	-	0.0	4.39E-02	-15.2	-	0.0
DY715711	gb|DY715711.1	EST_others	-	up	3.88E-05	up	-	-1.7	7.28E-04	up
DY720448	gb|DY720448.1	EST_others	-	0.00	2.44E-06	-48.0	-	0.0	-	up
DY720838	gb|DY720838.1	EST_others	-	0.00	-	-1.4	4.41E-02	-13.0	-	down
DY726856	gb|DY726856.1	EST_others	1.81E-04	up	-	up	-	1.1	-	0.0
DY729177	gb|DY729177.1	EST_others	1.34E-03	down	9.01E-02	down	1.28E-02	down	-	-1.6
DY735271	gb|DY735271.1	EST_others	-	-1.7	-	1.3	2.69E-02	-21.7	-	up
DY735321	gb|DY735321.1	EST_others	-	0.00	-	0.0	-	0.0	5.87E-02	24.1
DY735436	gb|DY735436.1	EST_others	-	1.2	-	0.0	8.79E-02	down	-	down
DY737082	gb|DY737082.1	EST_others	-	-1.3	-	-3.6	4.39E-02	-15.2	-	up
DW545057	gb|DW545057.1	EST_others	4.69E-02	down	-	1.2	-	2.8	-	1.1
GE767884	gb|GE767884.1	EST_others	-	-2.1	5.59E-02	down	5.88E-06	-48.9	-	down
GE775959	gb|GE775959.1	EST_others	-	8.7	-	3.1	7.05E-02	18.1	-	-1.1
GE780214	gb|GE780214.1	EST_others	-	-1.3	-	-2.4	8.79E-02	down	-	down
GE795073	gb|GE795073.1	EST_others	-	down	-	down	6.99E-02	down	-	0.0

GE783177	gb|GE783177.1	EST_others	-	0.00	-	down	8.79E-02	down	-	0.0
GE794330	gb|GE794330.1	EST_others	-	1.3	-	-2.0	-	-1.0	6.26E-06	60.2
GE792696	gb|GE792696.1	EST_others	-	-1.5	2.18E-02	-6.3	-	1.0	-	1.9
GE793450	gb|GE793450.1	EST_others	-	up	-	1.4	2.43E-02	up	-	0.0
GO044564	gb|GO044564.1	EST_others	1.61E-02	down	-	-1.8	9.10E-02	-5.6	-	down
GO045464	gb|GO045464.1	EST_others	-	-2.2	-	-2.6	8.30E-03	-17.0	-	-1.2
GO045318	gb|GO045318.1	EST_others	-	20.1	-	up	4.67E-03	down	-	1.5
GO054151	gb|GO054151.1	EST_others	-	down	3.54E-02	down	-	-1.6	-	-6.8
GO055970	gb|GO055970.1	EST_others	-	0.00	4.41E-02	up	-	up	-	8.4
GO063143	gb|GO063143.1	EST_others	-	down	-	down	3.90E-03	down	-	down
GO064603	gb|GO064603.1	EST_others	-	0.00	-	0.0	1.22E-03	down	-	0.0
GO061981	gb|GO061981.1	EST_others	-	-5.2	3.54E-02	-6.5	7.44E-06	-31.5	-	-8.6
GO057448	gb|GO057448.1	EST_others	-	-1.9	3.54E-02	up	6.99E-02	up	-	1.3
GO057929	gb|GO057929.1	EST_others	-	-6.8	-	-2.1	1.28E-06	down	-	down
GO062248	gb|GO062248.1	EST_others	-	-1.5	-	1.1	1.42E-03	-9.3	-	-1.6
GO059796	gb|GO059796.1	EST_others	-	0.00	-	down	3.90E-03	down	-	down
GO064045	gb|GO064045.1	EST_others	-	-4.2	-	-4.1	6.65E-06	-25.5	-	down
GO058122	gb|GO058122.1	EST_others	-	0.00	5.59E-02	up	-	0.0	-	-1.4
GO059623	gb|GO059623.1	EST_others	-	down	-	down	5.66E-03	down	-	down
GO060238	gb|GO060238.1	EST_others	-	-3.2	-	-7.9	2.51E-04	-37.3	-	0.0
EG757222	gb|EG757222.1	EST_others	-	down	-	0.0	3.76E-02	-11.4	-	0.0
EG757601	gb|EG757601.1	EST_others	-	-1.2	-	1.1	4.79E-06	up	-	1.7
BF228600	gb|BF228600.1	EST_others	-	up	-	1.9	8.79E-02	up	-	0.0
CK873433	gb|CK873433.1	EST_others	-	-2.5	7.27E-02	up	-	1.3	-	1.4
CK873617	gb|CK873617.1	EST_others	-	0.00	-	0.0	-	0.0	1.36E-04	down
CK876604	gb|CK876604.1	EST_others	-	3.6	-	2.4	-	down	7.44E-07	60.1
CK893686	gb|CK893686.1	EST_others	-	1.2	-	-10.2	2.47E-02	-11.3	-	1.0
CK899155	gb|CK899155.1	EST_others	-	down	-	down	5.66E-03	down	-	down
CK885162	gb|CK885162.1	EST_others	-	0.00	-	-1.5	2.73E-05	down	-	-1.0
CN181335	gb|CN181335.1	EST_others	-	-3.2	7.85E-02	5.2	-	1.2	-	-1.2
DW471606	gb|DW471606.1	EST_others	-	-3.4	-	-16.5	5.30E-03	-16.4	-	down
DW469525	gb|DW469525.1	EST_others	-	-4.8	-	down	7.05E-02	-17.0	-	down
DW472356	gb|DW472356.1	EST_others	5.86E-02	down	-	0.0	-	0.0	-	0.0
DW473174	gb|DW473174.1	EST_others	-	up	7.64E-03	down	-	-6.3	-	0.0
DW473451	gb|DW473451.1	EST_others	-	0.00	-	1.0	2.27E-04	down	-	0.0
GT129117	gb|GT129117.1	EST_others	-	down	-	0.0	-	-2.1	1.00E-02	up
CB501817	gb|CB501817.1	EST_others	4.69E-02	down	-	0.0	-	0.0	-	0.0
CA037858	gb|CA037858.1	EST_others	-	0.00	-	0.0	-	down	8.31E-04	down
CK990829	gb|CK990829.1	EST_others	-	0.00	-	0.0	-	0.0	7.68E-02	down
CK991140	gb|CK991140.1	EST_others	-	0.00	-	0.0	-	0.0	3.69E-05	down
CA039316	gb|CA039316.1	EST_others	-	0.00	2.04E-04	-65.3	-	0.0	-	0.0
CA038580	gb|CA038580.1	EST_others	1.61E-02	down	-	-1.1	-	0.0	-	-3.6
CB511060	gb|CB511060.1	EST_others	-	0.00	-	down	6.99E-02	down	-	up
CA042597	gb|CA042597.1	EST_others	-	3.2	-	1.9	3.65E-02	7.3	-	up
CA768031	gb|CA768031.1	EST_others	7.32E-02	down	-	down	3.59E-04	down	-	0.0
CA042008	gb|CA042008.1	EST_others	-	-1.6	5.49E-02	5.4	-	2.8	-	1.6
CA054965	gb|CA054965.1	EST_others	-	-1.7	-	1.3	7.56E-02	6.4	-	5.4
CA056117	gb|CA056117.1	EST_others	-	down	-	down	3.90E-03	down	-	down
CA057792	gb|CA057792.1	EST_others	-	down	-	-5.1	1.62E-03	-15.2	-	down
CA054786	gb|CA054786.1	EST_others	-	down	-	down	5.65E-02	down	-	down
CX353553	gb|CX353553.1	EST_others	-	down	-	down	1.42E-03	down	-	down
CA054240	gb|CA054240.1	EST_others	-	down	-	down	1.96E-03	down	-	0.0
CX357249	gb|CX357249.1	EST_others	-	up	-	-4.0	1.96E-02	down	5.60E-04	down
CA058601	gb|CA058601.1	EST_others	-	-6.8	5.76E-04	25.9	-	-2.5	-	down
CB517802	gb|CB517802.1	EST_others	-	3.4	-	down	1.59E-02	down	-	-2.2
CB516646	gb|CB516646.1	EST_others	-	down	-	down	1.59E-02	down	-	0.0
CA049024	gb|CA049024.1	EST_others	-	0.00	-	down	2.43E-02	down	-	up
CB499373	gb|CB499373.1	EST_others	-	down	-	down	2.51E-04	down	-	down
Locus_34577	-	-	-	2.1	-	-1.2	-	up	9.83E-03	14.2
Locus_32303	-	-	-	0.00	-	0.0	-	0.0	1.15E-02	-34.3
Locus_99680	-	-	-	0.00	-	down	-	0.0	1.15E-02	-35.6
Locus_9039	-	-	-	-4.3	-	-1.6	-	1.1	9.95E-02	down
Locus_112355	-	-	-	0.00	-	0.0	-	0.0	1.19E-02	down
Locus_44033	-	-	-	0.00	-	-5.9	-	up	5.91E-02	up
Locus_99603	-	-	-	0.00	-	0.0	-	0.0	3.22E-03	up
Locus_22516	-	-	-	up	-	1.2	-	1.4	3.74E-02	up
Locus_99354	-	-	-	down	-	up	-	up	4.73E-22	up
Locus_36047	-	-	-	1.0	-	1.0	1.28E-02	down	-	down
Locus_215	-	-	-	2.4	5.78E-02	-7.5	-	-1.3	-	-2.1
Locus_109383	-	-	-	-2.5	-	3.9	3.41E-02	-11.7	-	-1.2
Locus_17197	-	-	-	1.7	2.87E-03	down	-	-1.1	-	-1.1
Locus_91597	-	-	-	-1.2	6.48E-02	5.8	-	-1.4	-	1.1
Locus_16875	-	-	-	1.2	9.01E-02	-9.5	-	1.3	-	1.9
Locus_21416	-	-	-	down	-	0.0	2.43E-02	down	-	0.0
Locus_110699	-	-	-	0.00	-	0.0	4.49E-02	down	-	0.0
Locus_21692	-	-	-	0.00	-	0.0	4.49E-02	down	-	0.0
Locus_93618	-	-	-	0.00	-	0.0	1.04E-02	up	-	0.0
Locus_42929	-	-	1.47E-09	358.1	-	0.0	-	0.0	-	0.0
Locus_63842	-	-	3.78E-06	down	-	0.0	-	0.0	-	0.0
Locus_42919	-	-	9.35E-14	up	-	0.0	-	0.0	-	0.0
Locus_43483	-	-	3.91E-08	up	-	0.0	-	0.0	-	0.0
Locus_43115	-	-	1.45E-07	up	-	0.0	-	0.0	-	0.0
Locus_45068	-	-	2.87E-07	up	-	0.0	-	0.0	-	0.0
Locus_43319	-	-	8.82E-07	up	-	0.0	-	0.0	-	0.0
Locus_47644	-	-	2.14E-06	up	-	0.0	-	0.0	-	0.0
Locus_45727	-	-	5.37E-06	up	-	0.0	-	0.0	-	0.0
Locus_43795	-	-	8.08E-06	up	-	0.0	-	0.0	-	0.0
Locus_80358	-	-	1.21E-04	up	-	0.0	-	0.0	-	0.0
Locus_46093	-	-	7.42E-04	up	-	0.0	-	0.0	-	0.0
Locus_80642	-	-	1.15E-02	up	-	0.0	-	0.0	-	0.0
Locus_81054	-	-	1.61E-02	up	-	0.0	-	0.0	-	0.0
Locus_80712	-	-	7.32E-02	up	-	0.0	-	0.0	-	0.0
Locus_56467	-	-	-	2.8	-	1.0	6.04E-02	19.6	-	0.0
Locus_52222	-	-	-	4.7	2.56E-02	10.2	-	up	-	0.0
Locus_90954	-	-	-	0.00	9.17E-02	21.3	-	0.0	-	0.0
Locus_21308	-	-	3.14E-17	up	-	down	-	0.0	-	0.0
Locus_52406	-	-	-	0.00	1.03E-05	up	-	0.0	-	0.0
Locus_43970	-	-	1.76E-07	up	-	0.0	-	0.0	-	up
Locus_21331	-	-	2.80E-15	up	-	0.0	-	0.0	-	up

CHAPTER 3

Identification of conserved hepatic transcriptomic responses to estrogen using high-throughput sequencing in brown trout

Manuscript in preparation

Identification of conserved hepatic transcriptomic responses to estrogen using high-throughput sequencing in brown trout

Tamsyn M. Uren Webster, Janice A. Shears and Eduarda M. Santos

Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

Abstract

High-throughput RNA sequencing (RNA-seq) is a sensitive and reproducible tool for conducting transcriptomic analysis, but to date it has been rarely utilised in ecotoxicology. In particular, a major advantage of this technique is that it can be applied to species for which genomic information is limited. We aimed to apply RNA-seq to investigate the global effects of an endocrine disrupting chemical of environmental concern (17β-estradiol; E2) in an environmentally-relevant species, the brown trout (*Salmo trutta*). To do this, we exposed reproductively mature male brown trout to 1.94, 18.06 and 34.38 ng E2/L for four days, and sequenced three individual liver samples from each treatment on an Illumina HiSeq 2500 platform. Using a *de novo* approach, we assembled a hepatic transcriptome consisting of 172,688 transcripts. Subsequent expression analysis revealed 2113 differentially regulated transcripts in fish exposed to 34.38 ng E2/L, a concentration that has been reported in treated sewage effluent, and may be associated with peaks in agricultural pollution. Functional analysis revealed a strong up-regulation of known estrogen responsive transcripts, including vitellogenins, nothepsin, zona pellucida proteins and *esr1,* together with up-regulation of a number of processes associated with vitellogenesis including lipid transport and metabolism, cellular proliferation and growth, and ribosome biogenesis. This highly conserved response to estrogen exposure raises concerns for the sustainability of trout populations in some of the most contaminated rivers and demonstrates the potential for application of RNA-seq as a sensitive and robust tool for the assessment of the mechanistic effects of pollutants in species of ecological relevance.

Key words: RNA-seq, estrogen, Illumina, brown trout, transcriptomics, sequencing

Introduction

The major endogenous estrogen in vertebrates, 17β-estradiol (E2), is a significant contributor to the estrogenic contamination of surface waters (Desbrow et al. 1998). In addition to input via wastewater treatment work effluents, E2 also enters rivers in livestock and poultry waste (Shore and Shemesh 2003, Shappell et al. 2010). In water bodies, E2 can also act in conjunction with other natural and synthetic estrogenic chemicals (i.e. estrone; ethynylestradiol, phytoestrogens, alkylphenols and other industrial chemicals) to cause adverse effects in natural populations of fish. Reported effects include the induction of intersex in many species including roach (Jobling et al. 1998) and gudgeon (van Aerle et al. 2001), decreased reproductive success in wild fish (Jobling et al. 2002, Harris et al. 2011) and population collapses (Kidd et al. 2007), providing evidence for the risks that estrogens pose to the sustainability of wild fish populations.

The effects of E2, and other estrogenic contaminants, are mediated predominantly via genomic pathways through interaction with nuclear estrogen receptors, which are ligand-dependent transcription factors (Segner et al. 2013). Through this mechanism, estrogen exposure is associated with a highly conserved induction of a well characterised suite of responsive genes. Of these, vitellogenin induction (gene and protein), in male and juvenile fish, has been the most widely used. Estrogen receptor 1 (*esr1*) and zona pellucida proteins are also well characterised markers of estrogen exposure. The transcription of these genes in the liver is known to be strongly associated with stage of vitellogenesis in females, and regulated via estrogen signalling (Arukwe and Goksøyr 2003).

Estrogen receptors can also interact with, and activate, other transcription factors, inducing various downstream signalling cascades. In addition to regulating the reproductive system, they have a crucial role in a diverse range of other physiological processes including skeletal, muscular, cardiovascular, immune and ion-regulatory systems, all of which are therefore potential targets of estrogenic contaminants in fish (Hall et al. 2001, Segner et al. 2013). Transcriptomic approaches have been employed to characterise both the normal endogenous effects of estrogen signalling in females, and the effects of exposure to a number of estrogenic chemicals in male and juvenile fish using microarrays (e.g. Gunnarsson et al. 2007, Benninghoff and Williams 2008, Levi et al. 2009, Katsiadaki et al. 2010) and high-throughput sequencing (RNA-sage)

(Zheng et al. 2013). These studies have reported extensive transcriptional changes, reflecting the diverse range of genes and processes regulated by estrogens, including a number of broadly conserved pathways.

High-throughput RNA sequencing (RNA-seq) has recently emerged as a sensitive and reproducible tool for conducting transcriptomics but, as yet, this approach has rarely been applied to ecotoxicology. A major advantage of this technique is that it can be applied to quantify the transcriptome in species where existing genomic sequence information is limited, and can therefore be used to conduct a non-biased, global mechanistic analysis in any species of interest.

In this project, we employed RNA-seq on an Illumina HiSeq 2500 platform to profile the transcriptome of the brown trout, an ecologically and economically important native European species, known to be sensitive to environmental stressors, including estrogens. Despite its importance, little is known about the impacts of chemical stressors on this species and their relative contribution to population sustainability. E2, originating from agricultural pollution, is likely to be one of the most environmentally relevant chemical contaminants affecting brown trout populations, which typically inhabit, and spawn in, smaller streams within farmland catchments. Therefore, we aimed to characterise the global hepatic transcriptomic responses of male brown trout following exposure to E2, in order to conduct a mechanistic analysis of the effects of this model estrogen at environmentally relevant concentrations. This will provide valuable information for the assessment of the risk that estrogens may pose to natural brown trout populations. We additionally aimed to evaluate the potential of RNA-seq as a tool to evaluate the mechanistic toxicity of environmentally relevant chemicals in non-model, ecologically relevant species. This was achieved by assessing if conserved responses associated with estrogen exposure in other studies were identified among estrogen responsive genes in our experiment.

Materials and methods

Fish maintenance

Sexually mature male brown trout (2 years old) were obtained from a local aquaculture facility in late September, to correspond with the latter stages of reproductive maturation in this species, and maintained in 215 L tanks to allow for acclimation to laboratory conditions for three weeks prior to the start of the exposure. Each tank was aerated, supplied with 430 L/day de-chlorinated tap water (Na^+ = 390; K^+ = 47; Ca^{2+} = 598; Mg^{2+} = 152; Cl^- = 400 µM; pH 7.5), and maintained at 12 ± 0.2 °C. Fish were kept under a 16:8 h light:dark cycle (with 30 minute dawn/dusk transitional periods) and fed with pellet feed (8 mm, Biomar, Grangemouth, UK) at a rate of 2% body weight per day.

Chemical exposures and sampling

Chemical exposure was conducted via a flow through system for a period of 4 days. Fish were exposed to three nominal concentrations, 2.5, 25 and 250 ng E2/L (17β-estradiol ≥98% purity, Sigma) or a dilution water control. Each treatment group consisted of one tank containing 8 individual fish, and the control treatment was run in duplicate. Water samples were collected from each tank on day 3 of the exposure period and stored at -20 °C prior to chemical analysis, using an Enzyme Immunoassay for Estradiol kit (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's instructions. The measured concentrations of E2 in the water were 1.94, 18.06 and 34.38 ng E2/L. The relatively low concentration of E2 measured in the 250 ng E2/L treatment group is likely due to its poor water solubility, given that we performed the exposure without the use of solvents to increase its environmental relevance. Throughout this paper, we refer to the measured concentrations of E2 to indicate the exposure concentrations.

Fish were humanely sacrificed on day four of the exposure period by a lethal dose of benzocaine (0.5 g/L; Sigma-Aldrich) followed by destruction of the brain, in accordance with UK Home Office regulations. Wet weight and fork length were recorded and the condition factor (k= (weight (g) x 100)/ (fork length $(cm)^3$)) was calculated for individual fish. Sex and maturity of all fish was confirmed by observation of the gonads, and gonadsomatic index (GSI) (gonad weight (mg)/ total weight (mg)) x 100)) was

determined. Livers were dissected and weighed, and the hepatosomatic index (HSI) (liver weight (mg)/ total weight (mg)) x 100)) was determined for individual fish. Statistical analysis of morphological parameters was conducted using SigmaStat (version 12.0) for mature males only (n=3-6 per treatment group). All morphometric data met assumptions of normality and equal variance and was analysed using single factor one way analysis of variance (ANOVA). Portions of the liver were snap frozen in liquid nitrogen and stored at -80°C prior to transcript profiling.

RNA extraction, library preparation and sequencing

Transcript profiling was conducted in the livers of 3 sexually mature males per treatment group. RNA was extracted from livers using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions, then further purified and treated with DNase on RNeasy Mini extraction columns (Qiagen). The concentration, purity and integrity of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). All RNA input to library construction was of high quality with 260/280 and 260/230 ratios > 1.8 and RIN scores > 8. External RNA Controls Consortium (ERCC) spike-in control mixes (Ambion) were added to all individual RNA samples, according to the manufacturer's instructions. cDNA libraries from all 15 samples were then prepared using the Illumina TruSeq RNA Sample Preparation kit, multiplexed with 24 samples per lane (together with samples from another project) and sequenced using an Illumina HiSeq 2500 to generate 100 bp paired-end reads, according to the manufacturer's instructions.

Transcriptome Assembly and Annotation

To maximise sequence coverage depth and assemble an optimised male liver transcriptome for brown trout, sequence reads from all samples from the current study were combined with those from another project (Uren Webster and Santos *in preparation*). All analyses were carried out on a local server running under the NEBC Bio-Linux 7 environment. Contaminating Illumina adaptor sequences were removed and the first 12 bp of all raw sequence reads were trimmed to remove 5' bias caused by random hexamer priming using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). 3' sliding window quality trimming was performed (http://wiki.bioinformatics.ucdavis.edu/index.php/ Trim.slidingWindow.pl) and

all reads where < 90% bases had a Phred quality score >20, and those shorter than 15 bp, were discarded. Digital normalisation was performed to remove highly duplicated reads using the normalize-by-median.py script part of the khmer package described by Brown et al. (2012), with the recommended k-mer value of 20 and a coverage threshold of 200. This process reduces the computer memory requirements of transcriptome assembly, and also reduces the risk of potential sequencing error accumulation in abundant transcripts. All retained reads were then paired, separated into forward and reverse fastq files before *de novo* transcriptome assembly using Trinity (version r2013-02-25; (Grabherr et al. 2011), using the default parameters and specifying a minimum contig length of 200 bp). All transcripts were annotated using Blastx against Ensembl peptide databases (Release 71; April 2013) using an e-value cut off < $1e^{-15}$ and assigned in the following preferential order; zebrafish (*Danio rerio*); human (*Homo sapiens*) and mouse (*Mus musculus*); stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*) and cod (*Gadus morhua*). Additional annotation of previously un-annotated differentially expressed transcripts was performed using Blast (< $1e^{-15}$) against refseq, nr and nt databases.

Transcriptomic Analysis

Raw sequence reads from individual samples were mapped back against the assembled transcripts using Bowtie2 (version 2.1.0, (Langmead and Salzberg 2012)), using the -k 1 parameter to report a single best hit for each read and limit ambiguous mapping to redundant transcripts. Raw count data for each transcript was extracted using idxstats in samtools (version 0.1.18, (Li et al. 2009)) and input into edgeR (Robinson et al. 2010) for differential expression analysis. A criteria of at least one count in a minimum of three biological replicate samples (corresponding to the number of biological replicates per treatment group) was imposed, and tagwise dispersion was applied with the recommended prior.df =10. Pairwise comparisons were initially conducted between the two control groups to ensure that our analysis did not identify differential expression as a result of random variation between groups. Following this initial analysis, pairwise comparisons were conducted between the six individual fish from the combined control groups and 3 individuals from each of the other treatment groups. Transcripts were considered differentially expressed with a FDR < 0.05 (Benjamini-Hochberg correction). Hierarchical clustering was performed on all differentially expressed transcripts for all samples using an Euclidean distance metric,

in the Pheatmap package for R. Functional analysis was then performed for differentially expressed genes from each treatment using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.7; (Huang et al. 2008)), with the newly assembled brown trout male liver transcriptome as a background. Kegg pathways and Gene Ontology (GO) terms for Biological Processes, Cellular Components and Molecular Functions were considered significantly over-represented when $P < 0.05$. Canonical pathway and network analysis was conducted using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) based on the list of differentially expressed transcripts.

Results

Morphological parameters

The mean mass and length of all mature males were 472.3 ± 9.1 g and 34.3 ± 0.2 cm, respectively. There were no significant differences in size and condition factor (mean 1.17), HSI (mean 1.11) or GSI (mean 3.95) between treatment groups. Additionally, we observed no alteration of general health or behaviour during the exposure period.

Sequencing and transcriptome assembly

In total, we sequenced 225.3 million paired 100 bp reads from male brown trout liver samples (including those originating from a parallel study, also using mature male brown trout as a model species), and 208.1 million (92.4 %) of these were retained after processing and quality filtering. Highly duplicated reads were then removed by digital normalisation, and 46.73 million paired reads were retained for input into the *de novo* transcriptome assembly. The final transcriptome assembly consisted of 172,688 transcripts (107,095 loci) with a mean length of 767.5 bp and a N50 of 1292 bp. 62,236 transcripts were annotated using Blastx ($e < e\text{-}15$) against Ensembl peptide databases, and these included representation of 16,121 unique zebrafish transcripts (Figure 1).

Transcript expression analysis

A total of 137.6 million reads were obtained from the libraries generated from liver samples of E2 exposed and control male fish, averaging 9.2 million reads per individual sample, and 83.1 % of these were re-mapped against the transcriptome assembly.

Differential expression analysis between the control groups revealed only 3 differentially regulated transcripts, and pairwise comparisons for each E2 treatment group were conducted against the combined control groups. Exposure to 1.94 and 18.06 ng E2/L resulted in only 4 and 2 differentially expressed transcripts, respectively. Exposure to 34.38 ng E2/L, however, resulted in 2113 differentially expressed transcripts (Figure 2a; Table S4), including 808 unique annotations. Multidimensional scaling (MDS) plots and Euclidean cluster analysis based on all differentially regulated transcripts show that all three individual fish exposed to 34.38 ng E2/L have a very similar expression profile, clearly distinct from all other fish, whereas the control fish and those exposed to the lower concentrations of E2 cluster together (Figures 2b and c).

A list of the 20 most up- and down-regulated transcripts following exposure to 34.38 ng E2/L is shown in Table1. The greatest changes in expression were associated with up-regulated transcripts. This list is dominated by well characterised estrogen-responsive genes, including a number of vitellogenin transcripts (*vtg1, vtg1l, vtg2, vtg3, vtg6, vtg7*), of which *vtg1* was the most highly expressed. Additionally, a transcript encoding nothepsin (*nots*), was similarly expressed at very high levels in fish exposed to 34.38 ng E2/L. Transcripts encoding zona pellucida proteins (*zp2.2, zp2.5, zp3a.1, zp3a.2*) were also up-regulated (up to 70-230 fold) and estrogen receptor 1 (*esr1*) was up-regulated by up to 27 fold. Additionally, there was an apparent trend in up-regulation of *esr1, zp2.2* and *zp2.5* (2-4 fold) in the lower treatment groups compared to the control, but these results were not statistically significant.

Analysis of ERCC spike-in control data were conducted to determine the accuracy and dynamic range of the transcript expression measurements in this study, and are presented in the supporting information. For all individual samples, there was a strong correlation between the calculated FPKM values and the expected concentration of control transcripts (mean R^2 = 0.902±0.005) (Figure S1). The dynamic range was calculated for all samples individually, using the control transcripts that were detected in a minimum of three libraries as the lower cutoff limit (Table S1). The mean dynamic range in expression level for all 15 libraries was 26,753 FPKM. There was also a good correlation between the calculated and expected changes in transcript expression level between samples spiked with ERCC mix 1 and mix 2 (R^2=0.58). Together these results

provide strong technical validation for the quantitative expression profiling conducted in this study.

Functional analysis

Enriched Gene Ontology terms and Kegg pathways among up- and down-regulated transcripts following exposure to 34.38 ng E2/L are given in Tables S2 and S3. GO terms including *translation*, *ribosome*, *lipid metabolic processes* and *growth factor binding* were over-represented in the list of up-regulated transcripts. Regulated transcripts within these Gene Ontologies included RNA polymerases (*polr1a, polr3a)* for *transcription*; translation initiation factors (*eif1ad, eif3s10, eif4a2*) for *translation*; and ribosomal components and binding proteins (*rpl5a, rpl12, rpl15, rpl36a, rpl39, rplp0, rpp21, rps2, rps9, rps23, rpsa, rrbp1a*) for *ribosome.* Within *lipid metabolism*, differentially regulated transcripts included apolipoproteins (*apob, apobb, apof, apoc2*), lipoprotein receptor (*lrpap1*), glycolipid transfer proteins (*gltpd2*) and transcripts involved in PPAR signalling (*ppardb, acoxl*). In addition, insulin-like growth factor (IGF) signalling was also affected and transcripts encoding IGF binding proteins (IGFBPs) were up-regulated in some cases (*igfbp5a, igfbp2a, igfbp2b*) and down-regulated in others (*igfbp1a, igfbp1b*).

For down-regulated transcripts, the most over-represented GO terms related to amino acid metabolism and biosynthesis and associated processes including *organic acid biosynthesis*, *transaminase activity* and *pyridoxal phosphate binding*. Of note, a number of processes involved in cysteine and methionine metabolic pathways were enriched, whereby differentially regulated transcripts included betaine-homocysteine methyltransferase (*bhmt*), S-adenosylmethionine synthase (*sash1),* methionine adenosyltransferase (*mat2aa*) and cysteine dioxygenase (*cdo1*). *Apoptosis* and *programmed cell death* were also over-represented in the list of down-regulated transcripts.

Ingenuity pathway analysis identified a gene network involved in the response to E2 with functions relating to amino acid metabolism, cell death and survival, endocrine system development and small molecule biochemistry, and with *esr1* and the myelocytomatosis oncogene (*myc*) as central nodes (Figure 3).

Discussion

Despite the ecological and economic importance of brown trout, little is known about the responses of this species to key stressors affecting its freshwater habitat, which include endocrine disrupting chemicals. Here, for the first time, we have conducted global transcriptional profiling in the liver of sexually mature males exposed to E2, and identified very significant transcriptional changes at the highest concentration tested (34.38 ng/L). In contrast, concentrations of up to 18.06 ng E2/L induced few changes, which may correspond to changes associated with tank effects, given that similar numbers of differentially expressed transcripts were found between the two control tanks, indicating that the threshold for biological effects for short term exposures to E2 in this species occurs between 18 and 34 ng/L. E2 equivalent concentrations, which consider the combined activity of multiple estrogenic contaminants, have been reported to occur in this range in treated sewage effluent (Jobling et al. 2009, Green et al. 2013). Although these concentrations are higher than those regularly reported in surface waters, short-term peaks of E2 contamination in streams inhabited by this species can occur as a result of agricultural pollution (Shore and Shemesh 2003). Therefore, the extent of transcriptional change found in this study after a four day exposure to 34.38 ng E2/L raises concerns for populations of brown trout in these environments.

Conserved estrogen-responsive transcripts

Transcripts encoding six vitellogenin isoforms were strongly induced in males exposed to 34.38 ng E2/L, similarly to that reported in previous transcriptomic studies where *vtg* transcripts were amongst the most up-regulated following estrogen exposure (e.g. Levi et al. 2009, Zheng et al. 2013). The second most significantly up-regulated transcript encoded nothepsin (*nots*) which was highly expressed in fish exposed to 34.38 ng E2/L but not detected in the other treatment groups. Nothepsin, also known as liver-specific aspartic proteinase, is normally exclusively expressed in the livers of females where it plays a role in the proteolytic cleavage of the vitellogenin precursor, and has previously been shown to be induced in zebrafish males exposed to E2 (Riggio et al. 2002, Levi et al. 2009, Zheng et al. 2013). The threshold for vitellogenin induction in this study was also similar to previously reported values for salmonids. In juvenile rainbow trout exposed to E2 for 14 days, the median effective treatment concentration for plasma Vtg induction was in the range of 19-26 ng/L (Thorpe et al. 2003), while the lowest effective

concentration for both plasma Vtg and hepatic *vtg1* induction was found to be 14 ng/L (Thomas-Jones et al. 2003). In juvenile brown trout, the median EC50 for plasma Vtg induction following 7-day E2 exposure was 15 ng/L (Bjerregaard et al. 2008). Potentially, the relatively lower sensitivity to E2 exposure reported here reflects the shorter exposure period in our study.

Transcripts encoding four zona pellucida proteins and *esr1* were also amongst the most up-regulated transcripts in fish exposed to 34.38 ng E2/L, similarly to previous reports showing strong up-regulation of these transcripts in vitellogenic females and induction by E2 in males (Gunnarsson et al. 2007, Levi et al. 2009, Zheng et al. 2013). We found no evidence of differential regulation of the other estrogen receptors, suggesting that *esr1* is the dominant regulator of vitellogenesis, as previously reported for other species (Filby and Tyler 2005, Katsiadaki et al. 2010). Additionally, the apparent trend in up-regulation of *zp*s and *esr1* in the lower treatment groups suggests they may be particularly sensitive to estrogen exposure in brown trout, as previously reported in other species (Gunnarsson et al. 2007, Katsiadaki et al. 2010).

Estrogen-regulated hepatic processes

A number of signalling pathways and processes enriched in the list of differentially regulated transcripts were related to vitellogenesis. Functional analysis revealed enrichment of lipid transport, and also differential regulation of many other transcripts involved in lipid, fatty acid and cholesterol metabolism. These processes have been previously associated with vitellogenesis in females and E2 exposure in male fish (Levi et al. 2009, Zheng et al. 2013), and may reflect the incorporation of lipids into vitellogenins as they are synthesised in the liver.

Increased hepatic cellular growth and proliferation have also been extensively linked with vitellogenesis in maturing females, and estrogen-exposed males. Cell proliferation is regulated by a complex network of interacting signalling pathways. In particular, we found evidence of altered regulation of IGF signalling, which is an important regulator of cell proliferation and growth. We found evidence of up-regulation of IGFBP types 2 and 5 and down-regulation of IGFBP type 1. In mammals, IGF binding proteins regulate the transport and availability of IGF1 to bind to its receptors at target cells (Hwa et al. 1999). In fish, little is known about the relative binding affinities of the various IGFBP

isoforms, but the differential expression of IGFBPs observed here suggests regulation of the bioavailability of IGF1. Crosstalk between IGF and estrogen signalling pathways has been previously demonstrated, and the transcription of IGFBPs is known to be directly regulated by E2 (Hamelers and Steenbergh 2003, Kamangar et al. 2006).

The myelocytomatosis oncogene (MYC) is a transcription factor centrally involved in regulating cell proliferation and growth in mammalian cells. MYC signalling has been proposed as the dominant regulator of estrogen-induced cellular growth, and estrogen exposure induces *myc* transcription via upstream enhancer activation (Musgrove et al. 2008). We observed an up-regulation of *myc* by up to 10 fold following exposure to 34.38 ng E2/L. Additionally, pathway analysis highlighted its role as a central regulator, alongside *esr1*, of other differentially expressed genes involved in cell proliferation. Tissue homeostasis depends on a balance between cell death and cell survival, growth and proliferation, which are often controlled by the same interacting signalling pathways, including regulation by MYC and IGFs (Prendergast 1999). In parallel, *apoptosis* was among the down-regulated cellular processes, suggesting E2 exposure induced liver growth and proliferation and suppressed apoptosis.

Exposure to E2 also resulted in up-regulation of a number of transcripts with roles in transcription and translation. Furthermore, *ribosome* and *endoplasmic reticulum* were amongst the most enriched GO and Kegg pathway terms. Ribosome biogenesis in response to estrogen exposure has been previously linked to increased cell growth and proliferation, reflecting a general up-regulation of translation (Musgrove et al. 2008, Zheng et al. 2013). In fish, the observed induction of transcription and translation machinery is also likely to reflect the very significant increase in the synthesis and post-translational modification of vitellogenins and zona pellucida proteins. Ribosomal constituent over-expression has been previously reported in male zebrafish exposed to E2 (Ruggeri et al. 2008) and in female vitellogenic livers (Zheng et al. 2013). Together, induction of the expression of growth regulators, and of transcription and translation pathways, illustrates the very significant stimulatory effect of E2 on cell proliferation and protein synthesis in the livers of male brown trout.

Among the list of genes down-regulated as a result of exposure to E2, a number of processes involved in amino acid and carbohydrate metabolism were over-

represented, similarly to that reported by previous studies (Williams et al. 2007, Goetz et al. 2009). The down-regulation of these pathways in exposed males may reflect a compensatory response to the significant induction of vitellogenin and zona pellucida protein synthesis, and increase in cell proliferation and growth.

In addition, a differential regulation of processes and transcripts involved in methionine and cysteine metabolism was observed. This pathway plays an important role in regulating DNA methylation, whereby S-adenosylmethionine (SAM) acts as the key methyl group donor. Modulation of DNA methylation has been implicated in tumourgenesis, and reported to be altered by estrogen exposure (Baccarelli and Bollati 2009, Mirbahai et al. 2013). Additionally, studies in human cell lines have shown that reactive estrogen metabolites (quinones) bind homocysteine, which is a key intermediate in methionine and cysteine metabolism (Gaikwad 2013). Plasma concentrations of free homocysteine are also regulated by estrogen, and are lower in women of reproductive age (Dimitrova et al. 2002). Therefore, a reduction in homocysteine might contribute to the observed differential-regulation of these associated metabolic enzymes.

Application of RNA-seq in ecotoxicology

The present study provides evidence that RNA-seq has very significant potential for mechanistic analysis of chemical exposures in (non)-model organisms, offering a number of technical advantages over other methodologies to measure global transcript expression such as microarrays. Here, we conducted a *de novo* assembly of the hepatic transcriptome for an environmentally relevant fish species, and characterised the expression profile of 172,688 assembled transcripts, which represented over 16,000 unique zebrafish transcript annotations, following E2 exposure. Analysis of spike-in controls provided good technical validation for the accuracy of the expression analysis, and the mean calculated dynamic range in expression measured in our experimental data was 26,753, which far exceeds that typically found in microarray experiments (up to several hundred fold). Importantly, we found evidence of highly conserved responses to estrogen exposure in male brown trout, compared to that reported for other fish species. We measured a strong up-regulation of transcripts

encoding vitellogenins, zona pellucida proteins and *esr1*, which are classic biomarkers for estrogen exposure in fish. We also found evidence of regulation in a number of hepatic processes associated with the biochemical and morphological changes that accompany vitellogenesis. Together, our data highlights the potential of RNA-seq as a valuable, sensitive and robust tool in ecotoxicology which, crucially, is not reliant on pre-existing genomic resources for the species of interest.

Acknowledgements

We wish to thank Ronny van Aerle for advice on the bioinformatics, and Audrey Farbos, Karen Moore and Konrad Paszkiewicz for facilitating the sequencing experiments. We also thank Charles Tyler, Rod Wilson, Erin Reardon and Jo Rabineau for critical discussions and contributions towards setting up the fish exposure experiments.

Funding information

This work was supported by a Natural Environment Research Council CASE PhD studentship (Grant number NE/I528326/1) and the Salmon & Trout Association.

Supplementary data

The supporting information contains the results of the ERCC spike-in control analysis (Figure S1, Table S1), the enriched Gene Ontology terms and Kegg pathways (Table S2, Table S3) and a list of all differentially expressed transcripts (Table S4).

References

Arukwe, A., and A. Goksøyr. (2003). Eggshell and egg yolk proteins in fish, hepatic proteins for the next generation, oogenetic, population, and evolutionary implications of endocrine disruption. Comparative Hepatology **2**,4-12.

Baccarelli, A., and V. Bollati. (2009). Epigenetics and environmental chemicals. Current opinion in pediatrics **21**,243-249.

Benninghoff, A. D., and D. E. Williams. (2008). Identification of a transcriptional fingerprint of estrogen exposure in rainbow trout liver. Toxicological Sciences **101**,65-80.

Bjerregaard, P., P. R. Hansen, K. J. Larsen, C. Erratico, B. Korsgaard, and H. Holbech. (2008). Vitellogenin as a biomarker for estrogenic effects in brown trout, *Salmo trutta;* laboratory and field investigations. Environmental Toxicology and Chemistry **27**,2387-2396.

Brown, C. T., A. Howe, Q. Zhang, A. B. Pyrkosz, and T. H. Brom. (2012). A reference-free algorithm for computational normalization of shotgun sequencing data. arXiv,1203.4802 [q-bio.GN].

Desbrow, C., E. J. Routledge, G. C. Brighty, J. P. Sumpter, and M. Waldock. (1998). Identification of estrogenic chemicals in STW effluent.1. Chemical fractionation and in vitro biological screening Environ. Sci. Technol. **32**,1549-1558.

Dimitrova, K. R., K. DeGroot, A. K. Myers, and Y. D. Kim. (2002). Estrogen and homocysteine. Cardiovascular research **53**,577-588.

Filby, A. L., and C. R. Tyler. (2005). Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (Pimephales promelas). Biology of Reproduction **73**,648-654.

Gaikwad, N. W. (2013). Mass spectrometry evidence for formation of estrogens-homocysteine conjugates, Estrogens can regulate homocysteine levels. Free Radical Biology and Medicine ***In press***.

Goetz, F. W., M. L. Rise, M. Rise, G. W. Goetz, F. Binkowski, and B. S. Shepherd. (2009). Stimulation of growth and changes in the hepatic transcriptome by 17b-estradiol in the yellow perch (Perca flavescens). Physiological Genomics **38**,261-280.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, and Q. Zeng. (2011). Full-length

transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology **29**,644-652.

Green, C., R. Williams, R. Kanda, J. Churchley, Y. He, S. Thomas, P. Goonan, A. Kumar, and S. Jobling. (2013). Modelling of steroid estrogen contamination in UK and south Australian rivers predict modest increases in concentrations in the future. Environmental Science & Technology **47**,7224-7232.

Gunnarsson, L., E. Kristiansson, L. Forlin, O. Nerman, and D. G. J. Larsson. (2007). Sensitive and robust gene expression changes in fish exposed to estrogen - a microarray approach. BMC Genomics **8**, 149-155.

Hall, J. M., J. F. Couse, and K. S. Korach. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. Journal of Biological Chemistry **276**,36869-36872.

Hamelers, I., and P. Steenbergh. (2003). Interactions between estrogen and insulin-like growth factor signaling pathways in human breast tumor cells. Endocrine-Related Cancer **10**,331-345.

Harris, C. A., P. B. Hamilton, T. J. Runnalls, V. Vinciotti, A. Henshaw, D. Hodgson, T. S. Coe, S. Jobling, C. R. Tyler, and J. P. Sumpter. (2011). The consequences of feminization in breeding groups of wild fish. Environmental Health Perspectives **119**,306-311.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. (2008). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols **4**,44-57.

Hwa, V., Y. Oh, and R. G. Rosenfeld. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocrine reviews **20**,761-787.

Jobling, S., R. W. Burn, K. Thorpe, R. Williams, and C. Tyler. (2009). Statistical Modeling Suggests that Antiandrogens in Effluents from Wastewater Treatment Works Contribute to Widespread Sexual Disruption in Fish Living in English Rivers. Environmental Health Perspectives **117**,797-802.

Jobling, S., S. Coey, J. Whitmore, D. Kime, K. Van Look, B. McAllister, N. Beresford, A. Henshaw, G. Brighty, and C. Tyler. (2002). Wild intersex roach (Rutilus rutilus) have reduced fertility. Biology of Reproduction **67**,515-524.

Jobling, S., M. Nolan, C. R. Tyler, G. C. Brighty, and J. P. Sumpter. (1998). Widespread Sexual Disruption in Wild Fish. Environ. Sci. Technol. **32**,2498-2506.

Kamangar, B. B., J.-C. Gabillard, and J. Bobe. (2006). Insulin-like growth factor-binding protein (IGFBP)-1,-2,-3,-4,-5, and-6 and IGFBP-related protein 1 during rainbow trout postvitellogenesis and oocyte maturation, molecular characterization, expression profiles, and hormonal regulation. Endocrinology **147**,2399-2410.

Katsiadaki, I., T. D. Williams, J. S. Ball, T. P. Bean, M. B. Sanders, H. Wu, E. M. Santos, M. M. Brown, P. Baker, and F. Ortega. (2010). Hepatic transcriptomic and metabolomic responses in the Stickleback (*Gasterosteus aculeatus*) exposed to ethinyl-estradiol. Aquatic Toxicology **97**,174-187.

Kidd, K. A., P. J. Blanchfield, K. H. Mills, V. P. Palace, R. E. Evans, J. M. Lazorchak, and R. W. Flick. (2007). Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences **104**,8897-8903.

Langmead, B., and S. L. Salzberg. (2012). Fast gapped-read alignment with Bowtie 2. Nature methods **9**,357-359.

Levi, L., I. Pekarski, E. Gutman, P. Fortina, T. Hyslop, J. Biran, B. Levavi-Sivan, and E. Lubzens. (2009). Revealing genes associated with vitellogenesis in the liver of the zebrafish (Danio rerio) by transcriptome profiling. BMC Genomics **10**,141-158.

Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, and R. Durbin. (2009). The sequence alignment/map format and SAMtools. Bioinformatics **25**,2078-2079.

Mirbahai, L., A. D. Southam, U. Sommer, T. D. Williams, J. P. Bignell, B. P. Lyons, M. R. Viant, and J. K. Chipman. (2013). Disruption of DNA Methylation via S-Adenosylhomocysteine Is a Key Process in High Incidence Liver Carcinogenesis in Fish. Journal of Proteome Research **12**,2895-2904.

Musgrove, E. A., C. M. Sergio, S. Loi, C. K. Inman, L. R. Anderson, M. C. Alles, M. Pinese, C. E. Caldon, J. Schatte, and M. Gardiner-Garden. (2008). Identification of functional networks of estrogen-and c-Myc-responsive genes and their relationship to response to tamoxifen therapy in breast cancer. PLoS ONE **3**,e2987.

Prendergast, G. C. (1999). Mechanisms of apoptosis by c-Myc. Oncogene **18**,2967-2987.

Riggio, M., R. Scudiero, S. Filosa, and E. Parisi. (2002). Oestrogen-induced expression of a novel liver-specific aspartic proteinase in Danio rerio (zebrafish). Gene **295**,241-246.

Robinson, M. D., D. J. McCarthy, and G. K. Smyth. (2010). edgeR, a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26**,139-140.

Ruggeri, B., M. Ubaldi, A. Lourdusamy, L. Soverchia, R. Ciccocioppo, G. Hardiman, M. E. Baker, F. Palermo, and A. M. Polzonetti-Magni. (2008). Variation of the genetic expression pattern after exposure to estradiol-17 beta and 4-nonylphenol in male zebrafish (Danio rerio). General and Comparative Endocrinology **158**,138-144.

Segner, H., A. Casanova-Nakayama, R. Kase, and C. R. Tyler. (2013). Impact of environmental estrogens on fish considering the diversity of estrogen signaling. General and Comparative Endocrinology **191**,190-201.

Shappell, N. W., K. H. Elder, and M. West. (2010). Estrogenicity and nutrient concentration of surface waters surrounding a large confinement dairy operation using best management practices for land application of animal wastes. Environmental Science & Technology **44**,2365-2371.

Shore, L. S., and M. Shemesh. (2003). Naturally produced steroid hormones and their release into the environment. Pure and Applied Chemistry **75**,1859-1871.

Thomas-Jones, E., K. Thorpe, N. Harrison, G. Thomas, C. Morris, T. Hutchinson, S. Woodhead, and C. Tyler. (2003). Dynamics of estrogen biomarker responses in rainbow trout exposed to 17b-estradiol and 17a-ethinylestradiol. Environmental Toxicology and Chemistry **22**,3001-3008.

Thorpe, K. L., R. I. Cummings, T. H. Hutchinson, M. Scholze, G. Brighty, J. P. Sumpter, and C. R. Tyler. (2003). Relative potencies and combination effects of steroidal estrogens in fish. Environmental Science & Technology **37**,1142-1149.

Uren Webster, T. M., and E. M. Santos. *in preparation*. Global transcriptome profiling reveals mechanisms of toxicity of the herbicide Linuron in brown trout (*Salmo trutta*).

van Aerle, R., M. Nolanusan, S. Jobling, L. B. Christiansen, J. P. Sumpter, and C. R. Tyler. (2001). Sexual disruption in a second species of wild cyprinid fish (the gudgeon, Gobio gobio) in United Kingdom freshwaters. Environmental Toxicology and Chemistry **20**,2841-2847.

Williams, T. D., A. M. Diab, S. G. George, V. Sabine, and J. K. Chipman. (2007). Gene expression responses of European flounder (Platichthys flesus) to 17b estradiol. Toxicology Letters **168**,236-248.

Zheng, W., H. Xu, S. H. Lam, H. Luo, R. K. M. Karuturi, and Z. Gong. (2013). Transcriptomic Analyses of Sexual Dimorphism of the Zebrafish Liver and the Effect of Sex Hormones. PLoS ONE **8**,1-12.

Figure Legends

Table 1. List of the 20 most up-regulated and down-regulated transcripts in fish exposed to 34.38 ng E2/L. Values presented are fold changes and adjusted P-values relative to the control group. Where there were multiple differentially regulated transcripts assigned the same annotation, only the most significantly regulated transcript is included in this list.

Figure 1. Flow diagram illustrating the workflow employed during the bioinformatics procedures, including sequencing and assembling the transcriptome and conducting expression analysis to identify transcripts differentially regulated by E2 exposure.

Figure 2. Differentially expressed genes following exposure to E2 in the liver of mature male brown trout. **A)** Number of up-regulated and down-regulated transcripts in each treatment group calculated using EdgeR (FDR <0.05). **B)** Multidimensional scaling plot illustrating the very significant effect of exposure to 34.38 ng E2/L on the hepatic transcriptome of male brown trout (presented within the blue circle, for visualisation purposes) compared to all other groups, based on the expression of all differentially-regulated transcripts. Individual fish are represented by the following codes: c1, c2 and c3 represent the control individuals; le1, le2 and le3 represent individuals exposed to 1.94 ng E2/L; me1, me2 and me3 represent individuals exposed to 18.06 ng E2/L; he1, he2 and he3 represent individuals exposed to 34.38 ng E2/L. **C)** Heatmap illustrating the expression level of all differentially-regulated transcripts in all individual samples (individuals are represented by the same codes as in B. Data presented are log10 transformed read counts per transcript. The hierarchical clustering to generate gene and condition trees was conducted using an Euclidean distance metric in the pheatmap package in R.

Figure 3. Enriched gene network constructed using differentially expressed transcripts (FDR <0.05) following exposure to 34.38 ng E2/L in male brown trout. This was the most over-represented network generated by Ingenuity Pathway Analysis using default settings. Associated functions of this network include amino acid metabolism, cell death and survival, endocrine system development and small molecule biochemistry. Genes shaded red are significantly up-regulated and green are significantly down-regulated.

UP-REGULATED				**DOWN-REGULATED**			
Symbol	Name	Fold change	FDR	Symbol	Name	Fold change	FDR
vtg1	vitellogenin 1	↑ >5438	4.6E-119	*tat*	tyrosine aminotransferase	↓ 4.6	8.9E-9
nots	nothepsin	↑ >4475	5.4E-107	*tgm2l*	transglutaminase 2, like	↓ 186.1	3.3E-8
vtg6	vitellogenin 6	↑ >2000	1.3E-102	*cbln8*	cerebellin 8	↓ 5.7	2.6E-7
vtg2	vitellogenin 2	↑ >1100	7.3E-92	*hsd3b7*	hydroxy-delta-5-steroid dehydrogenase, 3beta- and steroid delta-isomerase	↓ 5.4	2.8E-7
si:dkey-4c23.3 *(vtg1-1)*	vitellogenin 1-1	↑ >220	9.6E-59	*errfi1*	ERBB receptor feedback inhibitor 1	↓ 6.3	5.5E-7
vtg3	vitellogenin 3	↑ >825	1.4E-56	*igfbp1a*	insulin-like growth factor binding protein 1a	↓ 50.7	6.2E-7
zp3a.2	zona pellucida 3a.2	↑ >149	1.6E-53	*slc3a2a*	solute carrier family 3, member 2a	↓ 10.4	6.8E-7
si:dkey-179j5.2 *(fam20c)*	family with sequence similarity 20, member C	↑ >185	5.0E-52	*faxdc2*	chromosome 5 open reading frame 4	↓ 4.8	1.8E-6
zp2.5	zona pellucida 2.5	↑ 77.6	1.1E-42	*pnp5a*	purine nucleoside phosphorylase 5a	↓ 27.1	2.3E-6
zp3a.1	zona pellucida 3a.1	↑ 161.5	1.1E-42	*epha8*	eph receptor A8	↓ >21	7.5E-6
vtg7	vitellogenin 7	↑ >107	8.3E-42	*pfkfb1*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1	↓ 30.1	8.1E-6
crot	carnitine o-octanoyltransferase	↑ 54.4	4.9E-40	*pptc7a*	PTC7 protein phosphatase homolog a	↓ 6.3	9.2E-6
esr1	estrogen receptor 1	↑ 25.7	1.6E-37	si:dkey-238o13.4	si:dkey-238o13.4	↓ 4.5	1.8E-5
zp2.2	zona pellucida 2.2	↑ 160.7	5.1E-31	*st3gal3b*	ST3 beta-galactoside alpha-2,3-sialyltransferase 3b	↓ 3.8	2.4E-5
aqp12	aquaporin 12	↑ 28.6	8.4E-31	*ret*	ret proto-oncogene receptor tyrosine kinase	↓ 8.5	4.3E-5
lrrc58b	leucine rich repeat containing 58b	↑ 20.6	1.0E-30	*ntng2a*	netrin g2a	↓ 4.9	4.9E-5
igfbp5a	insulin-like growth factor binding protein 5a	↑ >49	2.6E-30	*ulk1a*	unc-51-like kinase 1a	↓ 4.7	4.9E-5
rdh10a	retinol dehydrogenase 10a	↑ >108	8.8E-28	*grb7*	growth factor receptor-bound protein 7	↓ 26.3	1.2E-4
slc7a11	solute carrier family 7, member 11	↑ >51	8.9E-27	*cldn11a*	claudin 11a	↓ 6.5	1.3E-4
lpgat1	lysophosphatidylglycerol acyltransferase 1	↑ 26.0	3.0E-25	*slc25a29*	solute carrier family 25, member 29	↓ 4.8	1.4E-4

Table 1

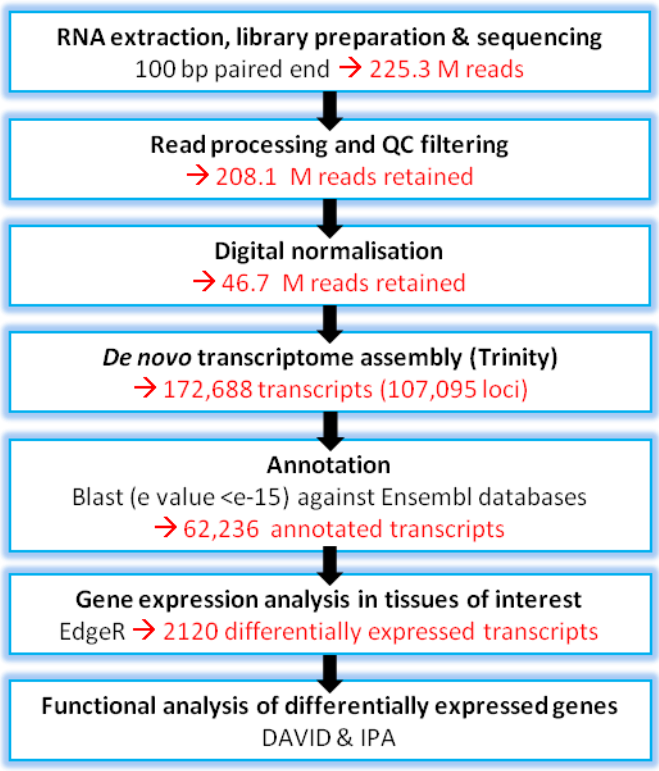

RNA extraction, library preparation & sequencing
100 bp paired end → 225.3 M reads
Read processing and QC filtering
→ 208.1 M reads retained
Digital normalisation
→ 46.7 M reads retained
De novo transcriptome assembly (Trinity)
→ 172,688 transcripts (107,095 loci)
Annotation
Blast (e value <e-15) against Ensembl databases
→ 62,236 annotated transcripts
Gene expression analysis in tissues of interest
EdgeR → 2120 differentially expressed transcripts
Functional analysis of differentially expressed genes
DAVID & IPA

Figure 1

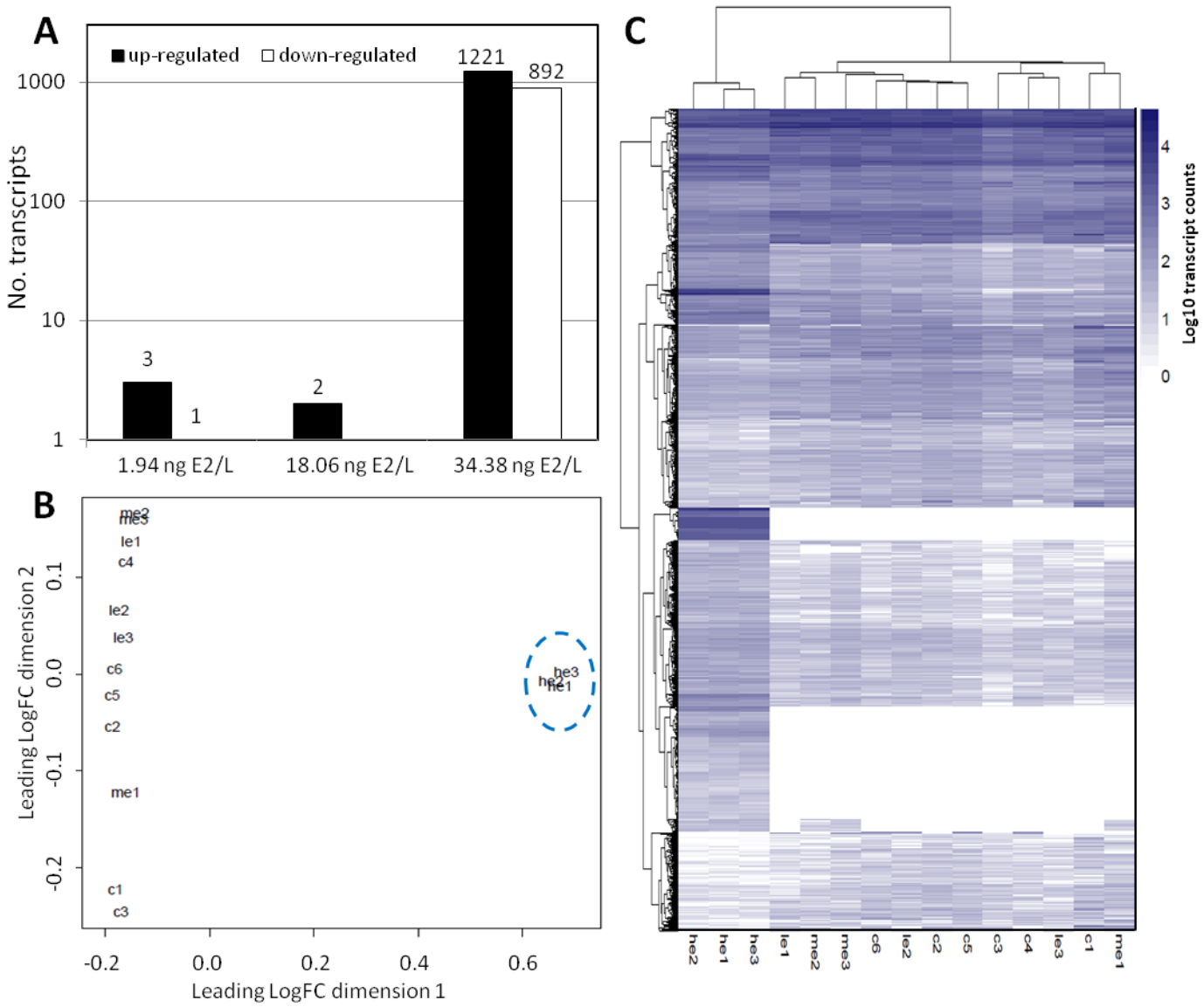

A
up-regulated
down-regulated
No. transcripts
1000
100
10
1
3
1
2
1221
892
1.94 ng E2/L
18.06 ng E2/L
34.38 ng E2/L
B
Leading LogFC dimension 2
Leading LogFC dimension 1
me2
me3
le1
c4
le2
le3
c6
c5
c2
me1
c1
c3
he2
he3
he1
-0.2
0.0
0.2
0.4
0.6
0.1
0.0
-0.1
-0.2
C
Log10 transcript counts
4
3
2
1
0
he2
he1
he3
le1
me2
me3
c6
le2
c2
c5
c3
c4
le3
c1
me1

Figure 2

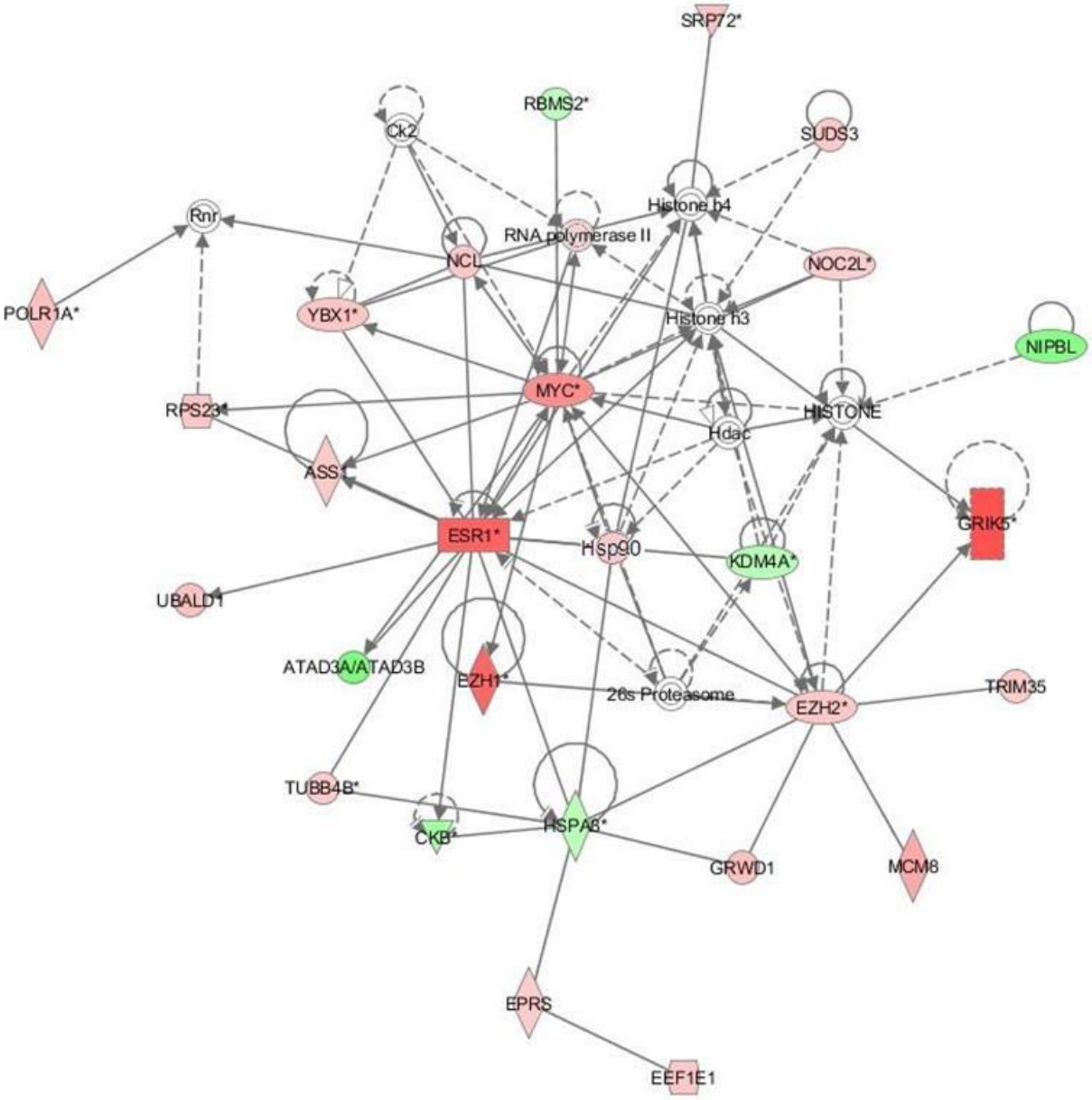

SRP72*
RBMS2*
Ck2
SUDS3
Rnr
Histone h4
RNA polymerase II
NCL
NOC2L*
POLR1A*
YBX1*
Histone h3
NIPBL
MYC*
RPS23*
HISTONE
Hdac
ASS1
GRIK5*
ESR1*
Hsp90
KDM4A*
UBALD1
ATAD3A/ATAD3B
EZH1*
26s Proteasome
EZH2*
TRIM35
TUBB4B*
CKB*
HSPA8*
GRWD1
MCM8
EPRS
EEF1E1

Figure 3

Supporting Information

Identification of conserved hepatic transcriptomic responses to estrogen using high-throughput sequencing in brown trout

Tamsyn M. Uren Webster*, Janice A. Shears and Eduarda M. Santos*

Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

* Corresponding authors

This Supporting Information contains:

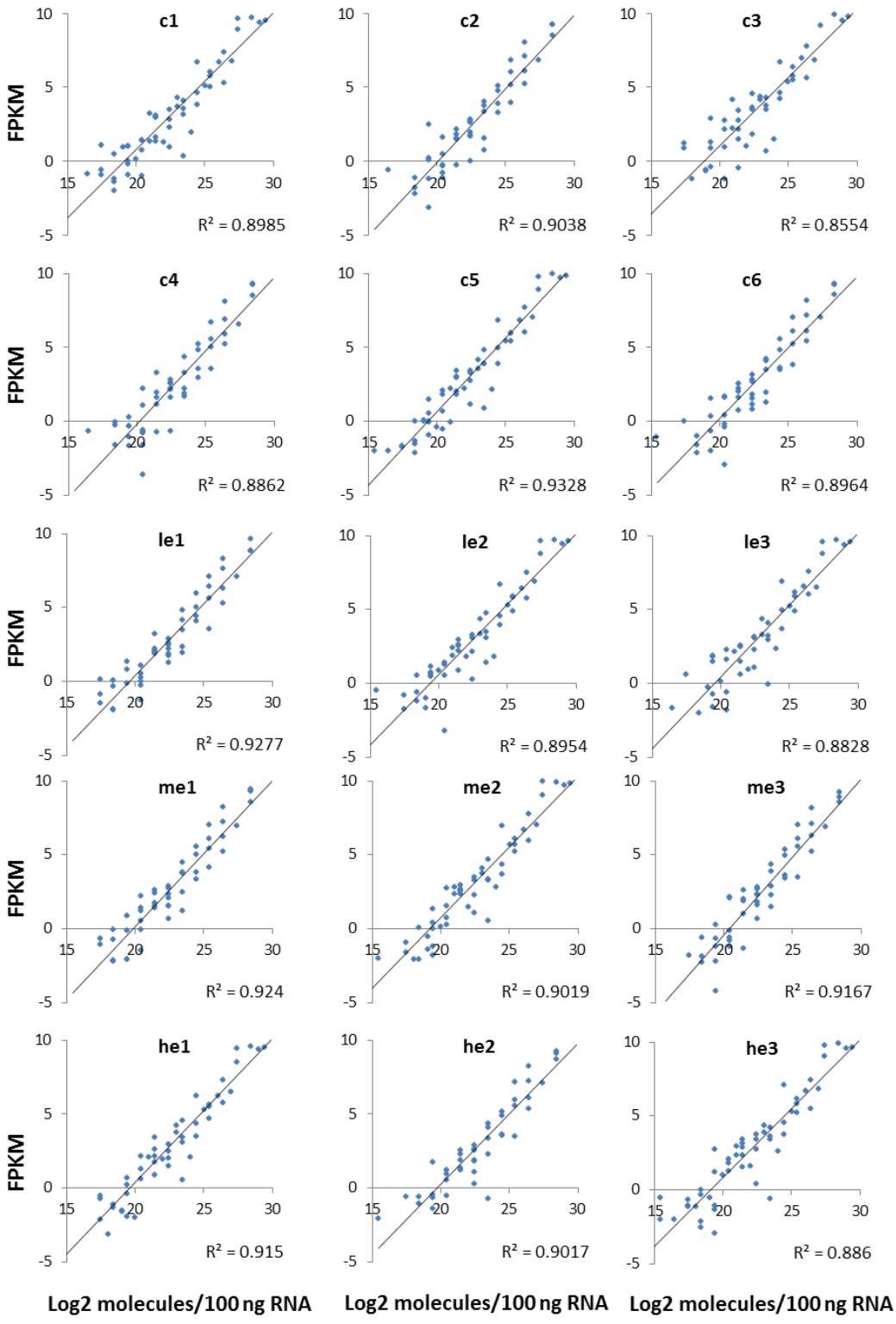


Figure S1. External RNA Controls Consortium **(**ERCC) spike-in control analysis for all individual liver samples sequenced in this project. Graphs show the relationship between the calculated expression level (FPKM) and the expected concentration of each control transcripts. Individual fish are represented by the following codes: c1, c2 and c3 represent the control individuals; le1, le2 and le3 represent individuals exposed to 1.94 ng E2/L; me1, me2 and me3 represent individuals exposed to 18.06 ng E2/L; he1, he2 and he3 represent individuals exposed to 34.38 ng E2/L.

Table S1. Dynamic range in transcript expression profiles for all individual samples included in this study. Values presented are log2 transformed maximum-minimum FPKM values calculated for ERCC spike-in control transcripts. Only transcripts that had at least 1 mapped read in a minimum of 3 replicate samples were included in the analysis. Individual fish are represented by the following codes: c1, c2 and c3 represent the control individuals; le1, le2 and le3 represent individuals exposed to 1.94 ng E2/L; me1, me2 and me3 represent individuals exposed to 18.06 ng E2/L; he1, he2 and he3 represent individuals exposed to 34.38 ng E2/L.

Sample	R^2	Log2 dynamic range (FPKM)
c1	0.90	15.00
c2	0.90	14.14
c3	0.86	14.52
c4	0.89	14.58
c5	0.93	15.00
c6	0.90	14.03
le1	0.93	13.68
le2	0.90	15.90
le3	0.88	14.74
me1	0.92	13.38
me2	0.90	15.07
me3	0.92	15.39
he1	0.92	15.73
he2	0.90	13.39
he3	0.89	16.07

Table S2. Gene Ontology Terms and Kegg Pathways over-represented in the list of up-regulated transcripts in fish exposed to 34.38 ng/L E2. Values presented are the number of transcripts associated with each term, and the P-values and adjusted P-values associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6 .7, using the *de novo* assembled liver transcriptome generated in this study as a background.

UP-REGULATED PROCESSES			
BIOLOGICAL PROCESS (BP FAT)	**Count**	**P-value**	**FDR**
translation	17	9.20E-04	1.20E-02
ribosome biogenesis	7	1.30E-03	5.00E-01
response to estrogen stimulus	3	2.40E-03	5.00E+00
ribonucleoprotein complex biogenesis	7	2.80E-03	2.60E+00
lipid transport	6	3.80E-03	3.50E+00
response to steroid hormone stimulus	3	7.70E-03	5.00E+00
ncRNA metabolic process	9	1.10E-02	3.80E+01
hemopoietic or lymphoid organ development	7	1.20E-02	4.00E+01
immune system development	7	1.30E-02	4.00E+01
hemopoiesis	6	3.00E-02	4.30E+01
response to hormone stimulus	3	3.80E-02	8.00E+01
CELLULAR COMPONENT (CC FAT)	**Count**	**P-value**	**FDR**
endoplasmic reticulum	20	4.70E-06	1.00E-03
small ribosomal subunit	4	3.30E-03	2.60E+00
ribonucleoprotein complex	16	3.50E-03	1.40E+00
ribosome	12	4.40E-03	1.90E+00
endoplasmic reticulum part	6	1.80E-02	4.30E+01
MOLECULAR FUNCTION (MF FAT)	**Count**	**P-value**	**FDR**
structural molecule activity	17	8.90E-05	2.00E-03
structural constituent of ribosome	12	3.10E-04	1.20E-02
lipid transporter activity	5	7.40E-04	2.60E+00
RNA binding	14	8.40E-03	1.20E+00
insulin-like growth factor binding	4	1.20E-02	4.30E+01
KEGG PATHWAY	**Count**	**P-value**	**FDR**
Ribosome	10	8.90E-05	2.00E-03
Aminoacyl-tRNA biosynthesis	6	1.40E-03	2.20E+00
One carbon pool by folate	3	3.90E-02	3.40E+01

Table S3. Gene Ontology Terms and Kegg Pathways over-represented in the list of down-regulated transcripts in fish exposed to 34.38 ng/L E2. Values presented are the number of transcripts associated with each term, and the P-values and adjusted P-values associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6 .7, using the de *novo* liver transcriptome assembly generated in this study as a background.

DOWN-REGULATED PROCESSES			
BIOLOGICAL PROCESS (BP FAT)	**Count**	**P-value**	**FDR**
sulfur amino acid metabolic process	6	8.00E-07	2.40E-04
cysteine metabolic process	4	8.40E-05	6.00E-02
sulfur metabolic process	6	9.60E-05	4.50E-02
sulfur amino acid biosynthetic process	4	1.50E-04	1.00E-01
organic acid biosynthetic process	7	2.10E-04	4.80E-02
cellular amino acid biosynthetic process	5	1.10E-03	2.00E-01
oxidation reduction	15	2.10E-03	1.80E-01
serine family amino acid metabolic process	4	3.50E-03	2.00E-01
aromatic amino acid family metabolic process	3	1.10E-02	2.00E+00
nucleoside metabolic process	4	2.60E-02	1.20E+01
apoptosis	5	2.80E-02	1.90E+01
cysteine biosynthetic process	2	3.30E-02	4.80E+01
CELLULAR COMPONENT (CC FAT)	**Count**	**P-value**	**FDR**
extracellular region	8	3.70E-02	5.00E+00
MOLECULAR FUNCTION (MF FAT)	**Count**	**P-value**	**FDR**
vitamin binding	10	3.50E-05	1.20E-02
transferase activity, transferring nitrogenous groups	6	1.50E-04	1.60E-01
pyridoxal phosphate binding	7	1.50E-04	2.80E-02
transaminase activity	5	3.90E-04	3.80E+00
iron ion binding	12	4.60E-04	3.00E-01
cofactor binding	10	5.50E-03	9.50E-01
oxidoreductase activity	2	4.00E-02	1.20E+01
electron carrier activity	7	4.00E-02	4.20E+01
amino acid binding	3	4.50E-02	6.80E+02
KEGG_PATHWAY	**Count**	**P-value**	**FDR**
Phenylalanine, tyrosine and tryptophan biosynthesis	4	6.50E-05	3.50E-02
Alanine, aspartate and glutamate metabolism	6	1.50E-04	4.00E-02
Cysteine and methionine metabolism	6	3.90E-04	6.00E-02
Arginine and proline metabolism	6	9.50E-04	7.00E-02
Glycine, serine and threonine metabolism	5	9.70E-04	7.00E-02
Phenylalanine metabolism	4	3.10E-03	2.00E+00
Primary bile acid biosynthesis	3	1.50E-02	2.50E+01

Table S4. List of all differentially expressed transcripts calculated in EdgeR (FDR <0.05). Values presented are Log2 transformed fold changes for each treatment group. Red shading indicates significant up-regulation and green shading represents significant down-regulation.

Name	Symbol	Database	Log2 FC 2.5 ng/L	Log2 FC 25 ng/L	Log2 FC 250 ng/L
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.43
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.34
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.34
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.34
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.34
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.32
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.32
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.31
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.30
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.30
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.30
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.30
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.29
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.29
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	14.28
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.22
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.21
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.20
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.20
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	14.17
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.85
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.85
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.81
nots	ENSDARG00000052792	Ensembl	0.00	0.00	13.56
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.55
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.54
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.48
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.45
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.43
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.40
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.39
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.38
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.36
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.36
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.35
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.34
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.33
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.33
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.25
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.20
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.20
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.19
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.19
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.14
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.12
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	13.11
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.07
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.04
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.04
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.03
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	13.02
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	13.02
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	13.01
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	13.01
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.98
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.98
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.98
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.90
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.85
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.83
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.82
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.81
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.73
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.71
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.68
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.67
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.62
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.54
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.51
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.49

vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.48
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.45
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.43
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.41
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.40
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.39
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.38
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.35
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.31
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	12.31
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.30
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.24
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.21
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.18
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.18
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.15
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	12.15
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.13
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.10
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.08
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.08
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	12.01
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	11.88
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.85
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	11.84
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.83
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	11.78
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.73
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.72
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.64
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.60
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.57
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.54
vtg1	ENSDARG00000092233	Ensembl	0.00	0.00	11.52
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.49
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.49
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.44
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.43
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	11.39
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.37
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	11.36
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.33
vtg6	ENSDARG00000016825	Ensembl	0.00	0.00	11.28
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.27
X92804.1	X92804.1	nt	0.00	0.00	11.22
vtg1-1	ENSDARG00000092028	Ensembl	0.00	0.00	11.14
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	11.12
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.95
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.94
EU221180.1	EU221180.1	nt	0.00	0.00	10.82
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.81
EU221176.1	EU221176.1	nt	0.00	0.00	10.71
vtg1-1	ENSDARG00000092028	Ensembl	0.00	0.00	10.67
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.63
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.60
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.57
si:dkey-50i6.5	ENSDARG00000093414	Ensembl	0.60	0.70	-10.39
EU025706.1	EU025706.1	nt	0.00	0.00	10.34
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.30
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	10.02
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	9.97
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	9.95
vtg7	ENSDARG00000092419	Ensembl	0.00	0.00	9.85
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	9.81
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	9.79
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	9.69
FJ969489.1	FJ969489.1	nt	0.00	0.00	9.65
vtg2	ENSDARG00000055809	Ensembl	0.00	0.00	9.61
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	9.60
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	9.59
CR936412.13	CR936412.13	nt	0.00	0.00	9.51
vtg7	ENSDARG00000092419	Ensembl	0.00	0.00	9.44
vtg3	ENSDARG00000016448	Ensembl	0.00	0.00	9.39
X92804.1	X92804.1	nt	0.00	0.00	9.27
vtg3	ENSDARG00000016448	Ensembl	0.00	0.00	9.19
NM_001126463.1	NM_001126463.1	refseq	0.00	0.00	9.06
igfbp5a	ENSDARG00000039264	Ensembl	0.00	0.00	9.03
BT060412.1	BT060412.1	nt	0.00	0.00	8.95
vtg3	ENSDARG00000016448	Ensembl	0.00	0.00	8.88
vtg7	ENSDARG00000092419	Ensembl	0.00	0.00	8.71
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	8.70
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	8.62

Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	8.52
NM_001124273.1	NM_001124273.1	refseq	0.00	0.00	8.52
vtg3	ENSDARG00000016448	Ensembl	0.00	0.00	8.52
FJ969489.1	FJ969489.1	nt	0.00	0.00	8.46
XM_003455714.1	XM_003455714.1	refseq	0.00	0.00	8.46
vtg3	ENSDARG00000016448	Ensembl	0.00	0.00	8.43
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	8.32
X92804.1	X92804.1	nt	0.00	0.00	8.31
vtg7	ENSDARG00000092419	Ensembl	0.00	0.00	8.08
CU367845.1	CU367845.1	nt	0.00	0.00	8.02
BT046279.1	BT046279.1	nt	0.00	0.00	7.98
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	7.89
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	7.88
FJ969489.1	FJ969489.1	nt	0.00	0.00	7.85
zp3a.2	ENSDARG00000042130	Ensembl	1.58	1.08	7.84
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	7.76
zp2.6	ENSDARG00000091409	Ensembl	0.00	0.00	7.74
zp3a.2	ENSDARG00000042130	Ensembl	1.94	0.78	7.64
epha8	ENSDARG00000023609	Ensembl	-3.04	-1.03	-7.56
tgm2l	ENSDARG00000093381	Ensembl	-2.02	0.53	-7.54
GU129140.1	GU129140.1	nt	0.00	0.00	7.51
zp3a.2	ENSDARG00000042130	Ensembl	1.95	0.85	7.50
ENSONIG00000014420	ENSONIG00000014420	Ensembl	0.00	0.00	7.49
BT060412.1	BT060412.1	nt	0.00	0.00	7.47
zp2.5	ENSDARG00000086522	Ensembl	1.68	1.61	7.41
CU367845.1	CU367845.1	nt	0.00	0.00	7.39
FAM20C	ENSG00000177706	Ensembl	0.00	0.00	7.39
pck1	ENSDARG00000013522	Ensembl	-6.41	-2.04	-7.38
DQ872852.1	DQ872852.1	nt	0.00	0.00	7.34
zp3a.1	ENSDARG00000042129	Ensembl	1.80	1.09	7.34
EU042125.1	EU042125.1	nt	0.00	0.00	7.33
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	7.33
zp2.2	ENSDARG00000091737	Ensembl	1.62	1.65	7.33
HM159469.1	HM159469.1	nt	0.00	0.00	7.31
EU861009.1	EU861009.1	nt	0.00	0.00	7.29
zp3a.2	ENSDARG00000042130	Ensembl	1.64	0.76	7.22
CU367845.1	CU367845.1	nt	0.00	0.00	7.19
FJ969489.1	FJ969489.1	nt	0.00	0.00	7.18
zp2.5	ENSDARG00000086522	Ensembl	1.38	1.37	7.15
zp3a.2	ENSDARG00000042130	Ensembl	1.65	0.97	7.15
zp2.2	ENSDARG00000091737	Ensembl	1.33	1.63	7.14
zp2.2	ENSDARG00000091737	Ensembl	1.20	1.50	7.12
zp2.2	ENSDARG00000091737	Ensembl	1.11	1.50	7.10
BT045832.1	BT045832.1	nt	0.00	0.00	7.08
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	6.95
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	6.88
BT072179.1	BT072179.1	nt	0.00	0.00	6.87
CR932977.11	CR932977.11	nt	0.00	0.00	6.79
BT049493.1	BT049493.1	nt	0.00	0.00	6.76
XM_003452872.1	XM_003452872.1	refseq	0.00	0.00	6.75
EU042125.1	EU042125.1	nt	1.23	1.29	6.58
CR352296.12	CR352296.12	nt	-0.51	-0.25	-6.58
EU621899.1	EU621899.1	nt	0.67	0.34	6.57
AC182739.1	AC182739.1	nt	1.12	0.71	-6.56
zp2.5	ENSDARG00000086522	Ensembl	0.82	1.55	6.56
EU042125.1	EU042125.1	nt	0.79	1.71	6.54
si:dkey-179j5.2	ENSDARG00000060325	Ensembl	0.00	0.00	6.38
zp2.5	ENSDARG00000086522	Ensembl	0.57	1.43	6.38
slc7a1	ENSDARG00000016439	Ensembl	0.00	0.00	6.32
slc7a11	ENSMUSG00000027737	Ensembl	0.00	0.00	6.31
DQ156149.1	DQ156149.1	nt	0.41	0.33	-6.28
zp2.5	ENSDARG00000086522	Ensembl	0.77	1.45	6.28
got2a	ENSDARG00000041068	Ensembl	-0.68	0.34	-6.27
AC203456.8	AC203456.8	nt	-0.42	-0.54	-6.25
zp2.5	ENSDARG00000086522	Ensembl	0.79	1.30	6.24
EU481821.1	EU481821.1	nt	0.00	0.00	6.19
apoc2	ENSDARG00000092155	Ensembl	-1.26	-1.33	-6.16
EU025718.1	EU025718.1	nt	-0.79	0.78	-6.08
crot	ENSDARG00000040352	Ensembl	0.82	1.30	5.96
EU621901.1	EU621901.1	nt	0.00	0.00	5.82
crot	ENSDARG00000040352	Ensembl	0.61	1.21	5.77
crot	ENSDARG00000040352	Ensembl	0.62	1.04	5.73
FJ969488.1	FJ969488.1	nt	0.39	0.82	-5.71
crot	ENSDARG00000040352	Ensembl	0.44	1.11	5.67
igfbp1a	ENSDARG00000014947	Ensembl	-1.92	-2.51	-5.66
EF427381.1	EF427381.1	nt	0.00	0.00	5.65
sulf2l	ENSDARG00000013838	Ensembl	-1.11	-1.06	-5.63
AY259111.1	AY259111.1	nt	0.00	0.00	5.58
junba	ENSDARG00000074378	Ensembl	-2.37	-1.92	-5.48
NM_001141007.1	NM_001141007.1	refseq	-1.86	0.40	-5.45
CU367845.1	CU367845.1	nt	0.00	0.00	5.40
EU481821.1	EU481821.1	nt	0.00	0.00	5.40

CU367845.1	CU367845.1	nt	0.00	0.00	5.31
grik5	ENSDARG00000075764	Ensembl	0.00	0.00	5.30
NM_001140006.1	NM_001140006.1	refseq	0.00	0.00	5.28
EU221176.1	EU221176.1	nt	0.09	-0.36	5.23
DQ979823.1	DQ979823.1	nt	0.00	0.00	5.22
aqp12	ENSDARG00000043279	Ensembl	0.00	0.00	5.22
EU008541.1	EU008541.1	nt	0.41	0.86	5.22
tgm2l	ENSDARG00000093381	Ensembl	-1.74	1.11	-5.20
rdh10a	ENSDARG00000058730	Ensembl	0.00	0.00	5.08
DQ246664.1	DQ246664.1	nt	-1.83	-1.98	-5.07
BT072179.1	BT072179.1	nt	1.59	-0.23	-5.04
aqp12	ENSDARG00000043279	Ensembl	0.72	0.17	5.03
diabloa	ENSDARG00000035323	Ensembl	-1.93	-2.35	-4.98
slc7a1	ENSDARG00000016439	Ensembl	0.00	0.00	4.94
EF210363.1	EF210363.1	nt	0.00	0.00	4.92
CU367845.1	CU367845.1	nt	0.00	0.00	4.92
ddit4	ENSDARG00000037618	Ensembl	-5.05	-2.27	-4.91
pfkfb1	ENSDARG00000037140	Ensembl	0.04	-1.73	-4.91
EU025715.1	EU025715.1	nt	-1.98	-2.81	-4.89
GU933433.1	GU933433.1	nt	-1.47	-1.45	-4.89
aqp12	ENSDARG00000043279	Ensembl	0.00	0.00	4.88
rpp21	ENSDARG00000043404	Ensembl	0.00	0.00	4.86
slc7a1	ENSDARG00000016439	Ensembl	0.00	0.00	4.86
esr1	ENSDARG00000004111	Ensembl	1.58	1.54	4.84
aqp12	ENSDARG00000043279	Ensembl	0.62	0.50	4.84
si:dkey-7f3.15	ENSDARG00000094929	Ensembl	0.00	0.00	4.83
EU816603.1	EU816603.1	nt	-1.37	-0.33	-4.83
EU042124.1	EU042124.1	nt	0.00	0.00	4.80
ccdc3	ENSDARG00000026052	Ensembl	0.00	0.00	4.80
esr1	ENSDARG00000004111	Ensembl	1.26	1.71	4.80
Fam20c	ENSMUSG00000025854	Ensembl	0.00	0.00	4.79
pnp5a	ENSDARG00000078619	Ensembl	-0.96	-1.86	-4.76
EU008541.1	EU008541.1	nt	0.32	-0.30	4.74
GRB7	ENSDARG00000042726	Ensembl	-0.95	-0.08	-4.72
HM159473.1	HM159473.1	nt	0.00	0.00	4.72
NM_001139631.1	NM_001139631.1	refseq	0.00	0.00	4.71
lpgat1	ENSDARG00000013542	Ensembl	0.14	0.67	4.70
aqp12	ENSDARG00000043279	Ensembl	0.48	-0.85	4.69
GQ505858.1	GQ505858.1	nt	-3.64	-3.15	-4.68
esr1	ENSDARG00000004111	Ensembl	1.44	1.47	4.68
ezh1	ENSDARG00000037894	Ensembl	0.00	0.00	4.67
lrrc58b	ENSDARG00000063509	Ensembl	0.16	0.49	4.66
diabloa	ENSDARG00000035323	Ensembl	-2.04	-1.62	-4.64
esr1	ENSDARG00000004111	Ensembl	1.08	1.30	4.63
slc7a1	ENSDARG00000016439	Ensembl	0.00	0.00	4.63
diabloa	ENSDARG00000035323	Ensembl	-1.82	-2.18	-4.62
aqp12	ENSDARG00000043279	Ensembl	-0.29	-0.24	4.61
HM159473.1	HM159473.1	nt	-2.28	-3.33	-4.59
AC203446.12	AC203446.12	nt	-0.69	-1.22	-4.57
esr1	ENSDARG00000004111	Ensembl	0.71	1.11	4.52
esr1	ENSDARG00000004111	Ensembl	1.53	1.60	4.52
diabloa	ENSGACG00000011316	Ensembl	-2.52	-1.67	-4.50
GQ505858.1	GQ505858.1	nt	-1.28	-2.70	-4.50
EU621899.1	EU621899.1	nt	-0.08	-0.21	4.48
EU025709.1	EU025709.1	nt	0.00	0.00	4.46
junba	ENSDARG00000074378	Ensembl	-2.17	-1.62	-4.45
GRB7	ENSDARG00000042726	Ensembl	-0.33	-0.50	-4.44
igfbp1b	ENSDARG00000038666	Ensembl	-0.67	-1.58	-4.43
GQ505858.1	GQ505858.1	nt	0.75	-0.05	4.42
krt18	ENSDARG00000018404	Ensembl	-0.60	1.34	-4.41
mpp1	ENSDARG00000031475	Ensembl	-0.75	0.38	-4.38
lrrc58b	ENSDARG00000063509	Ensembl	0.35	0.35	4.36
EU221179.1	EU221179.1	nt	0.00	0.00	4.35
EU025709.1	EU025709.1	nt	0.00	0.00	4.34
lpgat1	ENSDARG00000013542	Ensembl	-0.31	-1.06	4.32
U45968.1	U45968.1	nt	-0.77	-0.08	4.32
EU025708.1	EU025708.1	nt	-0.70	-1.43	-4.31
lrrc58b	ENSDARG00000063509	Ensembl	-0.05	-0.06	4.30
HM159473.1	HM159473.1	nt	-1.01	-2.06	-4.30
crot	ENSDARG00000040352	Ensembl	0.36	0.94	4.29
cyp7a1a	ENSDARG00000069018	Ensembl	-1.39	-3.33	-4.28
sgk1	ENSDARG00000025522	Ensembl	-2.44	-2.02	-4.26
NM_001173970.1	NM_001173970.1	refseq	-0.18	-0.77	-4.24
GQ505858.1	GQ505858.1	nt	-2.09	-2.65	-4.22
slc25a25a	ENSDARG00000010572	Ensembl	-1.37	-0.15	-4.21
foxg1b	ENSDARG00000032705	Ensembl	-1.50	-1.65	-4.21
ENSONIG00000002316	ENSONIG00000002316	Ensembl	-0.74	-1.40	-4.21
diabloa	ENSDARG00000035323	Ensembl	-1.93	-1.50	-4.19
FBLN7	ENSDARG00000089519	Ensembl	-2.66	0.07	-4.18
mmp9	ENSDARG00000042816	Ensembl	-0.13	-3.99	-4.14
pnp5a	ENSDARG00000078619	Ensembl	-0.88	-1.89	-4.11
GQ505858.1	GQ505858.1	nt	-1.78	-2.28	-4.11

ENSONIG00000017668	ENSONIG00000017668	Ensembl	0.00	0.00	4.10
upp2	ENSDARG00000036833	Ensembl	-2.82	-0.83	-4.10
EU025717.1	EU025717.1	nt	-1.76	-0.89	-4.07
gabarapl2	ENSDARG00000027200	Ensembl	-0.93	-5.29	-4.07
CCSER2	ENSDARG00000091535	Ensembl	0.00	0.00	4.03
X92804.1	X92804.1	nt	0.00	0.00	4.03
cpt1b	ENSDARG00000058285	Ensembl	0.52	-0.04	4.00
upp2	ENSDARG00000036833	Ensembl	-2.79	-1.34	-4.00
HM159473.1	HM159473.1	nt	-1.50	-2.23	-3.98
cpt1b	ENSDARG00000058285	Ensembl	0.63	0.15	3.98
pdk2	ENSDARG00000059054	Ensembl	-1.83	-1.26	-3.94
prodha	ENSDARG00000044804	Ensembl	-0.67	-0.86	-3.93
bty	ENSDARG00000040860	Ensembl	0.00	2.03	3.92
FJ969489.1	FJ969489.1	nt	-0.72	0.01	-3.89
cox4i2	ENSDARG00000022509	Ensembl	0.82	0.58	3.85
GQ505858.1	GQ505858.1	nt	-1.58	-2.33	-3.85
HM159473.1	HM159473.1	nt	-1.57	-2.39	-3.84
HM159473.1	HM159473.1	nt	0.00	0.00	3.83
fkbp9	ENSDARG00000005023	Ensembl	0.00	0.00	3.82
GQ505858.1	GQ505858.1	nt	-1.99	-2.38	-3.82
CR936412.13	CR936412.13	nt	-0.84	-1.81	3.78
ABCD2	ENSG00000173208	Ensembl	0.00	0.00	3.77
NM_001140849.1	NM_001140849.1	refseq	-0.58	-1.35	-3.77
yth2	ENSDARG00000014498	Ensembl	0.00	0.00	3.76
cpt1b	ENSDARG00000058285	Ensembl	0.57	0.48	3.76
hkdc1	ENSDARG00000038703	Ensembl	-1.10	-0.40	-3.74
AF312396.1	AF312396.1	nt	-1.08	-0.86	-3.74
GU129140.1	GU129140.1	nt	-1.10	0.14	-3.72
cpt1b	ENSDARG00000058285	Ensembl	-0.19	0.50	3.71
NM_001173782.1	NM_001173782.1	refseq	0.00	0.00	3.70
tsc22d1	ENSDARG00000038306	Ensembl	-2.11	-1.09	-3.70
rpp21	ENSDARG00000043404	Ensembl	0.36	-0.24	3.70
PFKFB1	ENSDARG00000074457	Ensembl	-0.01	-1.21	-3.70
AF375027.1	AF375027.1	nt	0.00	0.00	3.69
EU084728.1	EU084728.1	nt	-3.23	-2.16	-3.69
mertk	ENSDARG00000074695	Ensembl	0.67	1.30	3.66
Atad3a	ENSMUSG00000029036	Ensembl	-0.79	0.09	-3.65
NM_001173779.1	NM_001173779.1	refseq	0.00	0.00	3.63
dnal4b	ENSDARG00000088841	Ensembl	0.00	0.00	3.63
bty	ENSDARG00000040860	Ensembl	0.00	0.00	3.59
BT057528.1	BT057528.1	nt	0.25	0.00	3.59
ENSGACG00000014196	ENSGACG00000014196	Ensembl	-0.94	-0.52	-3.57
mthfr	ENSDARG00000053087	Ensembl	0.63	0.60	3.57
CR450814.1	CR450814.1	nt	-2.47	-0.19	-3.55
K03052.1	K03052.1	nt	-4.75	1.46	-3.55
DQ246664.1	DQ246664.1	nt	-3.98	-1.56	-3.55
BT072520.1	BT072520.1	nt	-0.14	0.90	3.53
ABCD2	ENSDARG00000087347	Ensembl	0.17	0.66	3.53
BT125319.1	BT125319.1	nt	-0.38	-1.47	-3.53
mthfr	ENSDARG00000053087	Ensembl	0.02	0.47	3.52
GU129139.1	GU129139.1	nt	1.21	0.42	3.51
EPS8L3	ENSDARG00000077296	Ensembl	-1.11	-0.28	-3.51
BT072179.1	BT072179.1	nt	2.11	-0.64	-3.50
GU129140.1	GU129140.1	nt	-0.74	-1.24	-3.49
BT072284.1	BT072284.1	nt	-1.25	-1.26	-3.48
ENSONIG00000002781	ENSONIG00000002781	Ensembl	0.00	0.89	3.47
NM_001141739.1	NM_001141739.1	refseq	-1.28	-0.28	-3.47
CU928220.2	CU928220.2	nt	0.00	0.00	3.45
myc	ENSDARG00000007241	Ensembl	0.00	0.21	3.45
JHDM1D	ENSDARG00000018559	Ensembl	0.00	0.00	3.45
Col5a2	ENSMUSG00000026042	Ensembl	-2.18	-0.71	-3.44
NM_001140006.1	NM_001140006.1	refseq	0.00	0.00	3.42
BX530056.17	BX530056.17	nt	0.00	0.00	3.42
got2a	ENSDARG00000041068	Ensembl	-1.26	-0.43	-3.40
FJ969488.1	FJ969488.1	nt	-0.82	-1.33	-3.40
EU481821.1	EU481821.1	nt	1.02	0.60	3.39
cpt1b	ENSDARG00000058285	Ensembl	0.27	-0.35	3.39
slc3a2a	ENSDARG00000036427	Ensembl	-1.42	0.25	-3.38
nipblb	ENSDARG00000061052	Ensembl	-3.87	-1.16	-3.37
EU221178.1	EU221178.1	nt	0.00	0.00	3.37
ulk1a	ENSDARG00000062518	Ensembl	-1.53	-0.59	-3.37
GU933433.1	GU933433.1	nt	-0.94	-1.17	-3.36
pim2	ENSDARG00000059001	Ensembl	0.93	0.71	3.35
si:dkey-200l5.2	ENSDARG00000039351	Ensembl	0.79	1.08	3.35
si:dkey-56d12.4	ENSDARG00000070845	Ensembl	-1.14	-0.07	-3.34
fam20a	ENSDARG00000079486	Ensembl	0.87	0.73	3.30
CU459095.1	CU459095.1	nt	0.49	0.82	3.30
DQ156150.1	DQ156150.1	nt	0.46	-0.03	-3.29
AC203456.8	AC203456.8	nt	0.00	1.28	3.28
NM_001173763.1	NM_001173763.1	refseq	-0.71	-1.59	-3.28
GU324549.1	GU324549.1	nt	0.00	0.39	3.28
nr0b2a	ENSDARG00000044685	Ensembl	1.21	1.06	3.27

abcg2b	ENSDARG00000079361	Ensembl	0.11	-1.30	-3.26
mthfr	ENSDARG00000053087	Ensembl	0.37	0.29	3.25
CU571382.2	CU571382.2	nt	-0.58	-0.41	-3.25
ugt5f1	ENSDARG00000054835	Ensembl	-0.24	0.23	-3.24
GRB7	ENSDARG00000042726	Ensembl	-0.72	-0.27	-3.23
GABRB1	ENSDARG00000076127	Ensembl	-0.93	0.26	-3.23
th2	ENSDARG00000038384	Ensembl	0.09	0.14	-3.23
gpcpd1	ENSDARG00000016011	Ensembl	-0.71	-0.74	-3.22
AJ295231.1	AJ295231.1	nt	0.00	0.00	3.22
FJ356099.1	FJ356099.1	nt	-0.68	-0.45	-3.22
ext1a	ENSDARG00000020373	Ensembl	0.00	0.00	3.21
PKP4	ENSG00000144283	Ensembl	-0.42	-0.97	-3.21
lpgat1	ENSDARG00000013542	Ensembl	0.00	0.00	3.20
tdh	ENSDARG00000002745	Ensembl	-0.62	0.63	-3.20
mthfr	ENSDARG00000053087	Ensembl	0.70	-0.08	3.20
got1	ENSDARG00000039093	Ensembl	-1.77	-0.17	-3.20
BT044658.1	BT044658.1	nt	0.00	0.00	3.20
ANK1	ENSDARG00000074777	Ensembl	1.16	-0.10	3.19
DQ156149.1	DQ156149.1	nt	-0.45	-0.53	-3.18
NM_001173925.1	NM_001173925.1	refseq	-0.41	-0.68	-3.18
BT072122.1	BT072122.1	nt	-0.63	0.46	-3.18
tmed5	ENSDARG00000008765	Ensembl	-0.28	0.55	3.18
tcnl	ENSDARG00000068088	Ensembl	-0.69	-2.67	-3.17
CU459095.1	CU459095.1	nt	0.83	0.78	3.17
FJ969488.1	FJ969488.1	nt	-1.15	-0.75	3.17
FQ310506.3	FQ310506.3	nt	-1.38	-0.90	-3.17
MAP3K6	ENSDARG00000069933	Ensembl	0.00	0.00	3.17
npsn	ENSDARG00000010423	Ensembl	-0.29	-1.55	-3.16
acsbg2	ENSDARG00000004094	Ensembl	-1.21	-0.70	-3.16
FQ310506.3	FQ310506.3	nt	-0.09	-2.15	-3.16
SYTL1	ENSDARG00000070094	Ensembl	0.00	0.00	3.15
zgc:165409	ENSDARG00000069528	Ensembl	0.00	0.00	3.13
SASH1	ENSDARG00000058853	Ensembl	1.53	1.55	3.13
ANK1	ENSDARG00000074777	Ensembl	0.00	0.00	3.13
NM_001141739.1	NM_001141739.1	refseq	-1.16	-0.29	-3.12
GABRB1	ENSDARG00000076127	Ensembl	-0.69	0.00	-3.12
eif4a2	ENSDARG00000016477	Ensembl	0.00	0.00	3.12
rpp21	ENSDARG00000043404	Ensembl	-0.96	-0.54	3.11
BT058872.1	BT058872.1	nt	1.14	0.84	3.11
GRB7	ENSDARG00000042726	Ensembl	-0.30	0.10	-3.10
ANK1	ENSDARG00000074777	Ensembl	0.86	0.21	3.09
NM_001141081.1	NM_001141081.1	refseq	-0.72	-0.61	-3.08
ret	ENSDARG00000055305	Ensembl	-1.15	-0.75	-3.08
zgc:92218	ENSDARG00000027851	Ensembl	-0.31	-1.45	-3.03
BT058872.1	BT058872.1	nt	0.10	0.46	3.03
BT049695.1	BT049695.1	nt	-2.56	-1.47	-3.03
EU221180.1	EU221180.1	nt	-0.46	-0.75	-3.02
DQ246664.1	DQ246664.1	nt	0.00	0.00	3.02
EU621899.1	EU621899.1	nt	-0.96	-0.64	-3.01
CR391962.1	CR391962.1	nt	-0.29	0.59	-3.01
BT060412.1	BT060412.1	nt	-0.58	-0.51	-3.01
GABRB1	ENSDARG00000076127	Ensembl	-0.35	0.13	-3.00
NM_001140307.1	NM_001140307.1	refseq	-1.26	0.68	-2.99
NM_001141336.1	NM_001141336.1	refseq	0.34	0.19	2.99
myc	ENSDARG00000007241	Ensembl	-0.95	-0.52	2.98
pnisr	ENSDARG00000069855	Ensembl	0.00	0.00	2.98
BT072803.1	BT072803.1	nt	0.00	0.00	2.98
EU025708.1	EU025708.1	nt	-0.63	-2.31	-2.97
rdh1	ENSDARG00000017882	Ensembl	-1.21	-0.43	-2.95
ulk1b	ENSDARG00000074481	Ensembl	-1.37	-0.32	-2.94
acsl3b	ENSDARG00000014674	Ensembl	0.00	0.00	2.94
myo9b	ENSDARG00000077410	Ensembl	0.00	0.00	2.94
EU325858.1	EU325858.1	nt	-0.77	-1.07	-2.94
pnp5a	ENSDARG00000078619	Ensembl	-1.15	-2.17	-2.94
EU025717.1	EU025717.1	nt	-0.45	-1.03	-2.93
HM159469.1	HM159469.1	nt	0.00	0.74	2.92
EU481821.1	EU481821.1	nt	0.68	0.81	2.92
AF256661.1	AF256661.1	nt	0.84	0.98	2.91
tmed5	ENSDARG00000008765	Ensembl	-0.11	0.71	2.91
psph	ENSDARG00000040314	Ensembl	0.48	0.28	2.91
mmp13a	ENSDARG00000012395	Ensembl	1.33	-1.17	-2.90
GU324549.1	GU324549.1	nt	0.00	0.00	2.90
GABRB1	ENSDARG00000076127	Ensembl	-0.69	0.11	-2.90
btd	ENSDARG00000006926	Ensembl	1.52	1.14	-2.90
CR391962.1	CR391962.1	nt	-0.14	0.70	-2.89
got2a	ENSDARG00000041068	Ensembl	-1.04	-0.14	-2.89
NM_001141336.1	NM_001141336.1	refseq	0.49	0.49	2.89
mrc1a	ENSDARG00000073928	Ensembl	0.00	0.00	2.89
HM159473.1	HM159473.1	nt	-1.30	-2.00	-2.88
DQ138301.1	DQ138301.1	nt	-1.10	-0.08	-2.88
crtac1a	ENSDARG00000059826	Ensembl	0.00	0.00	2.88
fam20a	ENSDARG00000079486	Ensembl	-0.23	0.05	2.87

EU221178.1	EU221178.1	nt	0.00	0.00	2.86
nedd4l	ENSDARG00000060006	Ensembl	-1.82	0.07	-2.86
hkdc1	ENSDARG00000038703	Ensembl	-0.16	0.21	-2.86
elovl5	ENSDARG00000004979	Ensembl	0.08	-0.39	2.86
EU221176.1	EU221176.1	nt	0.00	0.97	2.86
alg9	ENSDARG00000012840	Ensembl	0.00	0.00	2.84
fam20a	ENSDARG00000079486	Ensembl	-1.43	-0.97	2.84
GU129140.1	GU129140.1	nt	0.00	0.00	2.84
BT072419.1	BT072419.1	nt	1.13	0.38	2.83
ugt5c1	ENSDARG00000061444	Ensembl	-0.38	-0.01	-2.83
XM_695908.4	XM_695908.4	refseq	-0.08	-0.73	-2.83
ugt5g1	ENSDARG00000032862	Ensembl	-1.26	0.56	-2.82
BT059775.1	BT059775.1	nt	-0.36	0.57	2.82
psph	ENSDARG00000040314	Ensembl	-0.38	0.00	2.82
FBLN7	ENSDARG00000089519	Ensembl	-1.41	0.01	-2.80
foxo1a	ENSDARG00000063540	Ensembl	-0.52	0.02	-2.80
NM_001140188.1	NM_001140188.1	refseq	1.01	0.71	2.79
ANK1	ENSDARG00000074777	Ensembl	0.00	0.00	2.78
SYTL1	ENSDARG00000070094	Ensembl	0.00	0.00	2.78
EF427381.1	EF427381.1	nt	0.00	0.00	2.77
eif4a2	ENSDARG00000016477	Ensembl	0.00	0.00	2.76
pnp5a	ENSDARG00000078619	Ensembl	-1.12	-2.09	-2.76
fam20a	ENSDARG00000079486	Ensembl	0.11	-0.34	2.76
AC203446.12	AC203446.12	nt	0.00	0.00	2.76
btr21	ENSDARG00000013481	Ensembl	1.17	1.03	-2.75
pgbd5	ENSDARG00000011042	Ensembl	-0.28	-1.03	-2.75
chst2b	ENSDARG00000058585	Ensembl	-0.19	-0.19	2.75
EU221179.1	EU221179.1	nt	0.00	2.06	2.74
pptc7a	ENSDARG00000011122	Ensembl	-1.02	-0.63	-2.74
EU325858.1	EU325858.1	nt	-0.55	-1.41	-2.73
HM159471.1	HM159471.1	nt	0.00	-0.07	2.73
ANK1	ENSDARG00000074777	Ensembl	0.00	0.00	2.73
fam20a	ENSDARG00000079486	Ensembl	-0.46	-0.62	2.72
BT072284.1	BT072284.1	nt	-0.70	0.00	-2.71
BT059080.1	BT059080.1	nt	1.97	3.21	2.71
GQ903131.1	GQ903131.1	nt	0.30	-0.26	-2.71
AC203446.12	AC203446.12	nt	-0.26	0.58	2.70
ezh1	ENSDARG00000037894	Ensembl	0.92	1.03	2.70
XM_003451796.1	XM_003451796.1	refseq	-0.55	-0.70	-2.70
ANK1	ENSDARG00000074777	Ensembl	-0.06	-0.18	2.70
ntsr1	ENSDARG00000077577	Ensembl	0.00	0.00	2.70
cldn11a	ENSDARG00000020031	Ensembl	-1.20	-0.60	-2.69
BT049750.1	BT049750.1	nt	0.44	0.18	-2.69
NM_001140492.1	NM_001140492.1	refseq	-1.43	-0.59	-2.69
NM_001140092.1	NM_001140092.1	refseq	0.00	0.00	2.69
GQ505859.1	GQ505859.1	nt	0.18	0.59	2.69
BT059080.1	BT059080.1	nt	1.92	2.32	2.68
BT072138.1	BT072138.1	nt	-1.20	-0.50	-2.68
EU481821.1	EU481821.1	nt	0.00	0.69	2.67
cept1b	ENSDARG00000021177	Ensembl	-0.38	0.35	2.67
AB196459.1	AB196459.1	nt	0.58	0.63	2.67
EU621899.1	EU621899.1	nt	-0.95	-0.63	-2.67
si:dkey-23c22.5	ENSDARG00000068972	Ensembl	0.00	0.00	2.67
GU552297.1	GU552297.1	nt	0.00	0.00	2.66
pptc7a	ENSDARG00000011122	Ensembl	-0.83	-0.62	-2.66
errfi1	ENSDARG00000070171	Ensembl	-1.77	-1.09	-2.66
BT073892.1	BT073892.1	nt	0.27	0.47	2.66
DQ138301.1	DQ138301.1	nt	-0.94	-0.32	-2.65
BT059080.1	BT059080.1	nt	1.76	2.80	2.65
aldh18a1	ENSDARG00000061123	Ensembl	1.31	1.48	2.65
errfi1	ENSDARG00000070171	Ensembl	-1.54	-1.23	-2.65
nr0b2a	ENSDARG00000044685	Ensembl	1.46	0.87	2.65
btd	ENSDARG00000006926	Ensembl	1.42	0.98	-2.65
C2CD4C	ENSDARG00000079876	Ensembl	-0.36	0.70	-2.64
NM_001014308.1	NM_001014308.1	refseq	-2.60	0.25	-2.64
GU129140.1	GU129140.1	nt	0.00	0.00	2.64
GU129140.1	GU129140.1	nt	0.03	-0.55	-2.64
slc6a9	ENSDARG00000018534	Ensembl	-1.35	-0.23	-2.64
GQ505860.1	GQ505860.1	nt	0.96	2.05	2.62
aldh18a1	ENSDARG00000061123	Ensembl	1.69	2.37	2.62
EF467296.1	EF467296.1	nt	-0.24	0.58	-2.62
BC163282.1	BC163282.1	nt	0.00	0.00	2.61
HM210571.1	HM210571.1	nt	1.55	1.72	2.61
EU025718.1	EU025718.1	nt	-1.13	-0.33	-2.60
aldh18a1	ENSDARG00000061123	Ensembl	0.00	0.00	2.60
rrbp1a	ENSDARG00000013763	Ensembl	0.33	0.67	2.60
FBLN7	ENSDARG00000089519	Ensembl	-1.84	-0.25	-2.60
fam20a	ENSDARG00000079486	Ensembl	-0.11	0.06	2.60
si:dkey-56d12.4	ENSDARG00000070845	Ensembl	-1.00	-0.22	-2.59
ezh1	ENSDARG00000037894	Ensembl	0.00	0.00	2.59
ret	ENSDARG00000055305	Ensembl	-0.84	0.53	-2.58
EU325858.1	EU325858.1	nt	-0.76	-1.31	-2.58

NLRC3	ENSG00000167984	Ensembl	0.00	0.00	2.55
NM_001140025.1	NM_001140025.1	refseq	-0.71	0.32	-2.54
si:dkeyp-75b4.10	ENSDARG00000079043	Ensembl	0.13	0.66	2.54
EU481821.1	EU481821.1	nt	0.00	0.00	2.54
CR847523.7	CR847523.7	nt	0.00	0.00	2.54
si:dkey-56d12.4	ENSDARG00000070845	Ensembl	-1.08	0.07	-2.54
AC203446.12	AC203446.12	nt	0.54	-0.16	2.54
NM_001140006.1	NM_001140006.1	refseq	-2.51	-0.49	2.54
igl3v1	ENSDARG00000093258	Ensembl	-0.14	0.17	-2.54
nfasca	ENSDARG00000061099	Ensembl	0.34	-0.27	2.53
NM_001173711.1	NM_001173711.1	refseq	-1.55	-0.89	-2.53
fkbp5	ENSDARG00000028396	Ensembl	-0.51	0.17	-2.53
ENSORLG00000019699	ENSORLG00000019699	Ensembl	0.00	0.00	2.53
GRB7	ENSDARG00000042726	Ensembl	0.08	0.33	-2.53
NM_001141336.1	NM_001141336.1	refseq	0.09	0.43	2.53
cbln8	ENSDARG00000019294	Ensembl	-0.28	-0.43	-2.53
Ddit4l	ENSMUSG00000046818	Ensembl	2.04	1.94	2.52
rpp21	ENSDARG00000043404	Ensembl	-0.16	-0.19	2.52
elovl5	ENSDARG00000004979	Ensembl	0.15	-0.32	2.52
rdh1	ENSDARG00000017882	Ensembl	-1.99	-0.34	-2.51
rrbp1a	ENSDARG00000013763	Ensembl	-0.10	0.14	2.51
GQ505860.1	GQ505860.1	nt	0.63	1.78	2.50
ckba	ENSDARG00000069752	Ensembl	-0.57	0.48	-2.50
ckbb	ENSDARG00000043257	Ensembl	-2.23	0.79	-2.50
CABZ01072083.1	CABZ01072083.1	nt	0.00	1.24	2.50
AC203446.12	AC203446.12	nt	0.00	0.00	2.50
NM_001141336.1	NM_001141336.1	refseq	-0.06	0.26	2.50
MMP15	ENSDARG00000013072	Ensembl	-0.05	-0.86	2.49
AJ295231.1	AJ295231.1	nt	0.21	-0.07	2.49
EU481821.1	EU481821.1	nt	-0.38	0.72	-2.49
GQ502184.1	GQ502184.1	nt	1.03	0.62	2.49
ENSORLG00000004374	ENSORLG00000004374	Ensembl	-0.01	0.06	-2.49
rtn3	ENSDARG00000058028	Ensembl	0.37	0.63	2.49
abhd17b	ENSDARG00000035571	Ensembl	-0.67	-0.85	-2.48
EU325858.1	EU325858.1	nt	-0.42	-0.90	-2.48
BT058872.1	BT058872.1	nt	0.30	0.65	2.48
HGF	ENSDARG00000063316	Ensembl	0.00	0.00	2.48
gart	ENSDARG00000051855	Ensembl	0.89	1.27	2.48
errfi1	ENSDARG00000070171	Ensembl	-1.41	-1.12	-2.47
akap9	ENSDARG00000079610	Ensembl	0.00	0.00	2.47
hsd3b7	ENSDARG00000036966	Ensembl	-0.54	-0.01	-2.47
cog2	ENSDARG00000004037	Ensembl	-0.62	0.52	-2.47
NM_001173567.1	NM_001173567.1	refseq	-0.21	-0.28	-2.47
NM_001140457.1	NM_001140457.1	refseq	0.05	-1.35	-2.46
rrbp1a	ENSDARG00000013763	Ensembl	0.00	0.00	2.46
got2a	ENSDARG00000041068	Ensembl	-0.70	0.13	-2.46
ezh1	ENSDARG00000037894	Ensembl	0.00	0.00	2.46
GQ505860.1	GQ505860.1	nt	-0.25	-0.23	2.45
DQ156149.1	DQ156149.1	nt	0.19	-0.05	-2.45
chst12a	ENSDARG00000028786	Ensembl	0.18	-1.00	-2.45
errfi1	ENSDARG00000070171	Ensembl	-1.40	-1.18	-2.44
GU129139.1	GU129139.1	nt	-0.59	-0.12	-2.44
BT049003.2	BT049003.2	nt	0.25	1.32	2.44
FQ310506.3	FQ310506.3	nt	0.35	-1.32	-2.44
EU008541.1	EU008541.1	nt	-0.40	-0.32	-2.44
hsd3b7	ENSDARG00000036966	Ensembl	-0.56	-0.53	-2.44
SYTL1	ENSDARG00000070094	Ensembl	0.00	0.00	2.44
MMP15	ENSDARG00000013072	Ensembl	-0.95	-1.19	2.44
C3	LRG 27	Ensembl	0.34	-0.56	-2.43
ect2	ENSDARG00000007278	Ensembl	0.11	-1.65	-2.43
AB162342.1	AB162342.1	nt	-0.59	-0.21	-2.43
GRB7	ENSDARG00000042726	Ensembl	-0.32	0.23	-2.43
slc25a29	ENSDARG00000057352	Ensembl	-0.38	-0.04	-2.43
SYTL1	ENSDARG00000070094	Ensembl	1.15	0.58	2.43
cog2	ENSDARG00000004037	Ensembl	-0.43	0.33	-2.43
NM_001140826.1	NM_001140826.1	refseq	0.00	0.00	2.43
mat2aa	ENSDARG00000040334	Ensembl	1.27	2.18	2.42
pptc7a	ENSDARG00000011122	Ensembl	-0.97	-0.73	-2.42
pptc7a	ENSDARG00000011122	Ensembl	-0.96	-0.60	-2.42
BT057201.1	BT057201.1	nt	1.14	0.65	2.42
ENSONIG00000020881	ENSONIG00000020881	Ensembl	-0.40	-0.63	-2.42
errfi1	ENSDARG00000070171	Ensembl	-1.13	-1.01	-2.42
BT125436.1	BT125436.1	nt	0.17	-0.14	-2.41
sdf2	ENSDARG00000024026	Ensembl	0.13	0.36	2.41
ulk1b	ENSDARG00000074481	Ensembl	-1.36	-1.35	-2.41
slc1a4	ENSDARG00000000551	Ensembl	1.69	1.74	2.41
ezh1	ENSDARG00000037894	Ensembl	0.07	0.50	2.41
psph	ENSDARG00000040314	Ensembl	0.56	0.49	2.41
grik5	ENSDARG00000075764	Ensembl	0.00	0.00	2.41
HM159471.1	HM159471.1	nt	0.28	0.25	2.41
rwdd2b	ENSDARG00000055426	Ensembl	0.77	0.00	2.41
cog2	ENSDARG00000004037	Ensembl	-0.58	0.31	-2.40

ANK1	ENSDARG00000074777	Ensembl	-0.35	-0.34	2.40
chrne	ENSDARG00000034307	Ensembl	0.00	0.00	2.40
rrbp1a	ENSDARG00000013763	Ensembl	0.00	0.00	2.39
HM159469.1	HM159469.1	nt	-0.66	-0.21	-2.39
MYO9B	ENSDARG00000074413	Ensembl	-0.14	-0.78	2.39
crfb4	ENSDARG00000068711	Ensembl	0.05	0.60	-2.39
ENSONIG00000002783	ENSONIG00000002783	Ensembl	0.00	0.00	2.38
EU621898.1	EU621898.1	nt	0.00	0.00	2.38
npc1	ENSDARG00000017180	Ensembl	-1.25	-0.56	-2.38
BT057913.1	BT057913.1	nt	-0.94	-0.61	-2.38
AY550549.1	AY550549.1	nt	0.00	0.00	2.38
NM_001173879.1	NM_001173879.1	refseq	0.69	1.24	2.38
CABZ01072083.1	CABZ01072083.1	nt	0.00	0.00	2.38
myo9b	ENSDARG00000077410	Ensembl	0.00	-0.33	2.37
CR387996.2	CR387996.2	nt	0.00	0.00	2.37
NM_001124414.1	NM_001124414.1	refseq	0.20	-0.13	-2.37
fam20a	ENSDARG00000079486	Ensembl	-0.33	-0.52	2.36
ern1	ENSDARG00000013997	Ensembl	0.00	0.76	2.36
vwa1	ENSDARG00000075468	Ensembl	0.12	-0.64	-2.36
nfasca	ENSDARG00000061099	Ensembl	0.20	0.51	2.36
bhmt	ENSDARG00000013430	Ensembl	-1.64	-0.80	-2.35
NM_001140188.1	NM_001140188.1	refseq	0.68	-0.04	2.35
GU129139.1	GU129139.1	nt	-1.18	-0.19	-2.35
ezh1	ENSDARG00000037894	Ensembl	0.00	0.00	2.35
cept1b	ENSDARG00000021177	Ensembl	-0.05	-0.04	2.34
rp2	ENSDARG00000044339	Ensembl	0.00	0.77	2.34
slc25a28	ENSDARG00000074297	Ensembl	0.00	0.00	2.34
CR382327.2	CR382327.2	nt	0.00	1.68	2.34
NM_001173711.1	NM_001173711.1	refseq	-1.91	-1.01	-2.34
yth2	ENSDARG00000014498	Ensembl	0.02	0.31	2.34
tekt2	ENSDARG00000028973	Ensembl	0.00	0.87	2.34
per3	ENSDARG00000010519	Ensembl	1.84	0.00	2.34
NM_001140120.1	NM_001140120.1	refseq	-1.00	-1.23	2.34
slc6a9	ENSDARG00000018534	Ensembl	-1.46	-0.11	-2.33
si:dkey-238o13.4	ENSDARG00000078847	Ensembl	-1.06	-0.63	-2.33
Fam20c	ENSMUSG00000025854	Ensembl	0.34	0.47	2.33
cept1b	ENSDARG00000021177	Ensembl	-0.30	0.16	2.33
FJ969490.1	FJ969490.1	nt	0.34	0.18	2.33
cdkn1bb	ENSDARG00000088081	Ensembl	-1.17	-1.03	-2.33
errfi1	ENSDARG00000070171	Ensembl	-0.17	-1.29	-2.33
SASH1	ENSDARG00000058853	Ensembl	0.35	0.52	2.32
mpp1	ENSDARG00000031475	Ensembl	-1.65	-0.43	-2.32
rtn3	ENSDARG00000058028	Ensembl	-0.03	-0.13	2.32
ezh1	ENSDARG00000037894	Ensembl	0.12	0.00	2.31
FBLN7	ENSDARG00000089519	Ensembl	-1.75	0.11	-2.31
CABZ01072083.1	CABZ01072083.1	nt	0.00	0.00	2.31
cdkn1bb	ENSDARG00000088081	Ensembl	-1.56	-0.80	-2.31
ntng2a	ENSDARG00000077367	Ensembl	-0.64	0.15	-2.31
ezh1	ENSDARG00000037894	Ensembl	0.99	0.00	2.31
GQ925648.1	GQ925648.1	nt	-1.50	-0.52	-2.31
fam20a	ENSDARG00000079486	Ensembl	0.37	-0.18	2.31
BT045418.1	BT045418.1	nt	-0.42	-0.74	-2.30
polr3a	ENSDARG00000071269	Ensembl	0.53	1.37	2.30
GU129140.1	GU129140.1	nt	2.22	0.00	2.30
EU221178.1	EU221178.1	nt	-1.01	-0.73	-2.29
chst2b	ENSDARG00000058585	Ensembl	0.00	-0.33	2.29
BT045418.1	BT045418.1	nt	-0.40	-0.69	-2.29
ugt5c1	ENSDARG00000061444	Ensembl	-0.51	0.20	-2.29
fkbp5	ENSDARG00000028396	Ensembl	-0.62	0.29	-2.29
BT059181.1	BT059181.1	nt	-0.74	-0.65	-2.28
elovl5	ENSDARG00000004979	Ensembl	0.08	-0.39	2.28
yth2	ENSDARG00000014498	Ensembl	0.33	-0.37	2.28
ezh1	ENSDARG00000037894	Ensembl	0.07	-0.85	2.28
gltpd2	ENSDARG00000067889	Ensembl	0.50	-0.27	2.28
Fam20c	ENSMUSG00000025854	Ensembl	0.04	0.40	2.28
tk1	ENSDARG00000086561	Ensembl	-2.27	0.70	-2.28
klf13	ENSDARG00000061368	Ensembl	-0.34	0.44	2.28
PKP4	ENSG00000144283	Ensembl	-1.38	-0.91	-2.28
ENSGACG00000002729	ENSGACG00000002729	Ensembl	1.09	1.43	2.28
ube2h	ENSDARG00000000019	Ensembl	0.45	1.04	2.28
EU481821.1	EU481821.1	nt	0.00	0.00	2.27
faxdc2	ENSDARG00000023820	Ensembl	-1.23	-0.77	-2.27
CU638740.1	CU638740.1	nt	0.00	0.00	2.27
SYTL1	ENSDARG00000070094	Ensembl	-0.27	0.72	2.27
abcg2b	ENSDARG00000079361	Ensembl	0.19	-0.56	-2.27
HQ287745.1	HQ287745.1	nt	-1.25	0.06	-2.27
slc25a29	ENSDARG00000057352	Ensembl	-0.53	-0.34	-2.27
ap1s2	ENSDARG00000058504	Ensembl	-0.79	-0.81	-2.27
FAM149B1	ENSDARG00000061215	Ensembl	0.00	0.00	2.26
agt	ENSDARG00000016412	Ensembl	-0.67	-0.45	-2.26
BX511270.1	BX511270.1	nt	-0.94	-0.39	-2.26
MYO9B	ENSDARG00000074413	Ensembl	0.07	0.02	2.26

glulb	ENSDARG00000017339	Ensembl	-2.04	-0.94	-2.25
HM159472.1	HM159472.1	nt	-0.75	-0.75	-2.25
EU025709.1	EU025709.1	nt	0.61	0.02	2.25
arntl1a	ENSDARG00000006791	Ensembl	0.00	0.00	2.25
hsd3b7	ENSDARG00000036966	Ensembl	-0.35	-0.22	-2.25
GU129140.1	GU129140.1	nt	2.04	2.20	2.24
ulk1a	ENSDARG00000062518	Ensembl	-0.90	0.09	-2.24
tat	ENSDARG00000069630	Ensembl	-0.16	-0.34	-2.24
ulk1b	ENSDARG00000074481	Ensembl	-0.76	-1.04	-2.24
myo9b	ENSDARG00000077410	Ensembl	-0.14	-0.14	2.24
cept1b	ENSDARG00000021177	Ensembl	0.22	0.22	2.24
NM_001139680.1	NM_001139680.1	refseq	-0.20	0.38	2.24
fam5b	ENSDARG00000014302	Ensembl	-0.29	-0.93	-2.24
Fam20c	ENSMUSG00000025854	Ensembl	0.19	0.54	2.23
AC203456.8	AC203456.8	nt	-0.07	0.31	2.23
BT057448.1	BT057448.1	nt	0.40	-0.45	-2.23
AY544084.1	AY544084.1	nt	0.51	0.48	2.23
GU129140.1	GU129140.1	nt	-0.75	-0.38	-2.22
MYO9B	ENSDARG00000074413	Ensembl	-0.33	-0.10	2.22
DQ156151.1	DQ156151.1	nt	-0.53	-0.50	-2.22
tat	ENSDARG00000069630	Ensembl	-0.13	-0.42	-2.22
errfi1	ENSDARG00000070171	Ensembl	-0.71	-0.88	-2.22
BX936371.1	BX936371.1	nt	0.43	0.58	2.21
EU481821.1	EU481821.1	nt	-1.29	1.36	-2.21
bhmt	ENSDARG00000013430	Ensembl	-1.41	-0.73	-2.21
EU481821.1	EU481821.1	nt	-1.49	-1.48	-2.21
BT072559.1	BT072559.1	nt	0.00	0.00	2.21
errfi1	ENSDARG00000070171	Ensembl	-1.78	-1.10	-2.21
BT045302.1	BT045302.1	nt	-1.13	0.26	-2.20
slc25a48	ENSDARG00000021250	Ensembl	0.00	0.03	2.20
hsd3b7	ENSDARG00000036966	Ensembl	-0.35	0.01	-2.19
BT079691.1	BT079691.1	nt	-1.78	-0.42	-2.19
BX000999.4	BX000999.4	nt	0.00	0.00	2.19
Fam20c	ENSMUSG00000025854	Ensembl	0.12	0.09	2.19
ube2h	ENSDARG00000000019	Ensembl	0.99	0.11	2.19
ugt5f1	ENSDARG00000054835	Ensembl	-1.25	-0.10	-2.19
errfi1	ENSDARG00000070171	Ensembl	-0.17	-0.99	-2.18
EU481821.1	EU481821.1	nt	-1.02	-0.32	-2.18
BT043636.1	BT043636.1	nt	0.00	0.10	2.18
cog2	ENSDARG00000004037	Ensembl	0.01	0.17	-2.18
tat	ENSDARG00000069630	Ensembl	-0.21	-0.70	-2.18
CR376783.1	CR376783.1	nt	1.76	1.02	2.17
nfasca	ENSDARG00000061099	Ensembl	0.00	0.00	2.17
gltpd2	ENSDARG00000067889	Ensembl	-0.31	-0.22	2.17
dennd4c	ENSG00000137145	Ensembl	0.45	-0.76	2.17
HM159469.1	HM159469.1	nt	0.67	0.65	2.16
gltpd2	ENSDARG00000067889	Ensembl	0.31	-0.53	2.16
rdh1	ENSDARG00000017882	Ensembl	-1.64	-0.40	-2.16
si:dkey-238o13.4	ENSDARG00000078847	Ensembl	-0.74	-0.51	-2.16
GQ505858.1	GQ505858.1	nt	-0.89	-0.25	-2.16
axin2	ENSDARG00000014147	Ensembl	1.61	1.80	2.16
arl6ip1	ENSDARG00000054578	Ensembl	0.10	-0.28	2.16
cog2	ENSDARG00000004037	Ensembl	-0.61	0.27	-2.15
tat	ENSDARG00000069630	Ensembl	-0.40	-0.77	-2.15
ttll12	ENSDARG00000017407	Ensembl	0.38	0.18	2.15
BT044742.1	BT044742.1	nt	-0.51	-0.51	-2.15
EU888965.1	EU888965.1	nt	0.22	0.32	-2.15
kdelr3	ENSDARG00000040912	Ensembl	1.42	0.29	2.15
chrne	ENSDARG00000034307	Ensembl	0.61	0.00	2.14
Fam20c	ENSMUSG00000025854	Ensembl	0.22	0.11	2.14
slc25a48	ENSDARG00000021250	Ensembl	-0.86	-0.11	2.14
GU129140.1	GU129140.1	nt	0.63	0.80	2.14
EU069829.1	EU069829.1	nt	-0.55	-0.23	2.14
si:ch73-236c18.7	ENSDARG00000096516	Ensembl	-0.06	-0.36	2.14
rtn3	ENSDARG00000058028	Ensembl	0.10	0.09	2.14
cog2	ENSDARG00000004037	Ensembl	-0.34	0.47	-2.14
Fam20c	ENSMUSG00000025854	Ensembl	0.09	0.29	2.14
acin1b	ENSDARG00000026842	Ensembl	0.00	0.00	2.14
slc38a3a	ENSDARG00000027065	Ensembl	-1.17	0.20	-2.14
cbln8	ENSDARG00000019294	Ensembl	0.07	-0.24	-2.13
mfge8a	ENSDARG00000015349	Ensembl	0.00	0.00	2.13
tat	ENSDARG00000069630	Ensembl	0.05	-0.45	-2.13
tat	ENSDARG00000069630	Ensembl	-0.08	-0.32	-2.13
NM_001173711.1	NM_001173711.1	refseq	-0.49	-0.76	-2.13
ttll12	ENSDARG00000017407	Ensembl	0.38	1.07	2.13
EU025716.1	EU025716.1	nt	-1.18	-1.16	-2.12
atf5a	ENSDARG00000068096	Ensembl	1.08	0.91	2.12
cbln8	ENSDARG00000019294	Ensembl	0.08	-0.25	-2.12
psma5	ENSDARG00000003526	Ensembl	1.04	0.97	-2.12
prdm4	ENSDARG00000017366	Ensembl	0.01	0.38	2.12
errfi1	ENSDARG00000070171	Ensembl	-0.65	-0.91	-2.12
ulk1a	ENSDARG00000062518	Ensembl	-0.67	-0.98	-2.12

AJ829673.1	AJ829673.1	nt	-0.23	0.01	-2.12
GU129139.1	GU129139.1	nt	-0.37	-0.05	-2.11
HQ287749.1	HQ287749.1	nt	0.28	0.21	-2.10
CPNE4	ENSDARG00000040069	Ensembl	-1.00	0.02	-2.10
HM159473.1	HM159473.1	nt	-0.66	-0.66	-2.10
lyz	ENSDARG00000057789	Ensembl	0.94	-0.99	-2.10
HM208332.1	HM208332.1	nt	-0.55	-0.28	-2.09
EU025716.1	EU025716.1	nt	-1.61	-1.27	-2.09
tat	ENSDARG00000069630	Ensembl	-0.32	-0.90	-2.09
NR_030020.1	NR_030020.1	refseq	-0.27	0.93	-2.09
BX000999.4	BX000999.4	nt	0.55	1.16	2.09
GU129140.1	GU129140.1	nt	0.00	0.00	2.09
rrbp1a	ENSDARG00000013763	Ensembl	-0.48	-0.78	2.08
ret	ENSDARG00000055305	Ensembl	-1.31	-0.16	-2.08
errfi1	ENSDARG00000070171	Ensembl	-0.72	-1.40	-2.08
ap1s2	ENSDARG00000058504	Ensembl	-0.67	-0.85	-2.08
fkbp11	ENSDARG00000037000	Ensembl	0.72	0.37	2.08
BT072559.1	BT072559.1	nt	0.71	0.05	2.08
ugp2a	ENSDARG00000005578	Ensembl	0.47	-0.50	-2.08
dpydb	ENSDARG00000010267	Ensembl	-0.87	-0.29	-2.08
EU025718.1	EU025718.1	nt	-0.64	0.07	-2.08
ugt5g1	ENSDARG00000032862	Ensembl	-1.96	1.03	-2.07
CR388163.2	CR388163.2	nt	**-0.77**	**-0.62**	**-2.07**
pptc7a	ENSDARG00000011122	Ensembl	-0.73	-0.40	-2.07
pptc7a	ENSDARG00000011122	Ensembl	-0.90	-0.40	-2.07
rtn3	ENSDARG00000058028	Ensembl	-0.17	-0.32	2.07
kdelr3	ENSDARG00000040912	Ensembl	1.28	0.82	2.06
errfi1	ENSDARG00000070171	Ensembl	-1.24	-0.91	-2.06
GU552297.1	GU552297.1	nt	0.00	0.00	2.06
tat	ENSDARG00000069630	Ensembl	0.23	-0.33	-2.06
CR392001.1	CR392001.1	nt	-0.30	-0.28	2.06
cyp24a1	ENSDARG00000070420	Ensembl	-0.64	-1.19	-2.06
FJ969489.1	FJ969489.1	nt	0.93	-0.12	2.06
EU481821.1	EU481821.1	nt	-0.08	0.48	2.06
agxtb	ENSDARG00000018478	Ensembl	-1.06	-0.10	-2.06
tat	ENSDARG00000069630	Ensembl	-0.27	-0.74	-2.06
GQ505860.1	GQ505860.1	nt	0.41	1.05	2.06
slc25a29	ENSDARG00000057352	Ensembl	-0.39	0.30	-2.06
EU481821.1	EU481821.1	nt	-0.47	-0.11	2.05
nosip	ENSDARG00000037958	Ensembl	0.24	0.35	2.05
tat	ENSDARG00000069630	Ensembl	-0.19	-0.67	-2.05
BT072387.1	BT072387.1	nt	-0.44	-0.20	-2.05
polr3a	ENSDARG00000071269	Ensembl	0.40	-0.03	2.05
GU129140.1	GU129140.1	nt	0.11	0.50	2.05
cdkn1bb	ENSDARG00000088081	Ensembl	-1.29	-0.57	-2.05
rrbp1a	ENSDARG00000013763	Ensembl	-0.25	-0.70	2.05
NM_001139856.1	NM_001139856.1	refseq	-0.37	-0.58	-2.05
EU221179.1	EU221179.1	nt	0.61	0.80	2.05
EU481821.1	EU481821.1	nt	-0.08	0.85	2.04
agxtb	ENSDARG00000018478	Ensembl	-1.00	-0.34	-2.04
gnl2	ENSDARG00000053225	Ensembl	0.02	0.12	2.03
BT048266.1	BT048266.1	nt	-0.19	-0.21	-2.03
TGDS	ENSG00000088451	Ensembl	0.00	0.00	2.02
BT058978.1	BT058978.1	nt	-0.99	-0.23	-2.02
elac2	ENSDARG00000034060	Ensembl	0.60	0.51	2.02
riok2	ENSDARG00000035264	Ensembl	0.00	1.17	2.02
Fam20c	ENSMUSG00000025854	Ensembl	0.18	0.19	2.02
wdr46	ENSDARG00000078396	Ensembl	1.56	1.05	2.02
Fam20c	ENSMUSG00000025854	Ensembl	0.24	0.22	2.02
GU129139.1	GU129139.1	nt	0.25	0.32	2.01
GU129140.1	GU129140.1	nt	0.00	0.00	2.01
Fam20c	ENSMUSG00000025854	Ensembl	0.25	0.29	2.01
pdia4	ENSDARG00000018491	Ensembl	0.91	0.29	2.01
slc38a3a	ENSDARG00000027065	Ensembl	-0.91	0.34	-2.01
slc25a29	ENSDARG00000057352	Ensembl	-0.65	0.33	-2.00
nfasca	ENSDARG00000061099	Ensembl	0.00	1.00	2.00
errfi1	ENSDARG00000070171	Ensembl	-0.70	-0.86	-2.00
KDM4A	ENSDARG00000018782	Ensembl	0.16	-0.12	-2.00
Fam20c	ENSMUSG00000025854	Ensembl	0.42	0.37	2.00
BT058834.1	BT058834.1	nt	-1.24	-0.06	-2.00
mocs3	ENSDARG00000008239	Ensembl	0.00	0.00	1.99
cfhl3	ENSDARG00000094661	Ensembl	-0.03	-0.16	-1.99
BT075324.1	BT075324.1	nt	-0.58	-0.94	-1.99
cfhl3	ENSDARG00000094661	Ensembl	0.02	-0.14	-1.99
cfhl3	ENSDARG00000094661	Ensembl	0.13	-0.21	-1.99
kdelr2b	ENSDARG00000037361	Ensembl	0.42	-0.25	1.99
BT058795.1	BT058795.1	nt	0.76	0.76	1.99
BT056395.1	BT056395.1	nt	0.00	0.00	1.99
EU025707.1	EU025707.1	nt	0.57	0.73	1.99
slc38a3a	ENSDARG00000027065	Ensembl	-0.92	0.22	-1.98
NM_001141140.1	NM_001141140.1	refseq	-0.26	0.23	-1.98
tat	ENSDARG00000069630	Ensembl	0.06	-0.39	-1.98

pim3	ENSDARG00000055129	Ensembl	-0.52	-0.69	-1.98
cfhl3	ENSDARG00000094661	Ensembl	0.08	-0.24	-1.98
agxtb	ENSDARG00000018478	Ensembl	-1.03	-0.23	-1.98
BT072179.1	BT072179.1	nt	1.99	-0.26	-1.98
BX927253.1	BX927253.1	nt	-0.29	0.26	1.98
NM_001173865.1	NM_001173865.1	refseq	-0.90	-0.20	-1.98
tat	ENSDARG00000069630	Ensembl	-0.29	-0.91	-1.98
tmem167b	ENSDARG00000059400	Ensembl	0.44	0.40	1.98
EU025708.1	EU025708.1	nt	0.04	-0.12	1.97
AJ224693.1	AJ224693.1	nt	0.38	0.41	-1.97
msrb2	ENSDARG00000018459	Ensembl	0.98	-0.02	1.97
BT072559.1	BT072559.1	nt	0.58	-0.18	1.97
pdia4	ENSDARG00000018491	Ensembl	0.86	0.29	1.97
AY567793.3	AY567793.3	nt	-1.08	-0.44	1.97
nosip	ENSDARG00000037958	Ensembl	0.57	0.18	1.97
ube2h	ENSDARG00000000019	Ensembl	0.42	0.41	1.96
cyp24a1	ENSDARG00000070420	Ensembl	-0.67	-1.38	-1.96
CR391962.1	CR391962.1	nt	-0.64	-0.70	-1.96
igfbp2a	ENSDARG00000052470	Ensembl	0.72	0.58	1.96
cyp24a1	ENSDARG00000070420	Ensembl	-0.77	-1.28	-1.96
Ugt2b1	ENSMUSG00000035836	Ensembl	-0.39	-0.93	-1.95
map7d2a	ENSDARG00000068480	Ensembl	-0.08	0.24	-1.95
KDM4A	ENSDARG00000018782	Ensembl	0.30	0.27	-1.95
BX324155.1	BX324155.1	nt	0.00	0.00	1.95
cog2	ENSDARG00000004037	Ensembl	-0.59	0.13	-1.95
cfhl3	ENSDARG00000094661	Ensembl	0.08	-0.23	-1.94
slc25a29	ENSDARG00000057352	Ensembl	-0.28	0.40	-1.94
polr1a	ENSDARG00000029172	Ensembl	0.17	-1.30	1.94
ttll12	ENSDARG00000017407	Ensembl	0.00	0.00	1.94
cnn2	ENSDARG00000035858	Ensembl	-0.67	-0.22	-1.94
dennd4c	ENSMUSG00000038024	Ensembl	0.82	0.89	1.94
irf6	ENSDARG00000043296	Ensembl	-0.06	0.00	-1.93
bnip3lb	ENSDARG00000028067	Ensembl	0.91	0.95	1.93
S66606.1	S66606.1	nt	-0.07	-0.70	1.93
BT045802.1	BT045802.1	nt	-0.58	0.01	-1.93
ezh1	ENSDARG00000037894	Ensembl	0.23	0.00	1.93
MYLK4	ENSDARG00000091260	Ensembl	-0.36	-1.91	-1.93
nbr1	ENSDARG00000077297	Ensembl	0.00	0.00	1.92
FJ969490.1	FJ969490.1	nt	-0.94	-0.90	-1.92
tk1	ENSDARG00000086561	Ensembl	-1.30	0.76	-1.92
grhpra	ENSDARG00000068264	Ensembl	-0.85	0.35	1.92
ube2h	ENSDARG00000000019	Ensembl	1.17	0.65	1.92
st3gal3b	ENSDARG00000015252	Ensembl	-0.26	-0.04	-1.92
C24H18orf8	ENSDARG00000029307	Ensembl	0.00	0.00	1.92
BT072559.1	BT072559.1	nt	0.95	0.27	1.92
nosip	ENSDARG00000037958	Ensembl	-0.41	0.21	1.91
grwd1	ENSDARG00000004806	Ensembl	0.00	0.00	1.91
AC203456.8	AC203456.8	nt	-0.25	-1.30	-1.91
GU129140.1	GU129140.1	nt	0.78	0.59	-1.91
Fam20c	ENSMUSG00000025854	Ensembl	0.29	0.08	1.91
hspa8	ENSDARG00000068992	Ensembl	-0.72	0.76	-1.91
Tstd1	ENSMUSG00000091166	Ensembl	0.51	-0.47	-1.91
tat	ENSDARG00000069630	Ensembl	-0.16	-0.76	-1.91
BT059557.1	BT059557.1	nt	-0.70	-0.72	-1.90
ubald1a	ENSDARG00000002362	Ensembl	0.56	0.47	1.90
rtn3	ENSDARG00000058028	Ensembl	0.20	0.46	1.90
GU129140.1	GU129140.1	nt	2.33	2.62	1.90
zgc:123105	ENSDARG00000003127	Ensembl	-1.15	-1.69	-1.90
si:ch211-233a24.2	ENSDARG00000062330	Ensembl	0.00	0.00	1.90
gltpd2	ENSDARG00000067889	Ensembl	-0.46	-0.34	1.90
HM159471.1	HM159471.1	nt	0.62	0.73	1.89
KDM4A	ENSDARG00000018782	Ensembl	0.40	-0.02	-1.89
Ugt2a3	ENSMUSG00000035780	Ensembl	0.58	-0.33	-1.89
GU324549.1	GU324549.1	nt	0.00	0.00	1.89
cd63	ENSDARG00000025147	Ensembl	-0.63	-0.39	-1.89
nudt1	ENSDARG00000030573	Ensembl	-0.01	0.27	1.89
NM_001141633.1	NM_001141633.1	refseq	-0.79	0.30	-1.89
slc13a5	ENSDARG00000077691	Ensembl	-0.90	-0.39	-1.89
Phpt1	ENSMUSG00000036504	Ensembl	0.89	0.27	1.89
BT072559.1	BT072559.1	nt	0.78	0.16	1.88
tat	ENSDARG00000069630	Ensembl	-0.07	-0.93	-1.88
GU129140.1	GU129140.1	nt	-0.41	0.05	-1.88
pank1a	ENSDARG00000008192	Ensembl	-0.41	-0.86	-1.88
GU552297.1	GU552297.1	nt	0.35	0.78	1.88
PON2	ENSG00000105854	Ensembl	0.96	1.44	1.87
FQ310507.3	FQ310507.3	nt	0.38	1.04	1.87
polr1a	ENSDARG00000029172	Ensembl	-0.82	0.73	1.87
thbs4b	ENSDARG00000020072	Ensembl	-0.07	0.30	1.87
dennd4c	ENSMUSG00000038024	Ensembl	0.00	0.00	1.87
DQ156149.1	DQ156149.1	nt	-0.65	-0.90	-1.86
BX000999.4	BX000999.4	nt	0.59	1.03	1.86
tat	ENSDARG00000069630	Ensembl	-0.12	-0.71	-1.86

DQ156149.1	DQ156149.1	nt	0.45	-0.10	-1.86
nfasca	ENSDARG00000061099	Ensembl	-0.20	0.28	1.86
slc1a4	ENSDARG00000000551	Ensembl	0.89	1.29	1.86
MMP15	ENSDARG00000013072	Ensembl	-0.40	-0.63	1.86
cbsa	ENSDARG00000053500	Ensembl	-1.12	-0.34	-1.86
CABZ01072083.1	CABZ01072083.1	nt	0.46	0.32	1.86
si:dkey-238o13.4	ENSDARG00000078847	Ensembl	-0.85	-0.47	-1.85
st3gal3b	ENSDARG00000015252	Ensembl	-0.64	-0.40	-1.85
mhc1uba	ENSDARG00000075963	Ensembl	-0.71	-0.65	-1.85
rbms2b	ENSDARG00000056150	Ensembl	-1.27	-0.77	-1.85
agxtb	ENSDARG00000018478	Ensembl	-0.84	-0.30	-1.85
NM_001076652.2	NM_001076652.2	refseq	-0.35	-0.15	1.85
NM_001173863.1	NM_001173863.1	refseq	0.65	0.47	1.85
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.39	0.26	-1.84
ZFYVE1	ENSG00000165861	Ensembl	0.00	0.00	1.84
HM159471.1	HM159471.1	nt	1.33	0.77	1.83
foxred1	ENSDARG00000060790	Ensembl	-1.00	-0.90	1.83
myo3b	ENSDARG00000006892	Ensembl	0.00	0.00	1.83
irf6	ENSDARG00000043296	Ensembl	-0.43	-0.51	-1.83
st3gal3b	ENSDARG00000015252	Ensembl	-0.13	-0.08	-1.83
HQ287746.1	HQ287746.1	nt	-0.47	-0.40	-1.82
BX537133.3	BX537133.3	nt	-1.30	-0.56	-1.82
ncoa6	ENSDARG00000071272	Ensembl	0.52	-0.07	1.82
nosip	ENSDARG00000037958	Ensembl	-0.02	0.30	1.82
nupr1	ENSDARG00000094557	Ensembl	-0.76	-0.40	-1.82
tjp2a	ENSDARG00000063309	Ensembl	-0.51	-0.01	-1.82
dennd4c	ENSMUSG00000038024	Ensembl	0.99	-0.22	1.82
cog2	ENSDARG00000004037	Ensembl	-0.65	0.36	-1.82
slc13a5	ENSDARG00000077691	Ensembl	-0.89	-0.25	-1.82
AB370192.1	AB370192.1	nt	-0.16	0.77	-1.82
pdcd4a	ENSDARG00000021702	Ensembl	1.90	1.02	1.81
slc13a5	ENSDARG00000077691	Ensembl	-0.68	-0.24	-1.81
si:dkey-238o13.4	ENSDARG00000078847	Ensembl	-1.11	-0.62	-1.81
tlk1b	ENSDARG00000059190	Ensembl	-0.03	0.01	1.81
D32160.1	D32160.1	nt	0.78	-0.17	1.81
BT045120.1	BT045120.1	nt	0.11	-0.56	1.80
nosip	ENSDARG00000037958	Ensembl	0.06	-0.30	1.80
srp72	ENSDARG00000014139	Ensembl	0.01	0.83	1.80
zgc:123105	ENSDARG00000003127	Ensembl	-1.04	-1.57	-1.80
EF427381.1	EF427381.1	nt	-0.87	-1.28	-1.80
zgc:123105	ENSDARG00000003127	Ensembl	-1.09	-1.77	-1.80
AY217451.1	AY217451.1	nt	-0.32	-0.37	-1.80
NM_001140008.1	NM_001140008.1	refseq	-0.44	0.74	1.80
MMP15	ENSDARG00000013072	Ensembl	-0.65	-0.78	1.80
ube2h	ENSDARG00000000019	Ensembl	0.78	-0.09	1.80
XM_003442990.1	XM_003442990.1	refseq	1.02	0.24	1.80
NM_001173863.1	NM_001173863.1	refseq	0.52	-0.01	1.79
ENSORLG00000009238	ENSORLG00000009238	Ensembl	0.88	-0.08	1.79
KDM4A	ENSDARG00000018782	Ensembl	0.61	0.01	-1.79
hey1	ENSDARG00000070538	Ensembl	0.10	-0.43	-1.79
igfbp2b	ENSDARG00000031422	Ensembl	0.16	-0.10	1.79
BX005175.1	BX005175.1	nt	-0.35	-1.04	-1.79
cbln8	ENSDARG00000019294	Ensembl	-0.36	-0.04	-1.78
PLA2G10	ENSDARG00000074579	Ensembl	0.98	0.81	1.78
CR774179.2	CR774179.2	nt	0.77	0.07	-1.78
cbsb	ENSDARG00000010946	Ensembl	-1.21	-0.35	-1.78
EU621901.1	EU621901.1	nt	-0.07	-0.21	1.78
pdia4	ENSDARG00000018491	Ensembl	0.78	0.28	1.78
ENSORLG00000015212	ENSORLG00000015212	Ensembl	-0.65	-0.71	-1.78
BT071900.1	BT071900.1	nt	-0.37	-0.17	-1.77
BT058978.1	BT058978.1	nt	-0.93	0.26	-1.77
slc20a1b	ENSDARG00000010641	Ensembl	0.01	0.99	1.77
NM_001140148.1	NM_001140148.1	refseq	0.01	0.76	1.77
cyp8b1	ENSDARG00000053068	Ensembl	-0.66	-0.01	-1.77
CDO1	ENSG00000129596	Ensembl	-0.86	-0.38	-1.77
tmem30ab	ENSDARG00000043555	Ensembl	-1.50	-0.56	-1.77
SLC25A34	ENSG00000162461	Ensembl	0.16	-0.53	-1.77
btf3l4	ENSDARG00000070722	Ensembl	0.75	0.65	1.76
cyp8b1	ENSDARG00000053068	Ensembl	-0.49	-0.05	-1.76
riok2	ENSDARG00000035264	Ensembl	0.87	0.94	1.76
CU138575.8	CU138575.8	nt	-0.23	-0.02	-1.76
ip6k2	ENSDARG00000008310	Ensembl	-1.56	-0.60	-1.76
ybx1	ENSDARG00000004757	Ensembl	-0.04	-0.15	1.76
CYP2X12	ENSDARG00000068290	Ensembl	-1.28	-1.07	-1.75
rbms2b	ENSDARG00000056150	Ensembl	-1.35	-0.58	-1.75
EU221179.1	EU221179.1	nt	0.99	1.40	1.75
gpt2	ENSDARG00000012199	Ensembl	-0.90	-1.32	-1.75
igfbp2a	ENSDARG00000052470	Ensembl	0.94	0.45	1.75
gda	ENSDARG00000002986	Ensembl	-0.24	-0.74	-1.75
JHDM1D	ENSDARG00000018559	Ensembl	-0.28	-0.19	1.75
rps23	ENSDARG00000021838	Ensembl	0.66	0.08	1.75
CR388163.2	CR388163.2	nt	**-0.30**	**-0.21**	**-1.75**

EU221176.1	EU221176.1	nt	0.59	0.53	1.75
fam5b	ENSDARG00000014302	Ensembl	-0.27	-0.57	-1.75
NM_001173833.1	NM_001173833.1	refseq	-0.58	0.39	1.75
slc6a4a	ENSDARG00000061165	Ensembl	-0.76	0.05	-1.74
pdcd11	ENSDARG00000052480	Ensembl	0.00	0.00	1.74
NM_001173759.1	NM_001173759.1	refseq	-0.06	0.06	-1.74
GU129140.1	GU129140.1	nt	0.22	0.11	-1.74
lgals3bpa	ENSDARG00000037805	Ensembl	0.57	0.51	-1.74
im:7136021	ENSDARG00000054128	Ensembl	-2.10	-0.17	1.73
EU481821.1	EU481821.1	nt	2.01	2.18	1.73
idua	ENSDARG00000062904	Ensembl	-0.45	-0.57	-1.73
nupr1	ENSDARG00000094557	Ensembl	-0.89	-0.63	-1.73
NM_001141630.1	NM_001141630.1	refseq	0.52	1.39	1.73
BX537133.3	BX537133.3	nt	-0.36	0.01	-1.73
GQ505860.1	GQ505860.1	nt	-0.70	0.17	-1.73
AY217451.1	AY217451.1	nt	-0.31	-0.73	-1.73
nfasca	ENSDARG00000061099	Ensembl	-0.05	0.45	1.72
tlk1b	ENSDARG00000059190	Ensembl	-0.03	-0.21	1.72
NM_001139879.1	NM_001139879.1	refseq	0.58	0.78	1.72
CABZ01072083.1	CABZ01072083.1	nt	-0.14	-0.04	1.72
trim33	ENSDARG00000016181	Ensembl	0.00	0.00	1.72
CABZ01072083.1	CABZ01072083.1	nt	-0.29	-0.05	1.72
CABZ01072083.1	CABZ01072083.1	nt	0.14	-0.14	1.72
GU129140.1	GU129140.1	nt	-0.60	-0.04	-1.72
nfic	ENSDARG00000043210	Ensembl	-0.42	0.89	1.71
NM_001004593.1	NM_001004593.1	refseq	-0.39	-0.84	-1.71
nupr1	ENSDARG00000094557	Ensembl	-0.82	-0.62	-1.71
EU025708.1	EU025708.1	nt	1.14	0.70	1.71
FP103011.3	ENSG00000089060	Ensembl	-1.15	-0.33	-1.71
slc13a5	ENSDARG00000077691	Ensembl	-0.76	-0.35	-1.71
acoxl	ENSDARG00000020149	Ensembl	-0.35	-0.10	1.71
AC203456.8	AC203456.8	nt	-0.77	-0.32	1.71
ENSGACG00000002729	ENSGACG00000002729	Ensembl	0.39	1.14	1.71
BT046002.1	BT046002.1	nt	0.90	0.41	1.71
BX537350.1	BX537350.1	nt	0.24	-0.32	-1.71
fam98a	ENSDARG00000078391	Ensembl	0.13	0.99	1.70
kcnj2	ENSDARG00000019418	Ensembl	0.49	-0.33	1.70
Cdo1	ENSMUSG00000033022	Ensembl	-0.55	-0.19	-1.70
CU464087.2	CU464087.2	nt	1.41	2.30	1.70
NM_001173863.1	NM_001173863.1	refseq	-0.33	0.55	1.70
sash1a	ENSDARG00000007179	Ensembl	0.55	0.16	1.70
HPX	ENSDARG00000051912	Ensembl	0.85	0.22	-1.70
GU129140.1	GU129140.1	nt	0.21	0.07	-1.70
calua	ENSDARG00000045676	Ensembl	0.26	0.58	1.70
nupr1	ENSDARG00000094557	Ensembl	-0.74	-0.31	-1.70
ezh2	ENSDARG00000010571	Ensembl	-0.02	-0.35	1.70
wdr55	ENSDARG00000007217	Ensembl	-0.02	1.12	1.70
BT059271.1	BT059271.1	nt	-0.03	0.16	-1.70
nupr1	ENSDARG00000094557	Ensembl	-0.74	-0.39	-1.70
slc11a2	ENSDARG00000024295	Ensembl	0.39	0.26	1.70
slc13a5	ENSDARG00000077691	Ensembl	-0.83	-0.15	-1.70
acoxl	ENSDARG00000020149	Ensembl	0.46	-0.10	1.70
rhot1a	ENSDARG00000018130	Ensembl	0.35	0.94	1.69
ehd3	ENSDARG00000007869	Ensembl	0.40	0.14	-1.69
CABZ01072083.1	CABZ01072083.1	nt	-0.15	0.12	1.69
AF502957.1	AF502957.1	nt	0.37	-0.42	1.69
Cdo1	ENSMUSG00000033022	Ensembl	-0.53	-0.18	-1.69
acsl1	ENSDARG00000030514	Ensembl	0.19	0.15	1.69
thrap3b	ENSDARG00000003513	Ensembl	1.28	0.82	1.69
snrnp70	ENSDARG00000077126	Ensembl	0.19	0.37	1.69
BT072673.1	BT072673.1	nt	0.50	0.23	1.69
NM_001139920.1	NM_001139920.1	refseq	1.31	1.18	1.69
asap1b	ENSDARG00000039729	Ensembl	-0.20	0.34	1.68
GQ505859.1	GQ505859.1	nt	0.93	0.67	1.68
HELZ2	ENSDARG00000016527	Ensembl	1.37	0.13	-1.68
CT583687.1	CT583687.1	nt	0.90	0.75	1.68
EU025714.1	EU025714.1	nt	0.58	0.27	-1.68
rhot1a	ENSDARG00000018130	Ensembl	-0.15	0.35	1.68
AB258536.1	AB258536.1	nt	-0.47	-0.14	-1.68
NM_001140071.1	NM_001140071.1	refseq	-0.79	0.24	1.68
NM_001141007.1	NM_001141007.1	refseq	-0.39	0.52	1.68
ssh2b	ENSDARG00000077623	Ensembl	-0.68	-0.87	-1.68
ncl	ENSDARG00000002710	Ensembl	-0.28	0.03	1.68
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.29	0.18	-1.67
EU450668.1	EU450668.1	nt	0.47	0.27	1.67
col4a3bpa	ENSDARG00000024325	Ensembl	0.84	0.00	1.67
tmem183a	ENSDARG00000044899	Ensembl	0.19	1.05	1.67
EU025717.1	EU025717.1	nt	0.27	0.00	1.67
cct2	ENSDARG00000041754	Ensembl	-0.64	0.11	1.67
L24433.1	L24433.1	nt	0.56	0.09	-1.67
NM_001140351.1	NM_001140351.1	refseq	0.06	0.48	1.67
Arpc1a	ENSMUSG00000029621	Ensembl	1.41	0.21	-1.67

tat	ENSDARG00000069630	Ensembl	0.30	-0.80	-1.67
grhpra	ENSDARG00000068264	Ensembl	-0.85	0.46	1.67
pr2y4l	ENSDARG00000053570	Ensembl	0.64	0.92	1.67
gpd1b	ENSDARG00000043180	Ensembl	0.46	0.85	1.66
map7d2a	ENSDARG00000068480	Ensembl	0.22	0.46	-1.66
ubqln4	ENSDARG00000052975	Ensembl	0.61	0.08	1.66
GQ505860.1	GQ505860.1	nt	1.70	0.51	1.66
rcan2	ENSDARG00000021869	Ensembl	-1.28	0.64	1.66
EF210363.1	EF210363.1	nt	0.18	0.49	1.66
lonrf1	ENSDARG00000075048	Ensembl	-0.60	-0.47	-1.66
SLIRP	ENSG00000119705	Ensembl	0.45	-0.08	1.66
CABZ01072083.1	CABZ01072083.1	nt	-0.37	-0.13	1.66
ezh2	ENSDARG00000010571	Ensembl	-0.01	0.30	1.66
Cdo1	ENSMUSG00000033022	Ensembl	-0.28	-0.14	-1.66
BT059137.1	BT059137.1	nt	-0.12	-0.54	-1.65
BT059363.1	BT059363.1	nt	0.43	-0.03	1.65
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.44	0.28	-1.65
rcan2	ENSDARG00000021869	Ensembl	-0.91	0.56	1.65
nosip	ENSDARG00000037958	Ensembl	0.32	0.55	1.65
cdkn1bb	ENSDARG00000088081	Ensembl	-1.36	-0.72	-1.64
tfcp2l1	ENSDARG00000029497	Ensembl	-0.95	-0.25	-1.64
CABZ01072083.1	CABZ01072083.1	nt	-0.53	-0.16	1.64
L24433.1	L24433.1	nt	0.71	0.24	-1.64
slc13a5	ENSDARG00000077691	Ensembl	-0.62	-0.35	-1.64
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.31	0.20	-1.64
SAMD8	ENSG00000156671	Ensembl	-0.03	-0.51	-1.64
tgds	ENSDARG00000015622	Ensembl	0.00	0.00	1.64
st3gal3b	ENSDARG00000015252	Ensembl	-0.16	0.11	-1.64
HPX	ENSDARG00000051912	Ensembl	0.82	0.38	-1.63
BT048359.2	BT048359.2	nt	-0.93	-0.45	-1.63
lrig2	ENSDARG00000078561	Ensembl	-0.68	-0.63	-1.63
GU129140.1	GU129140.1	nt	0.19	0.05	-1.63
DQ156150.1	DQ156150.1	nt	-0.93	0.21	-1.63
nmd3	ENSDARG00000015676	Ensembl	0.36	0.62	1.63
EU221176.1	EU221176.1	nt	0.94	0.39	1.63
IP6K2	ENSDARG00000019613	Ensembl	-1.14	-0.55	-1.63
PBDC1	ENSG00000269056	Ensembl	-0.76	-0.55	-1.63
BT060099.1	BT060099.1	nt	-0.46	-0.28	-1.63
igfbp2a	ENSDARG00000052470	Ensembl	0.75	0.64	1.63
NM_001173833.1	NM_001173833.1	refseq	-1.75	-0.04	1.62
nfasca	ENSDARG00000061099	Ensembl	-0.57	0.28	1.62
NM_001173879.1	NM_001173879.1	refseq	0.43	1.04	1.62
GU129140.1	GU129140.1	nt	0.39	-0.05	-1.62
Cdo1	ENSMUSG00000033022	Ensembl	-0.24	-0.12	-1.62
Tubb4b	ENSMUSG00000036752	Ensembl	0.77	0.12	1.62
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.32	0.25	-1.62
EU025719.1	EU025719.1	nt	-0.31	-0.12	1.62
AC203456.8	AC203456.8	nt	-0.06	0.39	1.62
ENSONIG00000010940	ENSONIG00000010940	Ensembl	0.18	-0.47	-1.62
slc11a2	ENSDARG00000024295	Ensembl	-0.67	-0.03	1.62
BT058749.1	BT058749.1	nt	-0.72	-0.01	-1.62
XM_003444010.1	XM_003444010.1	refseq	-0.44	-1.23	-1.62
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.31	0.22	-1.62
nfasca	ENSDARG00000061099	Ensembl	-0.94	0.12	1.62
AY113693.1	AY113693.1	nt	-0.43	-0.20	-1.62
EU025717.1	EU025717.1	nt	0.21	-0.05	1.62
slc11a2	ENSDARG00000024295	Ensembl	-0.01	-0.17	1.62
cyp8b1	ENSDARG00000053068	Ensembl	-0.54	0.15	-1.61
fabp3	ENSDARG00000023290	Ensembl	0.01	-0.26	1.61
golga4	ENSDARG00000075331	Ensembl	-0.41	-0.24	1.61
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.39	0.26	-1.61
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.42	0.28	-1.61
tma16	ENSDARG00000008068	Ensembl	0.35	0.01	1.61
HM159471.1	HM159471.1	nt	0.47	0.45	1.60
ARG1	ENSDARG00000057429	Ensembl	-0.14	-0.23	-1.60
CD163L1	ENSG00000177675	Ensembl	0.47	-0.14	-1.60
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.29	0.23	-1.60
AJ224693.1	AJ224693.1	nt	0.34	0.33	-1.60
nupr1	ENSDARG00000094557	Ensembl	-0.59	-0.20	-1.59
ENSORLG00000018657	ENSORLG00000018657	Ensembl	-1.22	0.11	-1.59
CABZ01072083.1	CABZ01072083.1	nt	0.20	0.31	1.59
mapk14b	ENSDARG00000028721	Ensembl	0.10	-0.39	1.59
BT082996.1	BT082996.1	nt	-0.32	0.00	-1.59
lpcat3	ENSDARG00000075178	Ensembl	-0.61	-0.85	-1.59
CABZ01092943.1	CABZ01092943.1	nt	-0.58	-0.31	-1.59
ENSONIG00000020743	ENSONIG00000020743	Ensembl	0.22	0.47	1.59
NM_001124414.1	NM_001124414.1	refseq	0.20	-0.03	-1.59
BT082996.1	BT082996.1	nt	-0.11	0.11	-1.59
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.32	0.19	-1.59
AC147917.2	AC147917.2	nt	0.16	-0.08	1.58
slc13a5	ENSDARG00000077691	Ensembl	-0.88	-0.21	-1.58
GU129140.1	GU129140.1	nt	0.31	0.21	-1.58

calua	ENSDARG00000045676	Ensembl	0.13	0.49	1.58
FP102311.1	ENSMUSG00000094472	Ensembl	0.71	1.01	1.58
EU816603.1	EU816603.1	nt	-0.45	-0.50	-1.58
abcd3a	ENSDARG00000015167	Ensembl	0.38	0.17	1.58
abcd1	ENSDARG00000074876	Ensembl	-1.95	-0.40	1.58
nfasca	ENSDARG00000061099	Ensembl	-0.11	0.30	1.58
rbp4l	ENSDARG00000044684	Ensembl	-0.19	-0.38	-1.57
EEA1	ENSG00000102189	Ensembl	-0.85	-0.54	1.57
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.45	0.34	-1.57
nop58	ENSDARG00000058337	Ensembl	-0.51	0.42	1.57
BT072796.1	BT072796.1	nt	-0.05	0.35	-1.57
slc25a29	ENSDARG00000057352	Ensembl	-0.26	0.05	-1.57
gda	ENSDARG00000002986	Ensembl	-0.24	-0.47	-1.57
C4B	ENSG00000233312	Ensembl	0.33	0.21	-1.57
cdkn1bb	ENSDARG00000088081	Ensembl	-1.32	-0.79	-1.57
glud1b	ENSDARG00000002414	Ensembl	-0.87	-0.39	-1.56
BT072648.1	BT072648.1	nt	-0.14	0.54	1.56
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.37	0.25	-1.56
cpsf6	ENSDARG00000018618	Ensembl	0.21	-0.12	1.56
EU008541.1	EU008541.1	nt	0.47	0.48	-1.56
NM_001139920.1	NM_001139920.1	refseq	0.76	1.15	1.56
mmp16b	ENSDARG00000058876	Ensembl	0.10	-0.23	-1.56
CU207301.8	CU207301.8	nt	0.21	0.24	-1.56
cfhl3	ENSDARG00000094661	Ensembl	0.57	0.23	-1.56
C9H21orf33	ENSDARG00000020618	Ensembl	0.48	0.15	1.56
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.36	0.21	-1.56
NM_001140071.1	NM_001140071.1	refseq	0.30	0.39	1.55
EU816603.1	EU816603.1	nt	-1.15	-0.06	1.55
NM_001173863.1	NM_001173863.1	refseq	-0.02	0.02	1.55
pdia4	ENSDARG00000018491	Ensembl	0.88	0.27	1.55
NM_001139980.1	NM_001139980.1	refseq	0.70	0.29	-1.55
BX936371.1	BX936371.1	nt	-0.44	0.38	1.55
ZCCHC4	ENSDARG00000018810	Ensembl	-0.01	0.12	1.55
CABZ01072083.1	CABZ01072083.1	nt	0.03	-0.56	1.55
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.37	0.18	-1.55
nfil3-5	ENSDARG00000094965	Ensembl	-1.59	-1.22	-1.55
p4hb	ENSDARG00000052589	Ensembl	0.17	0.04	1.55
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.40	0.30	-1.55
cfhl3	ENSDARG00000094661	Ensembl	0.62	0.29	-1.55
C4B	ENSG00000233312	Ensembl	0.33	0.27	-1.55
p4hb	ENSDARG00000052589	Ensembl	0.34	-0.06	1.55
ybx1	ENSDARG00000004757	Ensembl	0.12	0.56	1.55
EU025714.1	EU025714.1	nt	-0.05	0.08	-1.54
COL4A3BP	ENSDARG00000063542	Ensembl	0.83	0.56	1.54
lmbrd1	ENSDARG00000052307	Ensembl	1.03	1.11	1.54
eprs	ENSDARG00000060494	Ensembl	0.55	0.69	1.54
bcl6a	ENSDARG00000070864	Ensembl	2.43	0.98	1.54
cfhl4	ENSDARG00000094496	Ensembl	-0.02	-0.42	-1.54
add3a	ENSDARG00000040874	Ensembl	-0.25	-0.46	-1.54
nptnb	ENSDARG00000043864	Ensembl	-0.39	-0.37	-1.54
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.31	0.28	-1.53
cfhl3	ENSDARG00000094661	Ensembl	0.51	0.22	-1.53
abcd3a	ENSDARG00000015167	Ensembl	-0.19	0.13	1.53
trim35-23	ENSDARG00000052276	Ensembl	0.19	0.58	1.53
aldh18a1	ENSDARG00000061123	Ensembl	-0.01	-0.15	1.53
glud1b	ENSDARG00000002414	Ensembl	-0.87	-0.29	-1.53
CR450778.7	CR450778.7	nt	0.53	0.01	1.53
wdr3	ENSDARG00000011079	Ensembl	0.04	-0.38	1.53
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.41	0.29	-1.53
cry5	ENSDARG00000019498	Ensembl	0.39	-1.06	1.53
HQ287746.1	HQ287746.1	nt	-0.09	0.36	-1.53
cfhl3	ENSDARG00000094661	Ensembl	0.47	0.21	-1.53
noc2l	ENSDARG00000001754	Ensembl	-0.10	0.10	1.52
tlk1b	ENSDARG00000059190	Ensembl	0.01	-0.20	1.52
st3gal3b	ENSDARG00000015252	Ensembl	-0.28	-0.12	-1.52
GU129140.1	GU129140.1	nt	-0.02	-0.16	-1.52
ENSONIG00000020396	ENSONIG00000020396	Ensembl	-0.67	-0.96	-1.52
BT071900.1	BT071900.1	nt	-0.02	-0.05	-1.52
map3k19	ENSDARG00000094272	Ensembl	0.51	0.30	-1.52
aplp1	ENSDARG00000004148	Ensembl	-0.31	-0.37	-1.52
ap1s2	ENSDARG00000058504	Ensembl	0.55	0.18	-1.52
cxcl14	ENSDARG00000056627	Ensembl	-0.70	-0.10	-1.52
DQ156150.1	DQ156150.1	nt	-0.42	-0.58	-1.52
rab20	ENSDARG00000005049	Ensembl	-0.23	-0.75	-1.52
fstl1b	ENSDARG00000039576	Ensembl	-0.01	0.40	1.52
ENSONIG00000021068	ENSONIG00000021068	Ensembl	-1.10	-0.38	-1.52
BX927253.1	BX927253.1	nt	0.49	0.13	1.52
HPX	ENSDARG00000051912	Ensembl	0.67	0.18	-1.52
GQ505858.1	GQ505858.1	nt	0.30	-0.11	1.51
ran	ENSDARG00000057026	Ensembl	-0.15	0.41	1.51
tma16	ENSDARG00000008068	Ensembl	0.05	0.18	1.51
ENSGACG00000013404	ENSGACG00000013404	Ensembl	0.02	-0.65	-1.51

EF427382.1	EF427382.1	nt	-0.10	-0.35	-1.51
ENSONIG00000010940	ENSONIG00000010940	Ensembl	0.20	-0.17	-1.51
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.39	0.20	-1.51
NM_001139980.1	NM_001139980.1	refseq	0.90	0.39	-1.51
nosip	ENSDARG00000037958	Ensembl	-0.06	0.23	1.51
tmem86b	ENSDARG00000038296	Ensembl	-0.21	0.47	1.50
actr3b	ENSDARG00000008790	Ensembl	-0.94	-0.82	-1.50
APCS	ENSDARG00000045089	Ensembl	0.11	-0.22	-1.50
EU481821.1	EU481821.1	nt	-0.04	-0.24	-1.50
tex15	ENSDARG00000088654	Ensembl	-0.50	-0.19	-1.50
EU008541.1	EU008541.1	nt	0.39	0.48	-1.50
cfhl3	ENSDARG00000094661	Ensembl	0.59	0.23	-1.50
acoxl	ENSDARG00000020149	Ensembl	0.26	-0.31	1.50
HPX	ENSDARG00000051912	Ensembl	0.83	0.42	-1.50
gda	ENSDARG00000002986	Ensembl	-0.09	-0.66	-1.50
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.32	0.21	-1.50
NM_001141724.1	NM_001141724.1	refseq	-0.24	0.76	-1.50
NM_001256664.1	NM_001256664.1	refseq	0.47	-0.13	-1.50
BT045802.1	BT045802.1	nt	-0.62	0.13	-1.49
upf1	ENSDARG00000016302	Ensembl	0.69	0.40	1.49
AF089860.1	AF089860.1	nt	0.36	0.39	-1.49
map3k19	ENSDARG00000094272	Ensembl	0.50	0.32	-1.49
calua	ENSDARG00000045676	Ensembl	0.55	0.88	1.48
p4hb	ENSDARG00000052589	Ensembl	0.39	0.17	1.48
AB162343.1	AB162343.1	nt	-0.22	0.01	-1.48
errfi1	ENSDARG00000070171	Ensembl	-0.72	-1.04	-1.48
msna	ENSDARG00000058128	Ensembl	0.01	-0.30	-1.48
HPX	ENSDARG00000051912	Ensembl	0.63	0.20	-1.48
EU025708.1	EU025708.1	nt	-0.85	-0.03	-1.48
gnmt	ENSDARG00000006840	Ensembl	0.06	-0.22	-1.47
fn1b	ENSDARG00000006526	Ensembl	0.15	0.50	-1.47
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.28	0.20	-1.47
BT050383.1	BT050383.1	nt	-0.16	-0.18	1.47
si:dkey-8k3.2	ENSDARG00000038424	Ensembl	0.39	0.27	-1.47
lpcat3	ENSDARG00000075178	Ensembl	-0.31	-0.43	-1.47
BX927253.1	BX927253.1	nt	-0.56	0.08	1.47
gda	ENSDARG00000002986	Ensembl	-0.15	-0.56	-1.47
Ubc	ENSMUSG00000008348	Ensembl	-0.70	-0.98	-1.47
apobb	ENSDARG00000022767	Ensembl	-0.19	-0.07	-1.46
tmem86b	ENSDARG00000038296	Ensembl	-0.37	0.19	1.46
kras	ENSDARG00000010844	Ensembl	0.25	-0.07	1.46
HPX	ENSDARG00000051912	Ensembl	0.81	0.31	-1.46
ENSONIG00000001860	ENSONIG00000001860	Ensembl	-0.22	0.35	1.46
DQ156149.1	DQ156149.1	nt	-0.10	-0.31	1.46
apobb	ENSDARG00000022767	Ensembl	-0.19	-0.06	-1.46
EU025708.1	EU025708.1	nt	-0.89	-0.11	-1.46
ppardb	ENSDARG00000009473	Ensembl	-0.88	-0.37	-1.46
cars	ENSDARG00000036164	Ensembl	0.86	0.75	1.46
HPX	ENSDARG00000051912	Ensembl	0.72	0.26	-1.46
ppan	ENSDARG00000022232	Ensembl	-0.34	0.47	1.46
ugp2b	ENSDARG00000008200	Ensembl	0.44	0.05	-1.46
BX321921.1	BX321921.1	nt	0.65	0.49	1.45
HPX	ENSDARG00000051912	Ensembl	0.86	0.51	-1.45
BT047240.1	BT047240.1	nt	0.38	0.15	1.45
pank1a	ENSDARG00000008192	Ensembl	0.20	-0.71	-1.45
slc11a2	ENSDARG00000024295	Ensembl	-0.54	-0.24	1.45
g6pca.1	ENSDARG00000031616	Ensembl	-0.46	-0.36	-1.45
CD163	ENSG00000177575	Ensembl	0.47	-0.08	-1.45
pam	ENSDARG00000042071	Ensembl	0.03	-0.47	1.45
gnl2	ENSDARG00000053225	Ensembl	0.14	0.05	1.44
NM_001140411.1	NM_001140411.1	refseq	-0.49	-0.16	-1.44
hsd17b3	ENSDARG00000023287	Ensembl	-0.57	-0.44	-1.44
tmem222b	ENSDARG00000074503	Ensembl	0.01	0.73	1.44
AF470013.1	AF470013.1	nt	-0.46	-0.38	-1.44
lss	ENSDARG00000061274	Ensembl	-0.76	-0.69	-1.44
minal	ENSDARG00000036359	Ensembl	-0.25	0.14	1.44
ccnd1	ENSDARG00000035750	Ensembl	0.98	0.21	1.44
cars	ENSDARG00000036164	Ensembl	0.35	0.90	1.44
NM_001139980.1	NM_001139980.1	refseq	0.81	0.40	-1.44
NM_001141300.1	NM_001141300.1	refseq	-0.37	-0.47	-1.44
tsr1	ENSDARG00000007744	Ensembl	0.13	0.41	1.44
CD163	ENSG00000177575	Ensembl	0.57	-0.03	-1.44
gda	ENSDARG00000002986	Ensembl	-0.37	-0.76	-1.43
rbp4l	ENSDARG00000044684	Ensembl	-0.40	-0.80	-1.43
BT045418.1	BT045418.1	nt	-0.34	-0.62	-1.43
HPX	ENSDARG00000051912	Ensembl	0.79	0.33	-1.43
HPX	ENSDARG00000051912	Ensembl	0.62	0.19	-1.43
HPX	ENSDARG00000051912	Ensembl	0.92	0.27	-1.43
sh3tc2	ENSDARG00000076300	Ensembl	0.06	1.23	1.43
HPX	ENSDARG00000051912	Ensembl	0.84	0.15	-1.43
glulb	ENSDARG00000017339	Ensembl	-0.91	-0.99	-1.43
apobb	ENSDARG00000022767	Ensembl	-0.18	-0.02	-1.43

gnmt	ENSDARG00000006840	Ensembl	0.02	-0.22	-1.43
cpne3	ENSDARG00000013175	Ensembl	-0.29	-0.02	1.43
HPX	ENSDARG00000051912	Ensembl	0.85	0.11	-1.43
BT045588.1	BT045588.1	nt	-0.28	0.46	1.43
abhd2a	ENSDARG00000025797	Ensembl	-0.01	-0.37	1.43
APCS	ENSDARG00000045089	Ensembl	0.13	-0.29	-1.43
tlk1b	ENSDARG00000059190	Ensembl	-0.37	0.26	1.43
tshr	ENSDARG00000037195	Ensembl	0.39	-0.18	-1.42
HPX	ENSDARG00000051912	Ensembl	0.77	0.09	-1.42
EU025707.1	EU025707.1	nt	-0.43	-0.18	1.42
mpp1	ENSDARG00000031475	Ensembl	-1.38	-0.16	-1.42
pnisr	ENSDARG00000069855	Ensembl	0.44	0.30	1.42
lrpap1	ENSDARG00000033604	Ensembl	0.35	0.12	1.42
cfhl4	ENSDARG00000094496	Ensembl	0.43	-0.06	-1.42
nfasca	ENSDARG00000061099	Ensembl	-0.82	0.05	1.42
abhd2a	ENSDARG00000025797	Ensembl	0.16	0.15	1.42
BT072227.1	BT072227.1	nt	-0.15	-0.27	-1.42
apobb	ENSDARG00000022767	Ensembl	-0.15	0.00	-1.41
BT058821.1	BT058821.1	nt	-1.33	-0.12	-1.41
gda	ENSDARG00000002986	Ensembl	-0.19	-0.52	-1.41
nupr1	ENSDARG00000094557	Ensembl	-0.57	-0.56	-1.41
lrrc8a	ENSDARG00000032188	Ensembl	-0.82	-0.20	-1.41
jarid2a	ENSDARG00000060925	Ensembl	0.60	0.42	1.41
atf6	ENSDARG00000012656	Ensembl	0.36	0.16	1.40
gda	ENSDARG00000002986	Ensembl	-0.16	-0.30	-1.40
ankrd28b	ENSDARG00000009023	Ensembl	0.89	0.47	1.40
akap1b	ENSDARG00000006062	Ensembl	0.11	0.46	1.40
APOB	ENSDARG00000042780	Ensembl	-0.09	-0.21	1.40
ddx54	ENSDARG00000016015	Ensembl	-0.40	-0.36	1.40
actr3b	ENSDARG00000008790	Ensembl	-0.47	-0.39	-1.40
cldn2	ENSDARG00000044387	Ensembl	0.41	0.20	1.40
nptnb	ENSDARG00000043864	Ensembl	-0.56	-0.44	-1.40
EU481821.1	EU481821.1	nt	-1.00	-0.20	-1.40
f8	ENSDARG00000015247	Ensembl	-0.54	-0.59	-1.40
L24433.1	L24433.1	nt	0.70	0.40	-1.39
BT044654.1	BT044654.1	nt	-0.80	-0.04	-1.39
fam114a1	ENSDARG00000008287	Ensembl	0.19	-0.08	1.38
CD163L1	ENSG00000177675	Ensembl	0.40	-0.26	-1.38
apobb	ENSDARG00000022767	Ensembl	-0.21	-0.11	-1.38
nmd3	ENSDARG00000015676	Ensembl	0.16	0.59	1.38
akap1b	ENSDARG00000006062	Ensembl	0.74	0.24	1.38
EF427382.1	EF427382.1	nt	-0.30	-0.29	-1.38
EF427382.1	EF427382.1	nt	0.18	-0.17	-1.38
BX321921.1	BX321921.1	nt	0.56	0.75	1.38
glulb	ENSDARG00000017339	Ensembl	-0.87	-1.05	-1.38
CYP2X12	ENSDARG00000068290	Ensembl	-1.15	-1.09	-1.38
sat1b	ENSDARG00000032272	Ensembl	-0.26	-0.09	-1.38
gda	ENSDARG00000002986	Ensembl	-0.15	-0.52	-1.38
BT072076.1	BT072076.1	nt	0.48	0.23	1.38
upf1	ENSDARG00000016302	Ensembl	0.07	0.39	1.37
prdx5	ENSDARG00000055064	Ensembl	-0.89	-0.36	-1.37
JN742065.1	JN742065.1	nt	-0.13	0.05	1.37
glud1b	ENSDARG00000002414	Ensembl	-0.82	-0.39	-1.37
ENSORLG00000013337	ENSORLG00000013337	Ensembl	0.02	0.00	1.37
nosip	ENSDARG00000037958	Ensembl	0.64	-0.11	1.37
gart	ENSDARG00000051855	Ensembl	-0.09	0.44	1.37
hsd17b3	ENSDARG00000023287	Ensembl	-0.59	-0.45	-1.36
CD163	ENSG00000177575	Ensembl	0.41	-0.24	-1.36
gda	ENSDARG00000002986	Ensembl	-0.09	-0.63	-1.36
gda	ENSDARG00000002986	Ensembl	-0.25	-0.46	-1.36
GOLGB1	ENSG00000173230	Ensembl	-0.38	-0.11	1.36
gprc5c	ENSDARG00000079092	Ensembl	-0.54	-0.11	-1.36
dkc1	ENSDARG00000016484	Ensembl	-0.17	0.01	1.36
NM_001140411.1	NM_001140411.1	refseq	-0.31	-0.07	-1.36
pgd	ENSDARG00000015343	Ensembl	-0.89	-0.28	-1.36
BT072643.1	BT072643.1	nt	0.88	0.66	1.36
BT072227.1	BT072227.1	nt	-0.87	0.05	-1.36
abcb4	ENSDARG00000010936	Ensembl	0.26	0.18	1.36
insig1	ENSDARG00000010658	Ensembl	0.39	-0.09	1.35
apobb	ENSDARG00000022767	Ensembl	-0.19	-0.06	-1.35
NM_001124565.1	NM_001124565.1	refseq	-0.34	-0.25	-1.35
apobb	ENSDARG00000022767	Ensembl	-0.16	0.04	-1.35
apobb	ENSDARG00000022767	Ensembl	-0.10	-0.04	-1.35
BT072643.1	BT072643.1	nt	0.77	0.47	1.35
apobb	ENSDARG00000022767	Ensembl	-0.14	-0.12	-1.35
gnl3	ENSDARG00000006219	Ensembl	-0.31	0.19	1.35
acsl1	ENSDARG00000030514	Ensembl	0.37	0.14	1.35
cfb	ENSDARG00000055278	Ensembl	0.63	0.27	-1.35
CD163	ENSG00000177575	Ensembl	0.52	-0.09	-1.35
gnl3l	ENSDARG00000020595	Ensembl	0.12	0.30	1.34
CABZ01072083.1	CABZ01072083.1	nt	-0.13	-0.17	1.34
BX004812.1	BX004812.1	nt	-0.18	-0.36	-1.34

sec61a1	ENSDARG00000021669	Ensembl	0.15	-0.08	1.34
pabpc4	ENSDARG00000059259	Ensembl	-0.52	0.85	1.34
sephs1	ENSDARG00000058292	Ensembl	0.12	0.05	1.34
casp3a	ENSDARG00000017905	Ensembl	-0.29	-0.03	-1.34
apobb	ENSDARG00000022767	Ensembl	-0.15	-0.04	-1.34
NM_001173562.1	NM_001173562.1	refseq	0.23	0.00	-1.34
DQ850637.1	DQ850637.1	nt	0.18	0.07	-1.34
ykt6	ENSDARG00000038308	Ensembl	-0.70	-0.15	1.34
gfpt1	ENSDARG00000057465	Ensembl	-0.34	-0.57	-1.34
CABZ01092943.1	CABZ01092943.1	nt	-0.84	-0.54	-1.33
BT076447.1	BT076447.1	nt	0.42	0.47	1.33
EU025709.1	EU025709.1	nt	-0.23	-0.37	-1.33
gda	ENSDARG00000002986	Ensembl	-0.27	-0.46	-1.33
gda	ENSDARG00000002986	Ensembl	-0.27	-0.57	-1.33
apobb	ENSDARG00000022767	Ensembl	-0.17	-0.02	-1.33
CU104700.1	CU104700.1	nt	-0.09	-0.11	-1.33
acsl1	ENSDARG00000030514	Ensembl	0.19	0.11	1.33
bhlhe40	ENSDARG00000004060	Ensembl	0.14	0.84	1.33
prdx4	ENSDARG00000069013	Ensembl	0.82	0.46	1.33
lrrc58b	ENSDARG00000063509	Ensembl	-0.71	-0.59	-1.33
nupr1	ENSDARG00000094557	Ensembl	-0.49	-0.43	-1.32
lpcat3	ENSDARG00000075178	Ensembl	-0.46	-0.51	-1.32
bida	ENSDARG00000069290	Ensembl	0.34	0.31	1.32
thnsl2	ENSDARG00000032584	Ensembl	0.13	0.09	1.32
Tubb4b	ENSMUSG00000036752	Ensembl	0.58	0.19	1.32
Hc	ENSMUSG00000026874	Ensembl	0.34	0.24	-1.32
apobb	ENSDARG00000022767	Ensembl	-0.16	0.00	-1.32
apobb	ENSDARG00000022767	Ensembl	-0.15	-0.04	-1.32
GQ925552.1	GQ925552.1	nt	-0.18	-0.28	-1.31
mcfd2	ENSDARG00000039757	Ensembl	0.26	-0.23	1.31
DDX17	ENSDARG00000010873	Ensembl	0.65	0.23	1.31
BX927289.12	BX927289.12	nt	-0.75	-0.45	-1.31
GU129139.1	GU129139.1	nt	0.07	0.46	-1.31
gnl3	ENSDARG00000006219	Ensembl	0.12	-0.01	1.31
th2	ENSDARG00000038384	Ensembl	-0.18	-0.32	-1.31
dis3	ENSDARG00000060559	Ensembl	0.24	0.20	1.31
cfb	ENSDARG00000055278	Ensembl	0.62	0.21	-1.30
ext1a	ENSDARG00000020373	Ensembl	-0.19	0.19	1.30
pdip5	ENSDARG00000009001	Ensembl	1.07	0.57	1.30
yipf3	ENSDARG00000020449	Ensembl	0.25	-0.54	1.30
thrap3b	ENSDARG00000003513	Ensembl	0.52	0.48	1.30
GQ505858.1	GQ505858.1	nt	0.73	0.47	1.30
mospd2	ENSDARG00000026024	Ensembl	-0.82	-0.28	-1.30
srp72	ENSDARG00000014139	Ensembl	-0.09	0.46	1.30
g6pca.1	ENSDARG00000031616	Ensembl	-0.48	-0.10	-1.30
slc26a5	ENSDARG00000022424	Ensembl	0.55	0.08	-1.30
serp1	ENSDARG00000025493	Ensembl	0.26	0.17	1.30
upf1	ENSDARG00000016302	Ensembl	-0.07	0.01	1.30
ENSORLG00000004374	ENSORLG00000004374	Ensembl	-0.23	-0.18	-1.29
FCF1	ENSDARG00000041373	Ensembl	-0.11	-0.03	1.29
EU025717.1	EU025717.1	nt	-0.08	0.10	-1.29
gda	ENSDARG00000002986	Ensembl	-0.32	-0.45	-1.29
pdip5	ENSDARG00000009001	Ensembl	0.91	0.34	1.29
rps23	ENSDARG00000021838	Ensembl	0.35	-0.25	1.29
apobb	ENSDARG00000022767	Ensembl	-0.11	0.00	-1.29
AB162343.1	AB162343.1	nt	-0.17	-0.01	-1.28
BT059137.1	BT059137.1	nt	-0.12	-0.47	-1.28
nosip	ENSDARG00000037958	Ensembl	-0.24	-0.08	1.28
nfil3-6	ENSDARG00000087188	Ensembl	-0.76	0.13	-1.28
Lyar	ENSMUSG00000067367	Ensembl	-0.26	-0.08	1.28
NM_001140313.1	NM_001140313.1	refseq	2.64	0.00	1.28
cfhl4	ENSDARG00000094496	Ensembl	0.27	-0.09	-1.28
slc11a2	ENSDARG00000024295	Ensembl	-0.62	-0.13	1.28
gda	ENSDARG00000002986	Ensembl	-0.15	-0.47	-1.28
pdia4	ENSDARG00000018491	Ensembl	0.70	0.20	1.28
adipor2	ENSDARG00000058688	Ensembl	-0.33	-0.40	-1.28
BT072144.1	BT072144.1	nt	0.26	0.05	1.28
CPN2	ENSDARG00000063518	Ensembl	0.52	0.07	-1.28
aatf	ENSDARG00000025467	Ensembl	0.63	0.23	1.27
CU928070.1	CU928070.1	nt	-0.40	-0.48	-1.27
pfdn5	ENSDARG00000035043	Ensembl	0.81	0.97	1.27
FJ969490.1	FJ969490.1	nt	-0.73	-0.59	-1.27
CR391962.1	CR391962.1	nt	0.00	-0.07	-1.27
POR	ENSDARG00000059035	Ensembl	-0.43	-0.36	-1.27
NM_001141300.1	NM_001141300.1	refseq	-0.72	-0.39	-1.27
hiat1a	ENSDARG00000013117	Ensembl	-0.41	-0.01	1.26
AL929104.3	AL929104.3	nt	-0.16	-0.42	-1.26
ENSONIG00000021374	ENSONIG00000021374	Ensembl	0.23	-0.08	1.26
alg5	ENSDARG00000061235	Ensembl	0.09	0.26	1.26
hrasa	ENSDARG00000070524	Ensembl	-0.02	-0.52	1.26
BT047994.2	BT047994.2	nt	0.36	0.80	1.26
vars	ENSDARG00000044575	Ensembl	-0.24	0.36	1.26

RPS6KL1	ENSDARG00000079024	Ensembl	-0.04	-0.24	-1.26
ip6k2	ENSDARG00000008310	Ensembl	-1.34	-0.47	-1.26
cfhl4	ENSDARG00000094496	Ensembl	0.29	-0.15	-1.25
NM_001139756.1	NM_001139756.1	refseq	-0.02	0.15	1.25
apof	ENSDARG00000090980	Ensembl	0.22	0.51	1.25
BT046002.1	BT046002.1	nt	0.08	0.29	1.25
bcl2l13	ENSDARG00000062370	Ensembl	0.24	0.45	1.25
mpc2	ENSDARG00000024478	Ensembl	-0.56	-0.63	-1.25
DQ246664.1	DQ246664.1	nt	0.05	-0.59	1.25
creld2	ENSDARG00000029071	Ensembl	0.65	0.12	1.25
RSL1D1	ENSDARG00000055868	Ensembl	-0.52	-0.33	1.25
BT057820.1	BT057820.1	nt	-0.04	0.08	1.25
utp3	ENSDARG00000056720	Ensembl	-0.25	0.37	1.25
GU129140.1	GU129140.1	nt	0.46	0.18	-1.25
nosip	ENSDARG00000037958	Ensembl	0.47	0.15	1.25
ghra	ENSDARG00000054771	Ensembl	-0.24	-0.48	-1.25
ern1	ENSDARG00000013997	Ensembl	-0.08	-0.30	1.24
abcd3a	ENSDARG00000015167	Ensembl	-0.22	-0.18	1.24
cfhl4	ENSDARG00000094496	Ensembl	0.30	-0.15	-1.24
aldh18a1	ENSDARG00000061123	Ensembl	-0.17	-0.21	1.24
NM_001140590.1	NM_001140590.1	refseq	0.86	0.53	1.24
sult2st2	ENSDARG00000053331	Ensembl	-0.61	-0.54	-1.24
ndrg1a	ENSDARG00000032849	Ensembl	-0.56	-0.77	-1.24
noc2l	ENSDARG00000001754	Ensembl	-0.10	0.35	1.23
ENSORLG00000004374	ENSORLG00000004374	Ensembl	-0.10	-0.22	-1.23
rrs1	ENSDARG00000003941	Ensembl	-0.32	0.30	1.22
amd1	ENSDARG00000043856	Ensembl	0.32	-0.42	1.22
nop2	ENSDARG00000043304	Ensembl	-0.38	0.09	1.22
TMEM82	ENSG00000162460	Ensembl	-0.03	0.15	1.22
PICALM	ENSG00000073921	Ensembl	-0.67	-0.37	-1.22
nosip	ENSDARG00000037958	Ensembl	0.42	-0.02	1.22
bxdc2	ENSDARG00000024511	Ensembl	-0.59	0.04	1.22
golgb1	ENSDARG00000061951	Ensembl	-0.51	-0.60	1.22
rpl39	ENSDARG00000036316	Ensembl	0.27	0.36	1.21
pawr	ENSDARG00000045486	Ensembl	-0.05	-0.02	-1.21
nosip	ENSDARG00000037958	Ensembl	0.44	-0.21	1.21
nosip	ENSDARG00000037958	Ensembl	0.50	-0.18	1.21
abcd3a	ENSDARG00000015167	Ensembl	-0.10	0.06	1.21
BT059266.1	BT059266.1	nt	0.54	0.70	1.21
HM159471.1	HM159471.1	nt	0.35	0.17	1.21
NM_001140411.1	NM_001140411.1	refseq	-0.38	-0.11	-1.21
slc23a1	ENSDARG00000015033	Ensembl	-0.17	-0.24	-1.21
man1b1	ENSDARG00000076592	Ensembl	-0.39	-0.36	-1.20
copz2	ENSDARG00000006786	Ensembl	0.21	-0.06	1.20
ftsj	ENSDARG00000076761	Ensembl	0.13	0.25	1.20
L41171.1	L41171.1	nt	0.26	0.46	1.19
macrod1	ENSDARG00000029609	Ensembl	0.09	-0.18	1.19
BT072384.1	BT072384.1	nt	-0.23	0.30	-1.19
pm20d1.2	ENSDARG00000062096	Ensembl	0.47	0.30	1.19
bcl2l13	ENSDARG00000062370	Ensembl	0.00	0.06	1.19
BT057538.1	BT057538.1	nt	-0.39	-0.67	-1.19
clip1a	ENSDARG00000078722	Ensembl	-0.18	0.25	1.18
abcd3a	ENSDARG00000015167	Ensembl	-0.08	0.03	1.18
th2	ENSDARG00000038384	Ensembl	-0.18	-0.30	-1.18
ghra	ENSDARG00000054771	Ensembl	-0.37	-0.30	-1.18
eif3s10	ENSDARG00000076815	Ensembl	0.53	0.31	1.18
atic	ENSDARG00000016706	Ensembl	0.17	0.11	1.17
apobb	ENSDARG00000022767	Ensembl	-0.17	-0.10	-1.17
rpl39	ENSDARG00000036316	Ensembl	0.18	0.32	1.17
Epb4.1l3	ENSMUSG00000024044	Ensembl	-0.52	0.11	1.17
apobb	ENSDARG00000022767	Ensembl	-0.20	-0.17	-1.17
gprc5c	ENSDARG00000079092	Ensembl	-0.29	0.07	-1.17
mib	ENSDARG00000087895	Ensembl	-0.42	-0.12	-1.17
ddost	ENSDARG00000037318	Ensembl	0.53	0.04	1.17
rps2	ENSDARG00000077291	Ensembl	0.13	0.37	1.17
gstal	ENSDARG00000090228	Ensembl	0.06	-0.41	-1.16
EU025708.1	EU025708.1	nt	-0.51	-0.09	-1.16
appb	ENSDARG00000055543	Ensembl	-0.52	-0.33	-1.16
copz2	ENSDARG00000006786	Ensembl	0.60	0.32	1.16
GU294488.1	GU294488.1	nt	0.53	1.22	1.16
bcl2l13	ENSDARG00000062370	Ensembl	0.04	0.13	1.16
apobb	ENSDARG00000022767	Ensembl	-0.17	-0.16	-1.16
pah	ENSDARG00000020143	Ensembl	-0.24	-0.25	-1.16
CU104700.1	CU104700.1	nt	0.29	0.29	-1.15
nars	ENSDARG00000061100	Ensembl	-0.25	-0.02	1.15
xbp1	ENSDARG00000035622	Ensembl	0.08	0.35	1.15
rps2	ENSDARG00000077291	Ensembl	0.09	0.34	1.15
hsp90b1	ENSDARG00000003570	Ensembl	0.71	0.27	1.15
apex1	ENSDARG00000045843	Ensembl	0.49	0.01	1.15
mpv17l2	ENSDARG00000056367	Ensembl	-0.15	-0.09	1.15
CU104700.1	CU104700.1	nt	-0.01	0.31	-1.15
dpydb	ENSDARG00000010267	Ensembl	-0.22	-0.04	-1.15

xirp2a	ENSDARG00000071113	Ensembl	0.01	-0.37	-1.15
eif1ad	ENSDARG00000016280	Ensembl	-0.13	-0.14	1.15
apobb	ENSDARG00000022767	Ensembl	-0.20	-0.13	-1.14
NM_001146360.1	NM_001146360.1	refseq	0.56	0.39	1.14
NM_001165344.1	NM_001165344.1	refseq	0.42	0.08	1.14
serpind1	ENSDARG00000021208	Ensembl	-0.09	0.16	-1.14
serp1	ENSDARG00000025493	Ensembl	0.32	0.16	1.14
zgc:153675	ENSDARG00000071555	Ensembl	0.42	0.58	1.13
copz2	ENSDARG00000006786	Ensembl	0.32	0.01	1.13
apobb	ENSDARG00000022767	Ensembl	-0.24	-0.15	-1.13
CPN2	ENSDARG00000063518	Ensembl	0.58	0.20	-1.13
nop56	ENSDARG00000012820	Ensembl	0.09	0.54	1.13
nosip	ENSDARG00000037958	Ensembl	0.41	-0.14	1.13
sdc2	ENSDARG00000002731	Ensembl	-0.05	-0.16	1.13
apobb	ENSDARG00000022767	Ensembl	-0.18	-0.08	-1.12
gnmt	ENSDARG00000006840	Ensembl	0.01	-0.32	-1.12
rad21b	ENSDARG00000035655	Ensembl	-0.36	-0.10	1.12
sdc2	ENSDARG00000002731	Ensembl	-0.07	-0.16	1.12
pah	ENSDARG00000020143	Ensembl	-0.15	-0.54	-1.12
rpl5a	ENSDARG00000020197	Ensembl	-0.05	0.29	1.12
S66606.1	S66606.1	nt	-0.17	-0.43	-1.12
nmd3	ENSDARG00000015676	Ensembl	-0.26	0.34	1.12
atic	ENSDARG00000016706	Ensembl	0.04	0.28	1.12
eif3s10	ENSDARG00000076815	Ensembl	-0.13	0.40	1.12
ugt5a1	ENSDARG00000016479	Ensembl	-0.56	-0.51	-1.11
rps2	ENSDARG00000077291	Ensembl	0.08	0.32	1.11
slc39a7	ENSDARG00000036388	Ensembl	0.28	0.26	1.11
apobb	ENSDARG00000022767	Ensembl	-0.19	-0.15	-1.11
apobb	ENSDARG00000022767	Ensembl	-0.17	-0.15	-1.11
cpne3	ENSDARG00000013175	Ensembl	0.94	0.14	1.11
bcl2l13	ENSDARG00000062370	Ensembl	0.05	0.22	1.10
phyhd1	ENSDARG00000029905	Ensembl	-0.06	-0.16	-1.10
pah	ENSDARG00000020143	Ensembl	-0.17	-0.50	-1.10
rrbp1a	ENSDARG00000013763	Ensembl	-0.31	0.02	1.10
rpl12	ENSDARG00000006691	Ensembl	0.28	0.17	1.09
BT050215.1	BT050215.1	nt	0.25	0.33	1.09
apobb	ENSDARG00000022767	Ensembl	-0.22	-0.14	-1.09
WARS	ENSDARG00000073930	Ensembl	-0.21	0.28	1.09
fbl	ENSDARG00000053912	Ensembl	-0.12	0.35	1.09
rps9	ENSDARG00000011405	Ensembl	0.11	0.14	1.09
copz2	ENSDARG00000006786	Ensembl	0.31	-0.48	1.09
Clec4e	ENSMUSG00000030142	Ensembl	-0.73	-0.37	-1.08
xirp2a	ENSDARG00000071113	Ensembl	0.02	-0.35	-1.07
pdia4	ENSDARG00000018491	Ensembl	0.93	0.28	1.07
larsb	ENSDARG00000019280	Ensembl	0.17	0.36	1.07
apobb	ENSDARG00000022767	Ensembl	-0.18	-0.14	-1.07
atic	ENSDARG00000016706	Ensembl	-0.10	0.25	1.06
apobb	ENSDARG00000022767	Ensembl	-0.20	-0.13	-1.06
pah	ENSDARG00000020143	Ensembl	-0.18	-0.50	-1.06
rplp0	ENSDARG00000051783	Ensembl	0.10	0.01	1.06
cbr1l	ENSDARG00000021149	Ensembl	0.34	-0.39	1.05
GU129140.1	GU129140.1	nt	0.22	0.12	-1.05
gars	ENSDARG00000059070	Ensembl	-0.30	-0.07	1.05
rab1ba	ENSDARG00000058044	Ensembl	-0.02	0.02	1.05
NM_001173721.1	NM_001173721.1	refseq	-0.11	-0.18	1.04
phyhd1	ENSDARG00000029905	Ensembl	-0.13	-0.29	-1.04
galnt14	ENSDARG00000023448	Ensembl	0.28	0.19	1.04
prdx4	ENSDARG00000069013	Ensembl	0.68	0.07	1.03
BT045832.1	BT045832.1	nt	0.43	0.49	1.02
bysl	ENSDARG00000001057	Ensembl	0.00	0.22	1.02
rpl36a	ENSDARG00000058105	Ensembl	-0.12	0.19	1.02
clic5b	ENSDARG00000077625	Ensembl	0.40	-0.43	1.02
rpl15	ENSDARG00000009285	Ensembl	0.40	0.33	1.01
sec61a1	ENSDARG00000021669	Ensembl	0.14	0.20	0.98
HABP4	ENSDARG00000025174	Ensembl	0.01	0.35	0.97
rpl12	ENSDARG00000006691	Ensembl	0.09	0.14	0.97
rpl15	ENSDARG00000009285	Ensembl	0.44	0.14	0.97
snd1	ENSDARG00000006766	Ensembl	0.26	0.35	0.96
ssr1	ENSDARG00000040518	Ensembl	0.44	0.43	0.95
rpsa	ENSDARG00000019181	Ensembl	0.16	0.24	0.95
rpsa	ENSDARG00000019181	Ensembl	0.18	0.32	0.94
ephx1l	ENSDARG00000008887	Ensembl	0.12	0.00	0.93
spns1	ENSDARG00000011925	Ensembl	-2.42	-0.31	-0.46
NM_001124310.1	NM_001124310.1	refseq	0.00	4.79	0.00
uncharacterised			-1.09	-3.27	-5.16
uncharacterised			-0.93	-1.16	-2.74
uncharacterised			-1.15	-1.22	-4.60
uncharacterised			0.03	0.37	-2.98
uncharacterised			-1.16	-0.77	-8.33
uncharacterised			-0.79	-0.67	-2.17
uncharacterised			-1.32	-0.67	-2.65
uncharacterised			-1.41	-0.95	-3.05

uncharacterised		-0.07	0.22	-2.88
uncharacterised		-0.76	-0.76	-2.28
uncharacterised		-0.84	-0.28	-3.08
uncharacterised		-0.47	0.09	-2.15
uncharacterised		0.28	-5.01	-4.96
uncharacterised		0.23	0.12	-1.73
uncharacterised		-0.54	-1.49	-3.04
uncharacterised		-2.35	-0.43	-4.34
uncharacterised		-0.66	-0.38	-2.29
uncharacterised		-0.62	-1.08	-2.75
uncharacterised		-0.72	-0.01	-4.57
uncharacterised		-0.71	-0.60	-1.67
uncharacterised		-1.48	-0.07	-6.12
uncharacterised		0.25	0.15	-1.77
uncharacterised		-2.06	1.03	-6.37
uncharacterised		-0.94	-1.74	-5.01
uncharacterised		-2.14	0.20	-3.92
uncharacterised		-1.28	-1.07	-2.54
uncharacterised		-0.49	-0.26	-1.79
uncharacterised		-0.28	-1.40	-2.91
uncharacterised		-0.36	-0.99	-2.36
uncharacterised		-1.88	-1.22	-2.48
uncharacterised		-0.41	-0.26	-1.67
uncharacterised		-0.31	-0.43	-2.12
uncharacterised		-0.46	0.26	-2.32
uncharacterised		-0.09	-0.36	-2.03
uncharacterised		0.28	-0.22	-6.47
uncharacterised		0.33	0.86	-4.10
uncharacterised		0.46	0.02	-1.56
uncharacterised		-1.11	-9.91	-9.92
uncharacterised		-0.76	0.04	-1.73
uncharacterised		0.28	0.13	-1.62
uncharacterised		0.23	0.13	-1.65
uncharacterised		-1.08	0.51	-3.64
uncharacterised		-0.60	-0.12	-1.98
uncharacterised		-0.12	-0.30	-6.21
uncharacterised		-0.53	-0.24	-1.86
uncharacterised		-0.27	0.31	-2.43
uncharacterised		-0.87	-0.84	-2.51
uncharacterised		-0.71	0.07	-1.49
uncharacterised		0.00	0.17	-2.59
uncharacterised		-1.77	0.10	-2.51
uncharacterised		-0.84	-1.80	-3.96
uncharacterised		-1.34	-0.75	-2.13
uncharacterised		-1.07	0.74	-2.35
uncharacterised		-0.26	-0.72	-1.76
uncharacterised		0.40	0.02	-1.38
uncharacterised		-0.09	-1.49	-4.40
uncharacterised		-0.56	-0.11	-1.63
uncharacterised		-2.52	-1.66	-3.84
uncharacterised		-1.82	-1.28	-3.96
uncharacterised		-1.73	-0.86	-3.49
uncharacterised		-1.56	1.25	-3.63
uncharacterised		-1.25	-8.92	-8.93
uncharacterised		-0.58	-0.58	-2.16
uncharacterised		-2.22	-1.90	-4.04
uncharacterised		0.49	0.57	-6.03
uncharacterised		-1.72	-0.73	-5.31
uncharacterised		-0.34	-1.56	-2.05
uncharacterised		-0.95	-1.01	-3.74
uncharacterised		0.37	0.26	-3.45
uncharacterised		-0.49	-0.39	-4.57
uncharacterised		0.24	0.06	-3.87
uncharacterised		-0.73	-0.21	-1.47
uncharacterised		-1.33	-0.89	-1.55
uncharacterised		-1.34	-0.61	-2.89
uncharacterised		-0.30	-0.75	-1.26
uncharacterised		-0.57	-0.04	-1.73
uncharacterised		-0.81	0.52	-6.11
uncharacterised		-0.44	-0.46	-2.65
uncharacterised		-0.27	-0.92	-1.96
uncharacterised		-0.96	-1.26	-2.01
uncharacterised		-0.11	-0.14	-1.63
uncharacterised		-0.46	-0.42	-2.65
uncharacterised		-0.75	0.36	-6.27
uncharacterised		0.12	-0.66	-6.09
uncharacterised		-0.84	-0.95	-1.73
uncharacterised		-0.54	-0.10	-1.54
uncharacterised		-0.51	-0.74	-1.78
uncharacterised		0.69	-0.40	-2.04
uncharacterised		-0.71	-0.05	-7.30
uncharacterised		-0.56	0.16	-2.68

uncharacterised		-0.53	-0.27	-1.33
uncharacterised		-0.22	-0.35	-1.36
uncharacterised		0.04	0.27	-1.57
uncharacterised		-0.11	-0.25	-1.59
uncharacterised		-0.89	-0.10	-2.69
uncharacterised		-2.80	-1.37	-2.03
uncharacterised		-0.27	0.19	-1.43
uncharacterised		-0.24	1.11	-2.73
uncharacterised		-0.83	0.37	-3.43
uncharacterised		0.01	0.56	-1.36
uncharacterised		-1.88	-0.13	-3.66
uncharacterised		-1.14	-1.12	-2.08
uncharacterised		-0.20	-0.57	-1.51
uncharacterised		0.15	0.01	-1.25
uncharacterised		-0.37	-0.50	-4.50
uncharacterised		-0.50	-1.91	-4.29
uncharacterised		-0.50	0.09	-2.39
uncharacterised		-0.70	0.93	-3.23
uncharacterised		-0.63	0.05	-2.16
uncharacterised		0.54	0.33	-1.98
uncharacterised		-0.84	-1.03	-3.37
uncharacterised		-0.46	-0.59	-1.24
uncharacterised		0.25	-0.77	-3.67
uncharacterised		0.07	-0.54	-1.93
uncharacterised		-0.55	-0.47	-1.38
uncharacterised		-0.18	-0.35	-1.39
uncharacterised		-0.16	0.21	-1.15
uncharacterised		-2.29	-1.45	-3.41
uncharacterised		-1.48	-2.09	-3.70
uncharacterised		-0.83	-0.46	-1.65
uncharacterised		-0.09	-0.06	-1.79
uncharacterised		-0.59	-0.06	-2.19
uncharacterised		-0.37	0.26	-2.24
uncharacterised		-0.25	-0.06	-1.17
uncharacterised		-1.84	0.61	-2.56
uncharacterised		-0.49	0.01	-2.68
uncharacterised		-0.68	-0.93	-2.91
uncharacterised		-0.46	-0.50	-2.28
uncharacterised		0.16	-0.91	-1.66
uncharacterised		-0.37	-1.06	-4.39
uncharacterised		-1.23	-0.28	-1.85
uncharacterised		-0.13	0.30	-1.15
uncharacterised		-0.37	-0.71	-3.18
uncharacterised		-0.31	-0.05	-2.18
uncharacterised		0.19	0.16	-1.73
uncharacterised		0.28	0.34	-1.31
uncharacterised		-0.16	-1.41	-3.30
uncharacterised		-1.35	-0.96	-2.07
uncharacterised		-0.15	0.03	-1.43
uncharacterised		-0.36	-0.51	-1.30
uncharacterised		-0.17	-1.00	-3.18
uncharacterised		-0.97	-0.79	-2.39
uncharacterised		-0.05	0.02	-1.23
uncharacterised		-1.99	-1.86	-3.08
uncharacterised		-1.74	-0.33	-1.85
uncharacterised		-0.28	-0.15	-1.40
uncharacterised		0.20	-0.69	-2.18
uncharacterised		-0.40	-0.52	-2.68
uncharacterised		-0.64	0.83	-2.34
uncharacterised		-0.81	-0.12	-2.03
uncharacterised		-0.26	-0.59	-1.62
uncharacterised		-0.58	-0.54	-2.48
uncharacterised		-0.68	-0.15	-1.28
uncharacterised		-1.02	-0.05	-2.09
uncharacterised		-0.93	0.34	-1.80
uncharacterised		-0.44	-0.14	-1.39
uncharacterised		0.00	0.00	9.20
uncharacterised		0.00	0.00	9.03
uncharacterised		0.00	0.00	9.17
uncharacterised		0.00	0.00	9.05
uncharacterised		0.00	0.00	9.88
uncharacterised		0.00	0.00	5.89
uncharacterised		0.00	0.00	7.16
uncharacterised		0.00	0.00	8.03
uncharacterised		0.00	0.00	6.22
uncharacterised		0.00	0.00	8.04
uncharacterised		0.00	0.00	6.46
uncharacterised		0.00	0.00	6.96
uncharacterised		0.00	0.00	9.08
uncharacterised		0.00	0.00	8.78
uncharacterised		0.00	0.00	6.32
uncharacterised		0.01	0.01	5.22

uncharacterised		0.53	0.00	4.08
uncharacterised		0.00	0.00	7.12
uncharacterised		0.22	0.27	3.51
uncharacterised		0.00	0.00	7.97
uncharacterised		0.00	0.00	5.40
uncharacterised		0.00	0.00	4.75
uncharacterised		-0.28	-0.61	3.81
uncharacterised		0.00	0.00	5.42
uncharacterised		0.00	0.00	7.80
uncharacterised		0.00	0.00	7.86
uncharacterised		0.00	0.00	7.76
uncharacterised		0.00	0.00	7.87
uncharacterised		0.00	0.00	5.38
uncharacterised		0.00	0.00	5.48
uncharacterised		0.00	0.00	7.63
uncharacterised		0.00	0.00	7.48
uncharacterised		0.00	0.00	7.63
uncharacterised		0.02	0.49	3.42
uncharacterised		0.00	0.00	7.46
uncharacterised		0.51	0.14	3.52
uncharacterised		0.00	0.00	5.84
uncharacterised		0.00	0.00	7.60
uncharacterised		0.43	0.23	3.45
uncharacterised		-0.98	-0.05	3.74
uncharacterised		0.00	0.00	7.46
uncharacterised		0.00	0.00	5.11
uncharacterised		0.00	0.00	7.21
uncharacterised		1.43	-0.10	2.77
uncharacterised		0.00	0.00	3.98
uncharacterised		0.80	1.28	3.04
uncharacterised		0.00	0.00	7.42
uncharacterised		0.00	0.00	5.06
uncharacterised		0.47	0.43	2.82
uncharacterised		0.00	0.00	4.24
uncharacterised		0.00	0.00	3.54
uncharacterised		0.00	0.00	3.99
uncharacterised		0.07	0.01	2.20
uncharacterised		0.00	0.00	3.90
uncharacterised		0.00	0.00	4.56
uncharacterised		0.00	0.00	7.00
uncharacterised		0.57	1.20	2.98
uncharacterised		0.00	0.00	4.42
uncharacterised		0.00	0.00	4.27
uncharacterised		0.00	0.00	7.13
uncharacterised		0.00	0.00	5.49
uncharacterised		0.00	0.00	3.67
uncharacterised		0.31	0.27	2.08
uncharacterised		0.00	0.00	5.66
uncharacterised		0.00	0.00	4.41
uncharacterised		1.11	1.49	3.12
uncharacterised		0.00	0.00	4.74
uncharacterised		0.00	0.00	6.94
uncharacterised		0.00	0.00	4.62
uncharacterised		0.00	0.00	5.24
uncharacterised		0.89	1.39	2.88
uncharacterised		0.00	0.00	4.67
uncharacterised		0.00	0.00	4.17
uncharacterised		0.00	0.00	6.66
uncharacterised		0.73	1.20	3.10
uncharacterised		0.42	0.63	1.86
uncharacterised		0.00	0.00	6.69
uncharacterised		0.00	0.00	3.57
uncharacterised		0.00	0.00	6.56
uncharacterised		0.00	0.00	4.53
uncharacterised		0.70	1.27	3.02
uncharacterised		0.00	0.00	6.54
uncharacterised		0.00	0.00	4.26
uncharacterised		0.84	0.31	2.40
uncharacterised		0.00	0.00	5.11
uncharacterised		0.67	0.94	2.87
uncharacterised		0.43	0.10	2.83
uncharacterised		0.00	0.00	3.28
uncharacterised		-0.30	0.35	1.94
uncharacterised		0.00	0.00	3.36
uncharacterised		0.73	1.26	2.75
uncharacterised		0.00	0.00	4.22
uncharacterised		0.56	0.67	3.07
uncharacterised		0.00	0.00	6.18
uncharacterised		0.79	0.98	2.88
uncharacterised		0.00	0.00	6.17
uncharacterised		0.00	0.00	4.09
uncharacterised		0.00	0.00	3.18

uncharacterised		0.78	0.83	2.71
uncharacterised		0.00	0.00	3.45
uncharacterised		0.00	0.00	5.99
uncharacterised		1.25	0.47	3.17
uncharacterised		0.00	0.00	3.28
uncharacterised		0.00	0.00	4.70
uncharacterised		-0.26	0.32	2.34
uncharacterised		0.49	0.76	2.24
uncharacterised		1.88	1.71	2.82
uncharacterised		0.00	0.00	3.43
uncharacterised		0.69	1.22	2.64
uncharacterised		0.51	1.13	2.92
uncharacterised		-0.40	-0.34	1.75
uncharacterised		-0.73	-0.40	3.52
uncharacterised		-0.28	0.07	2.21
uncharacterised		0.49	1.34	2.92
uncharacterised		0.00	0.00	2.86
uncharacterised		0.18	0.16	1.95
uncharacterised		0.00	0.88	2.71
uncharacterised		0.00	0.00	4.43
uncharacterised		0.00	0.00	3.56
uncharacterised		0.00	0.00	4.78
uncharacterised		2.21	2.03	2.84
uncharacterised		0.00	0.00	4.24
uncharacterised		0.30	-0.32	2.52
uncharacterised		0.00	0.00	3.13
uncharacterised		0.00	0.00	4.36
uncharacterised		0.00	0.00	4.02
uncharacterised		0.00	0.00	2.76
uncharacterised		0.00	0.00	4.13
uncharacterised		0.43	-0.60	2.27
uncharacterised		0.00	0.00	4.29
uncharacterised		-0.34	-0.12	2.25
uncharacterised		-0.19	0.32	2.34
uncharacterised		0.00	0.00	2.41
uncharacterised		0.00	1.52	2.68
uncharacterised		0.00	0.00	3.23
uncharacterised		0.00	0.00	2.26
uncharacterised		0.00	0.00	3.94
uncharacterised		0.00	0.00	2.58
uncharacterised		0.00	0.00	3.39
uncharacterised		0.47	0.00	2.09
uncharacterised		0.00	0.00	4.00
uncharacterised		1.13	1.25	2.62
uncharacterised		0.00	0.00	3.00
uncharacterised		0.70	0.28	1.55
uncharacterised		1.03	0.20	1.62
uncharacterised		0.00	0.00	4.04
uncharacterised		0.00	0.00	2.56
uncharacterised		0.00	0.00	3.98
uncharacterised		0.09	1.56	2.13
uncharacterised		0.32	0.81	1.74
uncharacterised		0.00	0.00	3.59
uncharacterised		0.00	0.00	3.51
uncharacterised		0.00	0.00	3.19
uncharacterised		0.00	0.00	3.54
uncharacterised		0.65	0.58	2.38
uncharacterised		-0.27	0.23	1.60
uncharacterised		1.34	1.56	1.94
uncharacterised		-0.02	0.17	1.75
uncharacterised		0.95	0.11	1.39
uncharacterised		-0.27	0.54	1.71
uncharacterised		0.00	0.00	3.25
uncharacterised		-0.01	-0.39	1.79
uncharacterised		0.00	0.00	2.34
uncharacterised		0.00	0.00	2.84
uncharacterised		-0.07	0.05	1.78
uncharacterised		0.00	0.00	3.97
uncharacterised		0.12	0.24	1.39
uncharacterised		0.00	0.00	3.31
uncharacterised		0.45	0.06	1.66
uncharacterised		0.00	0.00	2.96
uncharacterised		0.00	2.52	3.31
uncharacterised		0.08	-0.14	2.28
uncharacterised		0.86	0.52	1.86
uncharacterised		-0.26	0.29	1.28
uncharacterised		0.80	0.75	1.88
uncharacterised		0.00	0.00	3.10
uncharacterised		0.77	0.20	2.88
uncharacterised		0.00	2.24	2.53
uncharacterised		0.00	1.90	2.41
uncharacterised		0.21	-0.89	1.62

uncharacterised		0.70	0.68	2.02
uncharacterised		0.00	0.00	3.34
uncharacterised		0.00	0.00	3.23
uncharacterised		0.25	1.61	1.89
uncharacterised		0.00	0.00	2.49
uncharacterised		0.00	0.00	2.20
uncharacterised		-0.02	0.65	1.88
uncharacterised		0.00	0.37	1.95
uncharacterised		0.00	0.50	2.35
uncharacterised		0.00	0.00	2.60
uncharacterised		0.06	-0.02	1.34
uncharacterised		0.00	0.00	2.46
uncharacterised		0.00	0.00	3.59
uncharacterised		0.99	-0.23	2.31
uncharacterised		0.00	0.00	2.93
uncharacterised		0.00	0.00	2.29
uncharacterised		-0.07	0.55	1.27
uncharacterised		-1.10	-0.66	1.68
uncharacterised		0.00	0.00	2.72
uncharacterised		0.00	0.00	2.33
uncharacterised		0.00	0.00	3.16
uncharacterised		2.17	2.50	3.30
uncharacterised		-0.61	0.27	1.72
uncharacterised		0.00	0.00	2.30
uncharacterised		-0.44	0.49	1.94
uncharacterised		-1.25	-1.48	1.80
uncharacterised		1.89	1.48	1.91
uncharacterised		0.52	0.12	1.97
uncharacterised		0.13	0.05	1.15
uncharacterised		0.00	0.50	1.16
uncharacterised		-0.25	-1.47	1.84
uncharacterised		0.00	0.00	2.75
uncharacterised		0.76	0.71	1.93
uncharacterised		0.00	0.00	3.14
uncharacterised		0.00	0.00	2.17
uncharacterised		0.00	0.00	2.26
uncharacterised		0.00	0.00	2.39
uncharacterised		0.00	0.89	2.03
uncharacterised		-0.66	0.35	1.58
uncharacterised		1.87	0.98	2.39
uncharacterised		0.00	0.00	2.18
uncharacterised		0.35	0.32	1.23
uncharacterised		0.00	0.00	2.53
uncharacterised		0.00	1.75	2.71
uncharacterised		0.77	0.29	2.34
uncharacterised		0.00	0.00	2.04
uncharacterised		0.30	-0.16	1.37
uncharacterised		0.16	-0.13	1.54
uncharacterised		-1.23	0.03	1.80
uncharacterised		0.00	0.00	2.62
uncharacterised		-1.79	0.74	1.53
uncharacterised		0.00	0.00	3.07
uncharacterised		0.00	0.00	3.06
uncharacterised		0.00	0.00	3.07
uncharacterised		0.89	1.14	1.69
uncharacterised		0.06	0.41	1.81
uncharacterised		0.00	0.00	2.82
uncharacterised		0.00	0.00	2.11
uncharacterised		-0.03	-0.89	1.63
uncharacterised		0.00	0.00	1.98
uncharacterised		1.53	1.12	1.53
uncharacterised		1.84	0.00	2.34
uncharacterised		0.00	0.00	2.64
uncharacterised		0.00	0.00	2.59
uncharacterised		0.00	0.00	2.48
uncharacterised		-2.03	0.45	1.76
uncharacterised		0.17	-0.18	1.17
uncharacterised		0.00	0.00	2.48
uncharacterised		0.11	0.24	1.87
uncharacterised		1.22	0.38	1.79
uncharacterised		0.00	0.00	2.01
uncharacterised		0.00	0.00	2.65
uncharacterised		0.00	0.00	2.18
uncharacterised		0.00	0.00	2.21
uncharacterised		0.24	-0.07	1.24
uncharacterised		0.00	2.00	2.81
uncharacterised		1.15	0.00	1.74
uncharacterised		0.00	0.00	2.73
uncharacterised		0.51	0.15	1.41
uncharacterised		0.00	5.14	0.00

CHAPTER 4

Global transcriptome profiling reveals down-regulation of cholesterol biosynthesis and up-regulation of cellular stress response in brown trout (*Salmo trutta*) exposed to linuron.

Manuscript in preparation

Global transcriptome profiling reveals down-regulation of cholesterol biosynthesis and up-regulation of cellular stress response in brown trout (*Salmo trutta*) exposed to linuron

Tamsyn M. Uren Webster and Eduarda M. Santos

Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

Abstract

Linuron, a widely used phenylurea-based herbicide, has been measured in surface waters at concentrations in the low µg/L range, and has also been detected in food residues and drinking water. Previous research in mammals and fish has shown that linuron antagonistically binds the androgen receptor (AR) *in vitro,* shows anti-androgenic activity *in vivo*, and disrupts male rat reproductive development. However, there has been scarce research investigating the potential global toxicity of linuron to male fish, or considering the impacts of disruption of androgen signalling in tissues other than the gonad. Therefore, we aimed to characterise the global hepatic transcriptional response to linuron exposure using RNA-seq in mature male brown trout, an important environmentally relevant species. To do this, we exposed trout to three concentrations of linuron (2.5, 25 and 250 µg/L) for four days, and sequenced three replicate liver samples for each treatment on an Illumina HiSeq 2500 platform. We assembled the transcriptome using a *de novo* approach, and subsequent expression analysis revealed a total of 822 differentially regulated transcripts across all treatment groups. Functional analysis identified a striking down-regulation of the majority of the enzymes involved in the cholesterol biosynthesis pathway, possibly as a consequence of disruption of androgen signalling by linuron. In addition, we found evidence of an over-representation of transcripts involved in cellular stress response, including up-regulation of CYP1A by up to 560 fold following exposure to 250 µg linuron/L, as well as evidence of up-regulation of molecular chaperones and the antioxidant system. There was also evidence of similar, although less pronounced, transcriptional changes in fish exposed to 2.5 µg linuron/L which may raise concerns over the potential effects of this pesticide on natural brown trout populations following environmental exposure.

Introduction

Linuron is a widely used substituted phenylurea herbicide that disrupts photosynthesis by targeting protein D1, a central component of photosystem II, and inhibiting photo-dependent electron transport, leading to accumulation of reactive oxygen species (ROS) [1]. This compound is used to control a number of broadleaf and grass weeds in the cultivation of a variety of crop plants, particularly vegetables and cereals. Linuron is known to enter surface waters in agricultural runoff, particularly in association with sediment, and concentrations in rivers have been reported in the low µg/L range. One of the highest measured concentrations in the environment was 4.42 µg/L in Florida [2], and modelling approaches have predicted peak concentrations of 31.3 µg/L in surface waters associated with application on a nearby carrot crop, highlighting the potential for short-term peaks in contamination to occur [3]. Linuron has also been detected in drinking water, and in food residues [4, 5]. The potential for environmental exposure to this chemical therefore raises concerns about the risk linuron may pose to both human and wildlife health.

The majority of existing research investigating the toxicological effects of linuron has focused on its activity as an anti-androgenic compound. *In vitro* studies have shown that linuron acts as an anti-androgen in both mammals and fish, and competitively inhibits androgen binding to the androgen receptor (AR) [e.g. 6, 7-10]. Anti-androgenic activity has also been demonstrated *in vivo*. The Hershberger assay involves treating castrated or juvenile male rats with testosterone to induce controlled reproductive development, then simultaneously exposing them to suspected anti-androgens to investigate potential inhibitory effects on androgen-specific development. Using this method, exposure to linuron reduced the weight and development of androgen-sensitive reproductive tissues [11-13]. Using a similar principle in sticklebacks, the production of spiggin, an androgen-dependent glycoprotein normally produced by nest-building males, can be induced in females by androgen treatment and its subsequent inhibition by anti-androgens is assumed to occur specifically through AR antagonism [14]. Linuron was reported to suppress the production of dihydrotestosterone (DHT) induced spiggin protein in cultured kidney cells and, *in vivo,* concentrations of 100 µg/L and 250 µg/L reduced spiggin induction at both transcript and protein level [14-17].

Linuron was shown to have adverse impacts on male reproductive health in rats, including abnormal reproductive development following *in utero* exposure, Leydig cell tumourgenesis and reduced testosterone production *in vitro* and *in vivo* [11, 18-20]. It is hypothesised that in addition to its action as an AR antagonist, linuron may disrupt androgen synthesis and/or metabolism, but the relative contribution of each mechanism is unknown [20]. In addition, some evidence exists for anti-estrogenic effects of linuron in fish and amphibians [8, 21]. Recent transcriptomic and proteomic approaches in female fathead minnow and in zebrafish embryos have also demonstrated that the molecular signatures following exposure to linuron are more similar to that of the model anti-androgen, flutamide, than a model androgen (DHT) or oestrogen (ethinyl-estradiol)[22-24].

The anti-androgenic activity of linuron has therefore been relatively well established, although the detailed mechanisms by which this results in adverse reproductive health consequences following environmental exposure are unclear. Furthermore, in fish, there has been limited research investigating the impacts of linuron in males. Additionally, the majority of studies investigating the molecular mechanisms of anti-androgenic toxicity have focused on the gonads, but androgen signalling also has an important role in regulating a range of other biological processes in different tissues, including the liver [25]. Hepatic metabolism is the major mechanism responsible for steroid hormone degradation, as well as detoxification and elimination of xenobiotics, which makes the liver a sensitive and useful model for investigating toxic effect of chemical exposure. It is also possible that linuron has multiple mechanisms of toxicity, like other phenylurea-based herbicides, but the global effects of this chemical have seldom been investigated.

In this study, we conducted an exposure of mature male brown trout (*Salmo trutta*) to three concentrations of linuron (2.5, 25 and 250 µg/L), and employed RNA-seq to investigate the resulting global hepatic responses to this chemical. Brown trout are an ecologically and economically important native European species known to be sensitive to environmental stressors and are likely to be subject to pesticide exposure in their ecological niche, which includes rivers and streams in catchments

dominated by farmland. In addition, this species also may serve as a useful and sensitive model for the effects of exposure to linuron across vertebrate species.

Materials and methods

Chemical exposure

Sexually mature male brown trout were exposed to linuron via a flow through system for a period of 4 days. The treatment groups consisted of three concentrations of linuron; 2.5, 25 and 250 µg/L (Pestanol Analytical Standard, Sigma) or dilution water control alone. These concentrations were chosen to include a concentration within the range measured in the most contaminated surface waters (2.5 µg/L), an intermediate concentration representing concentrations that may potentially occur during peak contamination events, and a high concentration to facilitate a mechanistic investigation of molecular pathways disrupted by this chemical. Each treatment group consisted of one tank containing 8 individual fish, and the control treatment was run in duplicate. Water samples were collected from each tank on day 3 of the exposure period and were analysed using LC-MS by an accredited laboratory (South West Water, Exeter Laboratories). Full details on fish husbandry are given in the supporting information.

Sampling

Fish were humanely sacrificed on day four of the exposure period by a lethal dose of benzocaine (0.5 g/L; Sigma-Aldrich) followed by destruction of the brain, in accordance with UK Home Office regulations. For each individual fish, wet weight and fork length were recorded and the condition factor (k= (weight (g) x 100)/ (fork length $(cm)^3$)) was calculated. Sex and maturity of individuals was confirmed by observation of the gonads. Livers were dissected and weighed, and the hepatosomatic index (HSI) (liver weight (mg)/ total weight (mg)) x 100)) and gonadsomatic index (GSI) (gonad weight (mg)/ total weight (mg)) x 100)) were determined. Statistical analysis of morphological parameters was conducted for mature males only (n=3-6 per treatment group) in SigmaStat (version 12.0). All morphometric data met assumptions of normality and equal variance and was analysed using single factor one way analysis of variance (ANOVA). Portions of the

liver were snap frozen in liquid nitrogen and stored at -80°C prior to transcript profiling.

Illumina sequencing and transcriptomic analysis

Transcript profiling was conducted in the liver of three replicate fish per treatment group. RNA was extracted and spiked with External RNA Controls Consortium (ERCC) spike-in control mixes (Ambion) then prepared for sequencing using the Illumina TruSeq RNA Sample Preparation kit. The 15 individual libraries were multiplexed with 24 samples per lane (together with samples from another project [26]) and sequenced on an Illumina HiSeq 2500 platform to generate 100 bp paired-end reads. Sequence reads were combined with those from another project also employing mature male brown trout liver as a target tissue [26] in order to maximise sequence coverage depth for transcriptome assembly. Full details of the transcriptome assembly are given in [26] and in the supporting information. Briefly, raw sequence reads were subject to quality-related processing, filtering and digital normalisation, then a *de novo* transcriptome assembly was conducted using Trinity (version r2013-02-25; [27]), specifying default parameters. Transcripts were annotated using Blastx against Ensembl peptide databases using an e-value cut off $< 1e^{-15}$, and additional annotation of previously un-annotated differentially expressed transcripts was performed using Blast ($< 1e^{-15}$) against refseq, nr and nt databases.

Transcriptomic analysis was conducted according to [26] and full details are described in the supporting information. 83.2 % of raw sequence reads from each of the 15 individual libraries were re-mapped to the newly assembled brown trout transcriptome using Bowtie2 (version 2.1.0, [28]), specifying the -k 1 parameter. Raw count data for each transcript was extracted using idxstats in samtools (version 0.1.18, [29]) and input into edgeR [30] for differential expression analysis. Pairwise comparisons were initially conducted between the two control groups, which were also employed as controls in [26], and this revealed only 3 differentially regulated transcripts. Therefore we conducted pairwise comparisons between the three replicates in each linuron treatment group against the combined control groups (six replicates). Transcripts were considered differentially expressed with a FDR < 0.1 (Benjamini-Hochberg correction). Functional analysis was performed for differentially expressed genes from each treatment using the Database for Annotation,

Visualisation and Integrated Discovery (DAVID v6.7; [31]), and Ingenuity Pathways Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com).

Results

Water chemistry and morphological parameters

The measured concentrations of linuron on day 3 of the exposure were 1.7, 15.3 and 225.9 µg/L for the tanks exposed to nominal concentrations of 2.5, 25 and 250 µg/L, respectively. The measured values were 61-90 % of the nominal concentrations and, therefore, throughout this paper, we refer to the nominal concentrations of linuron to indicate the exposure concentrations. The mean mass and length of all mature males were 452.7 ± 14.3 g and 33.9 ± 0.3 cm. There were no significant differences in size and condition factor (mean 1.15 ± 0.01), HSI (mean 1.04 ± 0.04) or GSI (mean 3.82 ± 0.27) between treatment groups and we observed no alteration in the general health or behaviour of the exposed fish during the experiment.

Sequencing and transcriptomic analysis

The *de novo* transcriptome was assembled using a total of 225.3 million paired 100 bp reads from male brown trout liver (as described in [26]), and consisted of 172,688 transcripts (107,095 loci) with a mean length of 767.5 bp and a N50 of 1292 bp. 62,236 transcripts were annotated using blastx against Ensembl peptide databases, and these included representation of 16,121 unique zebrafish transcripts. Sequencing of the liver samples of fish exposed to linuron and associated controls generated a total of 137.9 million reads, averaging 9.2 million reads per sample, and 83.3 % of these re-mapped against the assembled transcriptome.

Pairwise expression analysis using EdgeR identified a total of 822 transcripts that were differentially expressed in one or more treatment group compared to the controls, 435 of which were up-regulated and 387 were down-regulated. The numbers of up- and down-regulated transcripts in each treatment group, and the overlaps between the lists of differentially expressed transcripts for each treatment group are shown in Figure 1. A full list of differentially expressed transcripts is presented in S Table 3. Heatmaps were generated using Euclidean cluster analysis

based on the expression levels of all transcripts that were differentially regulated in at least one treatment group. Clustering of all individual samples based on expression levels is presented in Figure 2, and on mean fold change for each treatment group compared to the control is shown in S Figure 1. Additionally, multidimensional scaling (MDS) plots illustrating the similarity of replicates within each treatment group compared to the controls are shown in Figure S2. These analyses indicated that the expression profiles of all individual fish exposed to 250 µg linuron/L were distinct from those in the control and in treatment groups exposed to lower concentrations of linuron, corresponding to the highest number of differentially expressed transcripts in this treatment group. Similarly, the expression profiles of individuals exposed to the lowest concentration of linuron (2.5 µg/L) also cluster together, but less distinctly from those in the control group, corresponding with a lower number of differentially-regulated transcripts in this group. In contrast, the individuals exposed to (25 µg linuron/L) did not cluster together, and it was clear that there was a more variable transcript expression profile in this group (Figure S2). This increased degree of biological variation between replicates reduced the statistical power for expression analysis, and subsequently resulted in the identification of fewer differentially expressed transcripts in this group.

The results from the ERCC-spike in control analysis provide strong technical validation of the quantitative expression profiling conducted in this study. There was a strong correlation between the calculated FPKM values and the expected concentration of control transcripts for all individual libraries (mean R^2 =0.901 ±0.005; Figure S3). Only ERCC control transcripts that were detected in a minimum of three individual libraries (at least one count) were included in the analysis. The dynamic range was calculated individually for all samples (Table S1), and the mean dynamic range in expression level across all libraries was 21, 062 FPKM. Additionally, there was a good correlation between the calculated and expected fold changes in expression between samples spiked with ERCC mix 1 and mix 2 (R^2=0.58). Together these results provide strong technical validation for the quantitative expression profiling conducted in this study.

Functional analysis

The list of over-represented Gene Ontology (GO) terms and Kegg pathways in fish exposed to 250 µg linuron/L is shown in S Table 2. Processes related to protein folding, lipid biosynthesis and metabolism, and response to stress and chemical stimulus were the most significantly enriched. There were no significantly over-represented GO terms or Kegg pathways for fish exposed to 2.5 or 25 µg linuron/L, possibly due to the relatively low number of differentially regulated transcripts in these groups.

Ingenuity pathway analysis identified a very significant over-representation of the cholesterol biosynthesis pathway in the lists of differentially expressed transcripts. This included down-regulation of 14 individual enzymes involved in this pathway in fish exposed to 250 µg linuron/L, as well as trends towards reduced expression of other enzymes. We also observed trends towards decreased expression of these enzymes in fish exposed to 2.5 and 25 µg linuron/L. A simplified schematic illustrating the down-regulation of this pathway is shown in Figure 3.

Discussion

Transcript expression analysis revealed a considerable degree of transcriptional change in fish exposed 250 µg/L of linuron. Fewer differentially expressed transcripts were observed in the 2.5 µg/L treatment group, but we observed broadly similar changes in the gene groups and pathways that were differentially regulated. This concentration of linuron is within the range measured in surface waters, highlighting the potential for adverse health impacts on fish following environmental exposure. The relatively low number of differentially regulated transcripts observed in the intermediate treatment group reflects the larger degree of biological variation between replicates in this group. In particular, one individual showed more pronounced transcriptional changes than the others. We hypothesise that this may reflect different threshold concentrations of response between individuals.

Cholesterol biosynthesis

Functional analysis revealed a striking down-regulation of transcripts encoding the majority of enzymes involved in the cholesterol biosynthesis pathway. Although most of these transcripts were only significantly down-regulated following exposure to the highest concentration of linuron (250 µg/L), there were clear trends towards reduced expression in the lower treatment groups. Cholesterol biosynthesis is a well characterised pathway consisting of a series of complex reactions involving more than 20 enzymes. Briefly, a precursor molecule, Acetyl CoA, is converted through the mevalonate pathway to lanosterol. This stage includes the synthesis of mevalonic acid by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which is generally regarded as the major irreversible, rate-limiting step in the biosynthesis of cholesterol. Lanosterol is then converted to cholesterol via a series of successive dimethylation reactions and double bond reductions, through either the Bloch or Kandutsch-Russell pathways [32, 33].

Members of the sterol regulatory element binding protein (SREBP) family of transcription factors are involved in controlling a number of aspects of lipid and sterol metabolism. Specifically, the isoform STREBP-2 is a major transcriptional regulator of cholesterol biosynthesis [32]. Highly regulated feedback mechanisms are responsible for controlling the activity of SREBPs. Sterol-sensing SREBP-cleavage-activating proteins (SCAPs) bind and retain inactive SREBP precursors. When cholesterol levels are depleted, SCAP dissociates from endoplasmic reticulum (ER) membranes and transports SREBP-2 to the Golgi complex where it is cleaved and activated by site 1 and site2 proteases. Activated SREBP-2 then moves to the nucleus and induces the transcription of target genes by binding sterol response elements (SREs) in their promoter regions, together with associated cofactors. Conversely, elevated levels of cholesterol stimulate the inactivation of SREBP through its re-association with SCAP in the ER membrane [34].

Nearly all genes encoding cholesterol biosynthesis enzymes have been shown to be regulated by SREBP-2 in mammalian studies; Sharpe and Brown [33] describe 22 enzymes involved in the cholesterol biosynthesis pathway, 21 of which are regulated by SREBP-2. Of these, 14 were significantly down-regulated by 250 ug linuron/L, while there were trends in reduced expression for the other seven. Furthermore, the

SREBP-2 encoding transcript (*strebf2*) was also significantly down-regulated. Our results, therefore, suggest that linuron specifically disrupts the regulation of cholesterol biosynthesis through SREBP-2.

We hypothesise that the anti-androgenic activity of linuron is a likely mechanism by which it down-regulates STREBP-2, and cholesterol biosynthesis. Androgens were found to directly stimulate the expression of SREBP transcripts and cholesterol biosynthesis enzymes in prostate cancer cell lines [35], and also enhance SCAP-mediated cleavage of precursor SREBP into the mature form [36]. *In vivo*, the expression of SREBP and cholesterol biosynthesis enzymes were reduced following castration in male rats, and restored following androgen treatment [37]. In fish, transcriptomic profiling of male fathead minnow exposed to pulp and paper mill effluent, which has been previously linked with androgenic activity, revealed an up-regulation of cholesterol biosynthesis enzymes [38]. Potentially, a similar mechanism of toxicity may be shared with other chemicals that antagonistically interact with the AR. Previous studies have reported that anti-androgen exposures modulate the expression and activity of individual enzymes involved in cholesterol metabolism, and alter serum cholesterol and lipid concentrations [e.g. 23, 39]. However, the down-regulation of cholesterol biosynthesis has not been previously widely recognised as a specific mechanism of anti-androgen toxicity.

Cholesterol is an essential component of cellular membranes. It is involved in the regulation of membrane fluidity and permeability, transmembrane transport and signalling, and is also the precursor of a number of other essential biological molecules including bile acids and steroid hormones. The liver is the primary organ responsible for vertebrate cholesterol production therefore the observed down-regulation of enzymes involved in cholesterol biosynthesis may have a number of potential health impacts in fish. One of the essential functions of the liver, and use of hepatic cholesterol, is formation of biliary acids. Bile synthesis is critical for the elimination of endogenous and xenobiotic metabolites [32]. We observed an increase in the expression level of *cyp7a1*, which encodes the rate limiting enzyme responsible for biliary acid formation from cholesterol, possibly suggesting a compensatory response to the putative reduction in cholesterol biosynthesis. Disruption of cholesterol homeostasis has also been implicated in a number of

human pathologies including prostate cancer, in which androgen signalling is also involved, dementia, diabetes and Alzheimer's disease. Although the majority of cholesterol for sex steroid production is synthesised in the gonads and adrenal gland, disruption of cholesterol biosynthesis may potentially contribute to the known anti-androgenic effects of linuron in reducing androgen synthesis, in particular if similar down-regulation of the cholesterol biosynthesis pathway is also occurring in those organs. To our knowledge the impact of linuron on cholesterol biosynthesis in the testis has not been investigated, although Ornostay et al. [23] report down-regulation of a number of transcripts encoding cholesterol biosynthesis enzymes in fathead minnow ovarian cell cultures exposed to linuron.

Androgen-signalling is also important in regulating other aspects of lipid metabolism, including through SREBP-1 and the nuclear liver X receptor (LXR) [35, 40]. We found significant up-regulation of SREBP-1 in the 250 µg linuron/L treatment group, but a less consistent response of known SREBP-1 regulated genes. However, a number of GO terms relating to wider lipid and fatty acid metabolic processes were also enriched. This is consistent we the results of previous studies that have reported alteration of the metabolism and transport of lipids to be among the most common processes regulated by androgens and anti-androgens in fish [25].

Stress response

Functional analysis revealed that the other major change in transcript expression following linuron exposure was related to cellular response to stress and chemical stimulus.

Transcripts encoding CYP1A were the most up-regulated (340-560 fold) in the 250 µg linuron/L treatment, and they were also significantly up-regulated in fish exposed to 2.5 µg linuron/L (4.1-6.7 fold). In addition, there were increasing trends in the expression of *cyp1a* in fish exposed to 25 µg linuron/L (4.6-8.5 fold), albeit not significantly. CYP1A is a primary phase 1 biotransformation enzyme involved in the detoxification or metabolic activation of a number of xenobiotics, as well as many endogenous compounds. It is amongst the most readily induced cellular proteins, and it is primarily regulated via aryl hydrocarbon receptor (AhR) signalling. Planar aromatic hydrocarbons, including PCBs and PAHs, are known to be the strongest agonists of the AhR, and CYP1A induction (gene and protein) has been extensively

used in ecotoxicology as a marker of exposure to these environmental contaminants [41]. Amongst commonly used pesticides, linuron was reported to be one of the most potent activators of AhR *in vitro* and also been shown to strongly induce CYP1A gene expression in mouse liver, and this was attributed to a structural feature, a dichlorophenyl residue [42]. In fish, CYP1A was induced by, and metabolised, linuron in Japanese eel liver [43]. Little is known about the toxicity of the products of linuron metabolism in fish or mammals. Some compounds are detoxified by CYP1A activity while others, such as PAHs, are bioactivated. The latter can generate highly reactive intermediates that subsequently induce cellular damage, including genotoxicity and carcinogenesis. CYP1A transcription can also be regulated by hormones, directly or indirectly via upsteam signalling pathways, which can affect the biological effects of xenobiotics. Androgens are known to inhibit CYP1A expression in mammals, and this is suggested to occur via AR-AhR interactions [44]. In addition to classical regulation via direct AhR activation, it is therefore possible than the anti-androgenic effects of linuron contributed to this large induction of CYP1A, either through antagonism of the AR and/or the reduction in androgen production which has been previously reported in mammalian studies.

Protein folding was the most significantly enriched GO Biological Process in the list of differentially expressed transcripts following exposure to 250 µg linuron/L, reflecting a consistent up-regulation of a number of transcripts encoding molecular chaperones which can bind and stabilise damaged proteins. The chaperonin containing CTP-1 complex (CCT) is made up of eight primary subunits, five of which (*cct2*, *cct3, cct4, cct5, cct6a , cct8*) were significantly up-regulated in the 250 µg linuron/L treatment group (by 2.3-3.6 fold). Additionally, there were trends towards increased expression of these transcripts in the lower treatment groups. CCT participates in normal protein metabolism by folding many proteins, particularly actin and tubulin, but has also been shown to be induced in response to stress, aiding cellular recovery. Examples include response induction by chemical stress in mammalian cells [45] and temperature stress in fish [46]. Transcripts encoding several heat shock proteins (HSPs) were also strongly up-regulated in the high treatment group (*hsp90aa1.2, hsp90ab1, hspa4a, hspa8, dnaja1l, dnaja4, dnajb1a, dnajb1b*). HSPs are probably the most well-known stress-inducible molecular chaperones, and have been extensively reported to respond to various

environmental stressors in fish, including many pesticides. HSP90 proteins are also known to have a role in chaperoning CYP1A [41]. This strong, and consistent, up-regulation of molecular chaperones suggests than linuron induces protein-damaging cellular stress.

A suite of transcripts encoding glutathione-related antioxidant enzymes (*gpx1b, gstal, gsto1, mgst1, mgst3*) and glutathione reductase (*gsr*) were up-regulated in fish exposed to 250 µg linuron/L, and the majority of these also showed increasing trends in expression in the lower treatment groups. Furthermore, two members of the nuclear factor erythroid-derived 2-like family (*nfe2l1b, nfe2l2a*) were also significantly up-regulated in the high treatment group. These transcription factors play a key role in regulating the response of the antioxidant system [47]. This suggests that linuron generates oxidative stress, through reactivity of the parent compound or metabolites produced by CYP1A. This is a specific mechanism of toxicity, but is common to many chemicals, particularly at high concentrations. Existing evidence linking linuron with generation of oxidative stress in fish, and other species, is scarce. However, other phenylurea-based pesticides with a similar chemical structure, including Diuron, have been shown to induce oxidative stress, and cellular damage [e.g. 48, 49]. Oxidative stress has been linked to pathological changes in the liver, including necrosis, apoptosis and carcinogenesis, and linuron exposure was previously found to cause lesions and a range of adverse effects on cellular components in the liver of rainbow trout exposed to concentrations of 30 µg/L and above for five weeks [50]. Although we only found significant differences in the regulation of these stress-responsive processes in fish exposed to 250 µg/L linuron, there was also evidence of similar trends occurring in the lower treatment groups.

Overall, using RNA-seq we have demonstrated that linuron induces considerable transcriptional changes in the liver of mature male brown trout. We found evidence of a striking down-regulation of a majority of the enzymes involved in the cholesterol biosynthesis pathway, likely via SREBP-2 regulation which was itself down-regulated. We hypothesise that the anti-androgenic activity of linuron is the likely mechanism responsible for this effect. This also suggests a novel mechanism of toxicity that might potentially be associated with other environmental anti-androgens. We also found differential regulation of a number of transcripts involved in cellular

stress response. In particular, these included a considerable up-regulation of CYP1A, up-regulation of molecular chaperones that bind and stabilise damaged proteins, and a number of enzymes involved in the antioxidant system. Although we generally only found evidence of significant changes in the regulation of these transcripts in fish exposed to the highest concentration of linuron (250 µg/L), we found trends towards similar changes in fish exposed to 2.5 µg linuron/L, which is representative of concentrations measured in the most contaminated surface waters. This highlights the potential for adverse impacts of linuron on the health of fish, and other species subject to environmental exposure.

Acknowledgements

We wish to thank Jan Shears for help with the fish husbandry, Ronny van Aerle for advice on the bioinformatics, and Audrey Farbos, Karen Moore and Konrad Paszkiewicz for facilitating the sequencing experiments. We also thank Charles Tyler, Rod Wilson, Erin Reardon and Jo Rabineau for critical discussions and contributions towards setting up the fish exposure experiments. This work was funded by a Natural Environment Research Council CASE PhD studentship (Grant number NE/I528326/1) and the Salmon & Trout Association.

Supporting Information

The supporting information contains: supplemental experimental details, a heatmap illustrating fold changes in transcript expression between treatments (Figure S1), MDS plots illustrating expression profiles for all treatments (Figure S2), ERCC spike-in control analysis (Figure S3, Table S1), enriched Gene Ontology terms and Kegg pathways (Table S2) and all differentially expressed transcripts (Table S3).

References

1. Arnaud, L.; Taillandier, G.; Kaouadji, M.; Ravanel, P.; Tissut, M., Photosynthesis inhibition by phenylureas: A QSAR approach. *Ecotoxicology and Environmental Safety* **1994,** *28*, (2), 121-133.

2. Schuler, L. J.; Rand, G. M., Aquatic risk assessment of herbicides in freshwater ecosystems of south Florida. *Archives of Environmental Contamination and Toxicology* **2008,** *54*, (4), 571-583.

3. Patterson, M. *Linuron- Analysis of Risks to Endangered and Threatened Salmon and Steelhead* 2004.

4. US EPA, Linuron R.E.D. Facts. *Prevention, Pesticdes and Toxic Substances (7508W)* **1995**.

5. Health Canada, Proposed Re-evaluation Decision PRVD2012-02, Linuron. **2012**.

6. Bauer, E. R.; Meyer, H. H.; Sauerwein, H.; Stahlschmidt-Allner, P., Application of an androgen receptor assay for the characterisation of the androgenic or antiandrogenic activity of various phenylurea herbicides and their derivatives. *Analyst* **1998,** *123*, (12), 2485-2487.

7. Wilson, V. S.; Cardon, M. C.; Gray, L. E.; Hartig, P. C., Competitive binding comparison of endocrine disrupting compounds to recombinant androgen receptor from fathead minnow, rainbow trout, and human. *Environmental Toxicology and Chemistry* **2007,** *26*, (9), 1793-1802.

8. Orton, F.; Lutz, I.; Kloas, W.; Routledge, E. J., Endocrine disrupting effects of herbicides and pentachlorophenol: in vitro and in vivo evidence. *Environmental Science & Technology* **2009,** *43*, (6), 2144-2150.

9. Freyberger, A.; Weimer, M.; Tran, H.-S.; Ahr, H.-J., Assessment of a recombinant androgen receptor binding assay: Initial steps towards validation. *Reproductive Toxicology* **2010,** *30*, (1), 2-8.

10. Kojima, H.; Katsura, E.; Takeuchi, S.; Niiyama, K.; Kobayashi, K., Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environmental Health Perspectives* **2004,** *112*, (5), 524-529.

11. Lambright, C.; Ostby, J.; Bobseine, K.; Wilson, V.; Hotchkiss, A.; Mann, P.; Gray, L., Cellular and molecular mechanisms of action of linuron: an antiandrogenic

herbicide that produces reproductive malformations in male rats. *Toxicological Sciences* **2000,** *56*, (2), 389-399.

12. Kang, I. H.; Sik Kim, H.; Shin, J.-H.; Kim, T. S.; Moon, H. J.; Kim, I. Y.; Choi, K. S.; Kil, K. S.; Park, Y. I.; Dong, M. S., Comparison of anti-androgenic activity of flutamide, vinclozolin, procymidone, linuron, and DDE in rodent 10-day Hershberger assay. *Toxicology* **2004,** *199*, (2), 145-159.

13. Freyberger, A.; Schladt, L., Evaluation of the rodent Hershberger bioassay on intact juvenile males testing of coded chemicals and supplementary biochemical investigations. *Toxicology* **2009,** *262*, (2), 114-120.

14. Pottinger, T.; Katsiadaki, I.; Jolly, C.; Sanders, M.; Mayer, I.; Scott, A.; Morris, S.; Kortenkamp, A.; Scholze, M., Anti-androgens act jointly in suppressing spiggin concentrations in androgen-primed female three-spined sticklebacks-prediction of combined effects by concentration addition. *Aquatic Toxicology* **2013,** *140*, 145-156.

15. Jolly, C.; Katsiadaki, I.; Morris, S.; Le Belle, N.; Dufour, S.; Mayer, I.; Pottinger, T. G.; Scott, A. P., Detection of the anti-androgenic effect of endocrine disrupting environmental contaminants using in vivo and in vitro assays in the three-spined stickleback. *Aquatic Toxicology* **2009,** *92*, (4), 228-239.

16. Hogan, N. S.; Gallant, M. J.; van den Heuvel, M. R., Exposure to the pesticide linuron affects androgen-dependent gene expression in the three-spined stickleback (Gasterosteus aculeatus). *Environmental Toxicology and Chemistry* **2012,** *31*, (6), 1391-1395.

17. Katsiadaki, I.; Morris, S.; Squires, C.; Hurst, M. R.; James, J. D.; Scott, A. P., Use of the three-spined stickleback (Gasterosteus aculeatus) as a sensitive in vivo test for detection of environmental antiandrogens. *Environmental Health Perspectives* **2006,** *114*, (S-1), 115-121.

18. Cook, J.; Mullin, L.; Frame, S.; Biegel, L., Investigation of a mechanism for Leydig cell tumorigenesis by linuron in rats. *Toxicology and Applied Pharmacology* **1993,** *119*, (2), 195-204.

19. Hotchkiss, A.; Parks-Saldutti, L.; Ostby, J.; Lambright, C.; Furr, J.; Vandenbergh, J.; Gray, L., A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. *Biology of Reproduction* **2004,** *71*, (6), 1852-1861.

20. Wilson, V. S.; Lambright, C. R.; Furr, J. R.; Howdeshell, K. L., The herbicide linuron reduces testosterone production from the fetal rat testis during both in utero and in vitro exposures. *Toxicology Letters* **2009,** *186*, (2), 73-77.

21. Marlatt, V. L.; Lo, B. P.; Ornostay, A.; Hogan, N. S.; Kennedy, C. J.; Elphick, J. R.; Martyniuk, C. J., The effects of the urea-based herbicide linuron on reproductive endpoints in the fathead minnow *Pimephales promelas*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **2012,** *157*, 24-32.

22. Schiller, V.; Wichmann, A.; Kriehuber, R.; Schafers, C.; Fischer, R.; Fenske, M., Transcriptome alterations in zebrafish embryos after exposure to environmental estrogens and anti-androgens can reveal endocrine disruption. *Reproductive Toxicology* **2013,** *42*, 210-223.

23. Ornostay, A.; Cowie, A. M.; Hindle, M.; Baker, C. J.; Martyniuk, C. J., Classifying chemical mode of action using gene networks and machine learning: A case study with the herbicide linuron. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* **2013,** *8*, (4), 263-274.

24. Martyniuk, C. J.; Alvarez, S.; Lo, B. P.; Elphick, J. R.; Marlatt, V. L., Hepatic protein expression networks associated with masculinization in the female fathead minnow (Pimephales promelas). *Journal of Proteome Research* **2012,** *11*, (8), 4147-4161.

25. Martyniuk, C. J.; Denslow, N. D., Exploring androgen-regulated pathways in teleost fish using transcriptomics and proteomics. *Integrative and Comparative Biology* **2012,** *52*, (5), 695-704.

26. Uren Webster, T. M.; Shears, J. A.; Santos, E. M., Identification of conserved hepatic transcriptomic responses to estrogen in brown trout using a High Throughput Sequencing approach. *Submitted*.

27. Grabherr, M. G.; Haas, B. J.; Yassour, M.; Levin, J. Z.; Thompson, D. A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q., Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **2011,** *29*, (7), 644-652.

28. Langmead, B.; Salzberg, S. L., Fast gapped-read alignment with Bowtie 2. *Nature Methods* **2012,** *9*, 357-359.

29. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R., The sequence alignment/map format and SAMtools. *Bioinformatics* **2009,** *25*, (16), 2078-2079.

30. Robinson, M. D.; McCarthy, D. J.; Smyth, G. K., edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010,** *26*, (1), 139-140.

31. Huang, D. W.; Sherman, B. T.; Lempicki, R. A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **2008,** *4*, (1), 44-57.

32. Ikonen, E., Cellular cholesterol trafficking and compartmentalization. *Nature reviews Molecular cell biology* **2008,** *9*, (2), 125-138.

33. Sharpe, L. J.; Brown, A. J., Controlling cholesterol synthesis beyond 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). *Journal of Biological Chemistry* **2013,** *288*, (26), 18707-18715.

34. Bengoechea-Alonso, M. T.; Ericsson, J., SREBP in signal transduction: cholesterol metabolism and beyond. *Current Opinion in Cell Biology* **2007,** *19*, (2), 215-222.

35. Swinnen, J. V.; Ulrix, W.; Heyns, W.; Verhoeven, G., Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins. *Proceedings of the National Academy of Sciences* **1997,** *94*, (24), 12975-12980.

36. Heemers, H.; Maes, B.; Foufelle, F.; Heyns, W.; Verhoeven, G.; Swinnen, J. V., Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway. *Molecular Endocrinology* **2001,** *15*, (10), 1817-1828.

37. Heemers, H.; Vanderhoydonc, F.; Roskams, T.; Shechter, I.; Heyns, W.; Verhoeven, G.; Swinnen, J. V., Androgens stimulate coordinated lipogenic gene expression in normal target tissues in vivo. *Molecular and Cellular Endocrinology* **2003,** *205*, (1), 21-31.

38. Costigan, S. L.; Werner, J.; Ouellet, J. D.; Hill, L. G.; Law, R. D., Expression profiling and gene ontology analysis in fathead minnow (Pimephales promelas) liver following exposure to pulp and paper mill effluents. *Aquatic Toxicology* **2012,** *122*, 44-55.

39. Johnson, K. J.; McDowell, E. N.; Viereck, M. P.; Xia, J. Q., Species-specific dibutyl phthalate fetal testis endocrine disruption correlates with inhibition of

SREBP2-dependent gene expression pathways. *Toxicological Sciences* **2011,** *120*, (2), 460-474.

40. Krycer, J. R.; Brown, A. J., Cross-talk between the Androgen Receptor and the Liver X Receptor implications for cholesterol homeostasis. *Journal of Biological Chemistry* **2011,** *286*, (23), 20637-20647.

41. Bucheli, T. D.; Fent, K., Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology* **1995,** *25*, (3), 201-268.

42. Takeuchi, S.; Iida, M.; Yabushita, H.; Matsuda, T.; Kojima, H., In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron. *Chemosphere* **2008,** *74*, (1), 155-165.

43. Uno, T.; Kaji, S.; Goto, T.; Imaishi, H.; Nakamura, M.; Kanamaru, K.; Yamagata, H.; Kaminishi, Y.; Itakura, T., Metabolism of the herbicides chlorotoluron, diuron, linuron, simazine, and atrazine by CYP1A9 and CYP1C1 from Japanese eel (Anguilla japonica). *Pesticide Biochemistry and Physiology* **2011,** *101*, (2), 93-102.

44. Monostory, K.; Pascussi, J.-M.; Kabori, L.; Dvorak, Z., Hormonal regulation of CYP1A expression. *Drug Metabolism Reviews* **2009,** *41*, (4), 547-572.

45. Yokota, S. i.; Yanagi, H.; Yura, T.; Kubota, H., Upregulation of cytosolic chaperonin CCT subunits during recovery from chemical stress that causes accumulation of unfolded proteins. *European Journal of Biochemistry* **2000,** *267*, (6), 1658-1664.

46. Buckley, B. A.; Gracey, A. Y.; Somero, G. N., The cellular response to heat stress in the goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis. *Journal of Experimental Biology* **2006,** *209*, (14), 2660-2677.

47. Kobayashi, M.; Yamamoto, M., Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxidants & redox signaling* **2005,** *7*, (3-4), 385-394.

48. Ihlaseh, S. M.; Bailey, K. A.; Hester, S. D.; Jones, C.; Ren, H.; Cardoso, A. P. F.; Oliveira, M. L. C.; Wolf, D. C.; de Camargo, J. L. V., Transcriptional profile of diuron-induced toxicity on the urinary bladder of male Wistar rats to inform mode of action. *Toxicological Sciences* **2011,** *122*, (2), 330-338.

49. Akcha, F.; Spagnol, C.; Rouxel, J., Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos. *Aquatic Toxicology* **2012,** *106*, 104-113.
50. Oulmi, Y.; Negele, R.; Braunbeck, T., Cytopathology of liver and kidney in rainbow trout Oncorhynchus mykiss after long-term exposure to sublethal concentrations of linuron. *Diseases of Aquatic Organisms* **1995,** *21*, 35-35.

Figure Legends

Figure 1. Venn diagrams displaying the numbers of differentially expressed transcripts (FDR<0.1) in each treatment group obtained using EdgeR. Red and green numbers represent up- and down-regulated transcripts, respectively.

Figure 2. Heatmap illustrating the expression level of all differentially-regulated transcripts in all individual samples. Individual fish are represented by the following codes: c1, c2 and c3 represent the control individuals; ll1, ll2 and ll3 represent individuals exposed to 2.5 µg linuron/L; ml1, ml2 and ml3 represent individuals exposed to 25 µg linuron/L; hl1, hl2 and hl3 represent individuals exposed to 250 µg linuron/L. Data presented are log10 transformed read counts per transcript. The hierarchical clustering to generate gene and condition trees was conducted using an Euclidean distance metric using the pheatmap package in R.

Figure 3. Schematic illustrating the cholesterol biosynthesis pathway. Green surrounding boxes indicate differential transcript expression of enzymes. Each colour-coded bar represents mean transcript expression fold change in each treatment compared to the control (left to right: 2.5, 25, 250 µg/L), asterisks signify significant down-regulation (FDR <0.1).
ACAT2 (acetyl-CoA acetyltransferase 2), *HMGCS* (3-hydroxy-3-methylglutaryl-Coenzyme A synthase), *HMGCR* (3-hydroxy-3-methylglutaryl-Coenzyme A reductase), *MVK* (mevalonate kinase), *PMVK* (phosphomevalonate kinase), *MVD* (mevalonate decarboxylase), *IDI1* (isopentenyl-diphosphate delta isomerase 1), *FDPS* (farnesyl diphosphate synthase), *GGPS1* (geranylgeranyl diphosphate synthase 1), *FDFT1* (farnesyl-diphosphate farnesyltransferase 1), *SQLE* (squalene epoxidase), *LSS* (lanosterol synthase), *CYP51A1* (cytochrome P450,family 51), *TM7SF2* (transmembrane 7 superfamily member 2), *SC4MOL* (methylsterol monooxygenase 1), *NSDHL* (NAD(P) dependent steroid dehydrogenase-like), *HSD17B7* (hydroxysteroid (17-beta) dehydrogenase 7), *EBP* (sterol isomerase) , *SC5D* (sterol C5 desaturase), *DHCR7* (7-dehydrocholesterol reductase), *DHCR24* (24-dehydrocholesterol reductase).

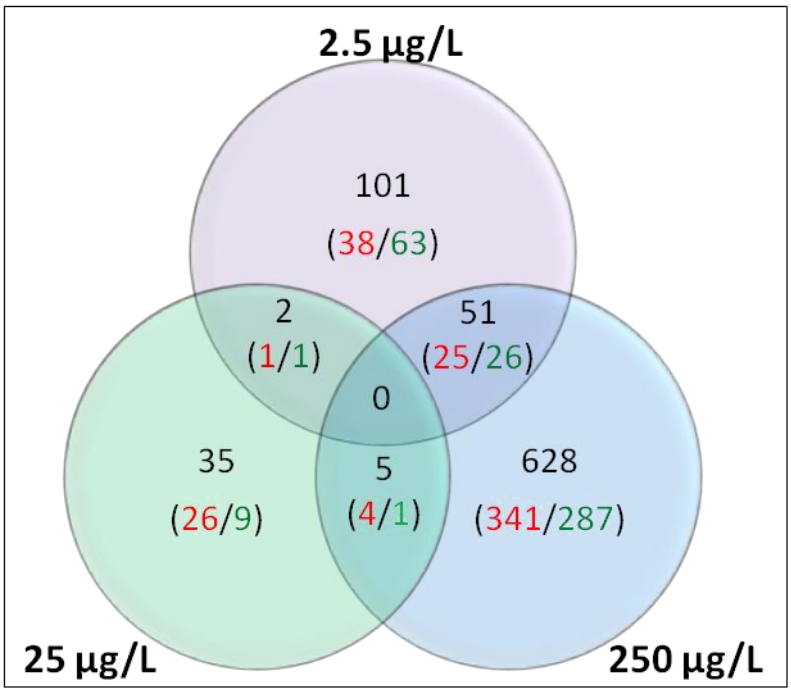

2.5 µg/L
101
(38/63)
2
(1/1)
51
(25/26)
0
35
(26/9)
5
(4/1)
628
(341/287)
25 µg/L
250 µg/L

Figure 1

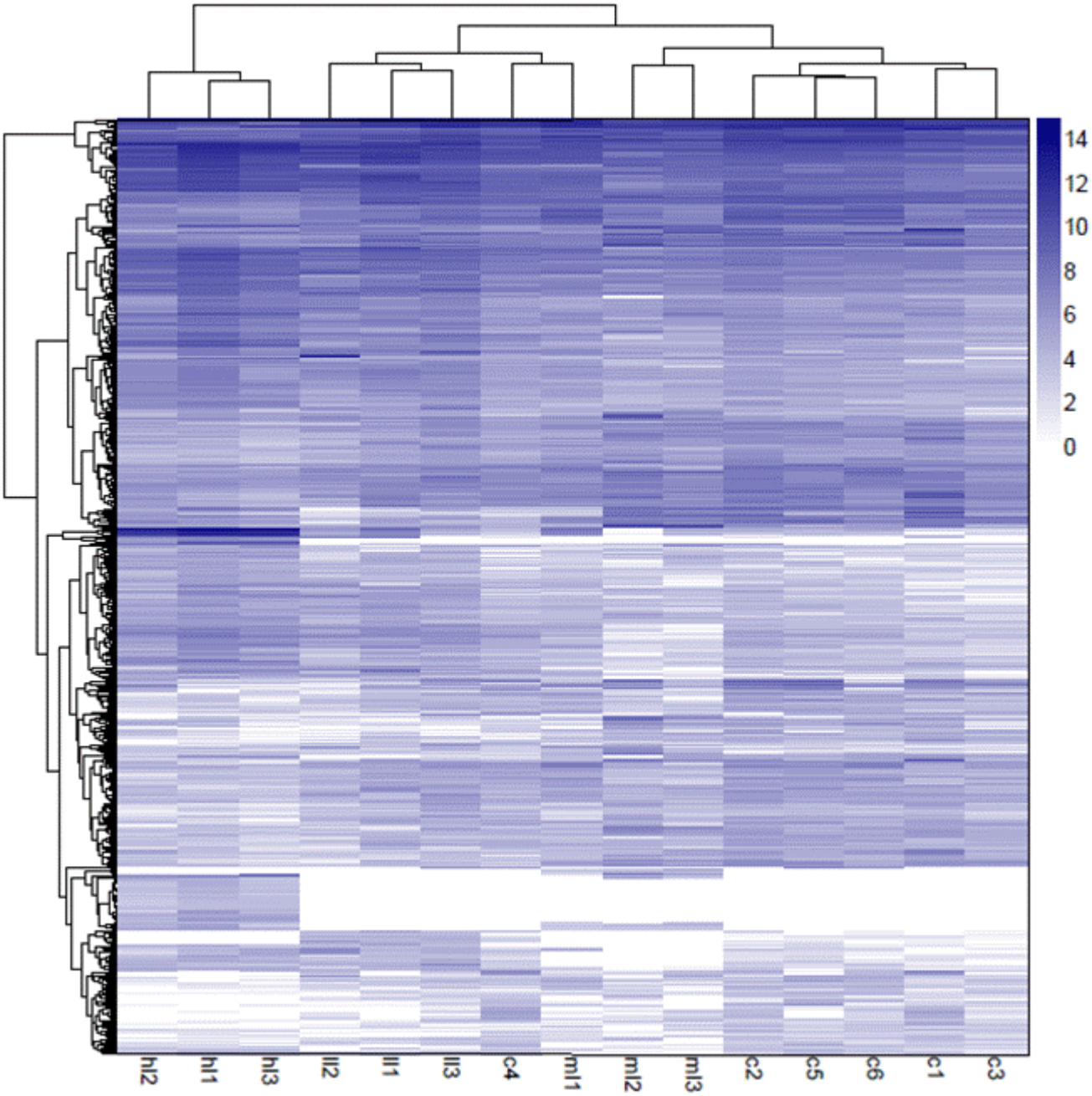

14
12
10
8
6
4
2
0
hl2
hl1
hl3
ll2
ll1
ll3
c4
ml1
ml2
ml3
c2
c5
c6
c1
c3

Figure 2

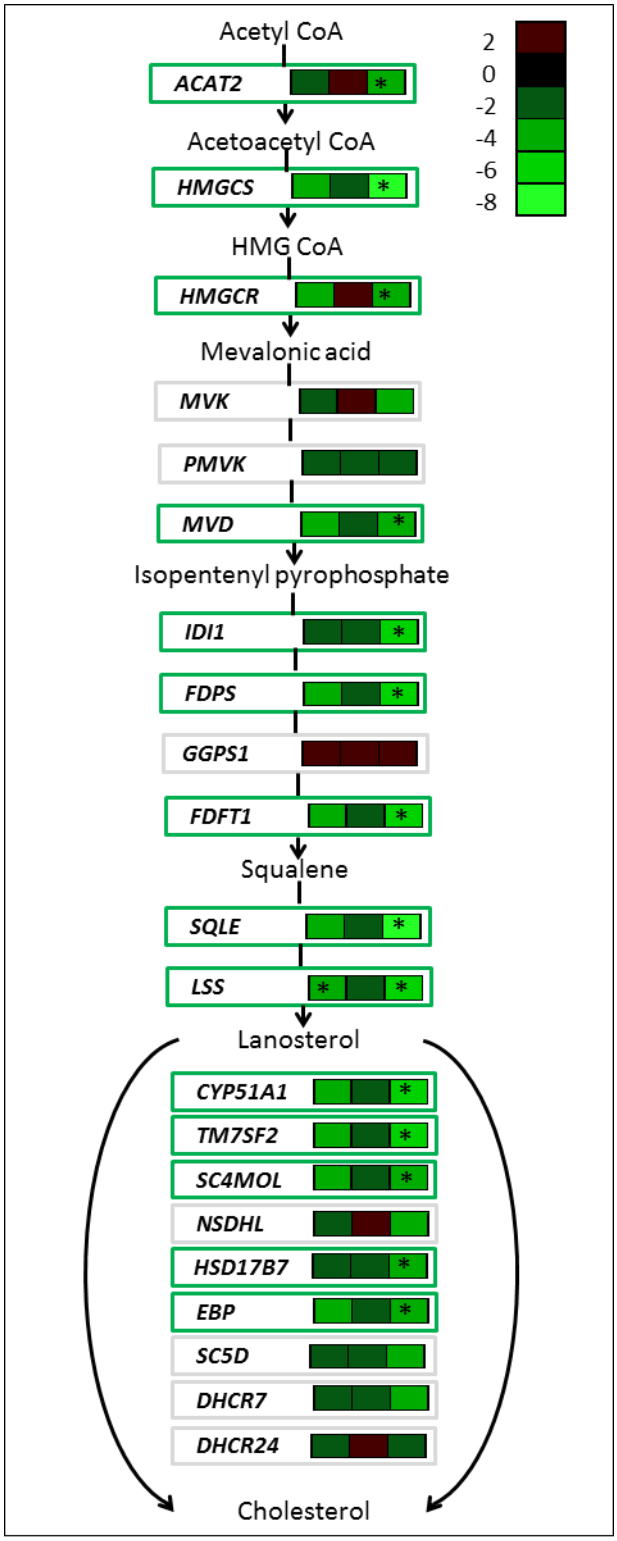

Acetyl CoA
ACAT2
Acetoacetyl CoA
HMGCS
HMG CoA
HMGCR
Mevalonic acid
MVK
PMVK
MVD
Isopentenyl pyrophosphate
IDI1
FDPS
GGPS1
FDFT1
Squalene
SQLE
LSS
Lanosterol
CYP51A1
TM7SF2
SC4MOL
NSDHL
HSD17B7
EBP
SC5D
DHCR7
DHCR24
Cholesterol
2
0
-2
-4
-6
-8

Figure 3

Supporting Information

Global transcriptome profiling reveals down-regulation of cholesterol biosynthesis and up-regulation of cellular stress response in brown trout (*Salmo trutta*) exposed to linuron

Tamsyn M. Uren Webster and Eduarda M. Santos

Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

This Supporting Information contains:

Supplemental Experimental Information

Fish husbandry

Sexually mature male brown trout (2 years old; originating from a local aquaculture facility) were maintained in 215 L tanks, and acclimated to laboratory conditions for three weeks prior to exposure. Each tank was aerated, supplied with 430 L/day of de-chlorinated tap water, and maintained at 12 ± 0.2 °C, pH 7.5. Fish were kept under a 16:8 h light:dark cycle (with 30 minute dawn/dusk transitional periods) and fed with pellet feed (8 mm, Biomar, Grangemouth, UK) at a rate of 2% body weight per day.

RNA extraction, library preparation and sequencing

Transcript profiling was conducted in the livers of 3 fish per treatment group. RNA was extracted from livers using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions, then treated with DNase on RNeasy Mini extraction columns (Qiagen). The concentration, purity and integrity of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). All RNA input to library construction was of high quality with 260/280 and 260/230 ratios > 1.8 and RIN scores > 8. ERCC spike-in control mixes (Ambion) were added to all individual RNA samples, according to the manufacturer's instructions. cDNA libraries from all 15 samples were then prepared using the Illumina TruSeq RNA Sample Preparation kit, multiplexed with 24 samples per lane (together with samples from another project) and sequenced using an Illumina HiSeq 2500 to generate 100 bp paired-end reads, according to the manufacturer's instructions.

Transcriptome Assembly and Annotation

To maximise sequence coverage depth and assemble an optimised male liver transcriptome for brown trout, sequence reads from all samples from the current project were combined with those from another project, also employing mature male brown trout as a model organism [1]. All analyses were carried out on a local server running under the NEBC Bio-Linux 7 environment [2]. Contaminating Illumina adaptor sequences were removed and the first 12 bp of all raw sequence reads were trimmed to remove 5' bias caused by random hexamer priming [3] using the FASTX-

Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). 3' sliding window quality trimming was performed (http://wiki.bioinformatics.ucdavis.edu/index.php/Trim.slidingWindow.pl) and all reads where < 90% bases had a Phred quality score >20, and those shorter than 15 bp, were discarded. Digital normalisation was performed to remove highly duplicated reads using the normalize-by-median.py script part of the khmer package described by Brown et al. [4], with the recommended k-mer value of 20 and a coverage threshold of 200. This process reduces the computer memory requirements of transcriptome assembly, and also reduces the risk of potential sequencing error accumulation in abundant transcripts [5]. All retained reads were then paired, separated into forward and reverse fastq files before *de novo* transcriptome assembly using Trinity (version r2013-02-25; [6], using the default parameters and specifying a minimum contig length of 200 bp). All transcripts were annotated using Blastx against Ensembl peptide databases (Release 71; April 2013) using an e-value cut off < $1e^{-15}$ and assigned in the following preferential order; zebrafish (*Danio rerio*); human (*Homo sapiens*) and mouse (*Mus musculus*); stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*), tilapia (*Oreochromis niloticus*) and cod (*Gadus morhua*). Additional annotation of previously un-annotated differentially expressed transcripts was performed using Blast (< $1e^{-15}$) against refseq, nr and nt databases.

Transcriptomic Analysis

Raw sequence reads from individual samples were mapped back against the assembled transcripts using Bowtie2 (version 2.1.0, [7]), using the -k 1 parameter to report a single best hit for each read and limit ambiguous mapping to redundant transcripts. Raw count data for each transcript was extracted using idxstats in samtools (version 0.1.18, [8]) and input into edgeR [9] for differential expression analysis. In edgeR, a criteria of at least 1 count from 3 samples was imposed and tagwise dispersion was applied with the recommended prior.df =10. Pairwise comparisons were initially conducted between the two control groups to ensure that our analysis did not identify differential expression as a result of random variation between groups. Following this initial analysis, pairwise comparisons were conducted between the six individual fish from the combined control groups and 3 individuals from each of the other treatment groups. Transcripts were considered differentially expressed with a FDR < 0.1 (Benjamini-Hochberg correction).

Hierarchical clustering was performed on all differentially expressed transcripts using an Euclidean distance metric, in the Pheatmap package for R. Functional analysis was then performed for differentially expressed genes from each treatment using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.7; [10]), with our brown trout male liver transcriptome as a background. Kegg pathways and Gene Ontology (GO) terms for Biological Process, Cellular Component and Molecular Function were considered significantly over-represented when $P < 0.05$. Canonical pathway analysis and network analysis was conducted using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com) based on the list of differentially expressed transcripts.

References

1. Uren Webster, T. M.; Shears, J. A.; Santos, E. M., Identification of conserved hepatic transcriptomic responses to estrogen in brown trout using a High Throughput Sequencing approach. *Submitted*.
2. Field, D.; Tiwari, B.; Booth, T.; Houten, S.; Swan, D.; Bertrand, N.; Thurston, M., Open software for biologists: from famine to feast. *Nature Biotechnology* 2006, *24*, (7), 801-804.
3. Hansen, K. D.; Brenner, S. E.; Dudoit, S., Biases in Illumina transcriptome sequencing caused by random hexamer priming. *Nucleic Acids Research* 2010, *38*, (12), e131.
4. Brown, C. T.; Howe, A.; Zhang, Q.; Pyrkosz, A. B.; Brom, T. H., A reference-free algorithm for computational normalization of shotgun sequencing data. *arXiv:1203.4802 [q-bio.GN]* 2012.
5. Francis, W. R.; Christianson, L. M.; Kiko, R.; Powers, M. L.; Shaner, N. C.; Haddock, S. H., A comparison across non-model animals suggests an optimal sequencing depth for de novo transcriptome assembly. *BMC Genomics* 2013, *14*, (1), 167.
6. Grabherr, M. G.; Haas, B. J.; Yassour, M.; Levin, J. Z.; Thompson, D. A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q., Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* 2011, *29*, (7), 644-652.
7. Langmead, B.; Salzberg, S. L., Fast gapped-read alignment with Bowtie 2. *Nature Methods* 2012, *9*, 357-359.
8. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R., The sequence alignment/map format and SAMtools. *Bioinformatics* 2009, *25*, (16), 2078-2079.
9. Robinson, M. D.; McCarthy, D. J.; Smyth, G. K., edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 2010, *26*, (1), 139-140.
10. Huang, D. W.; Sherman, B. T.; Lempicki, R. A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* 2008, *4*, (1), 44-57.

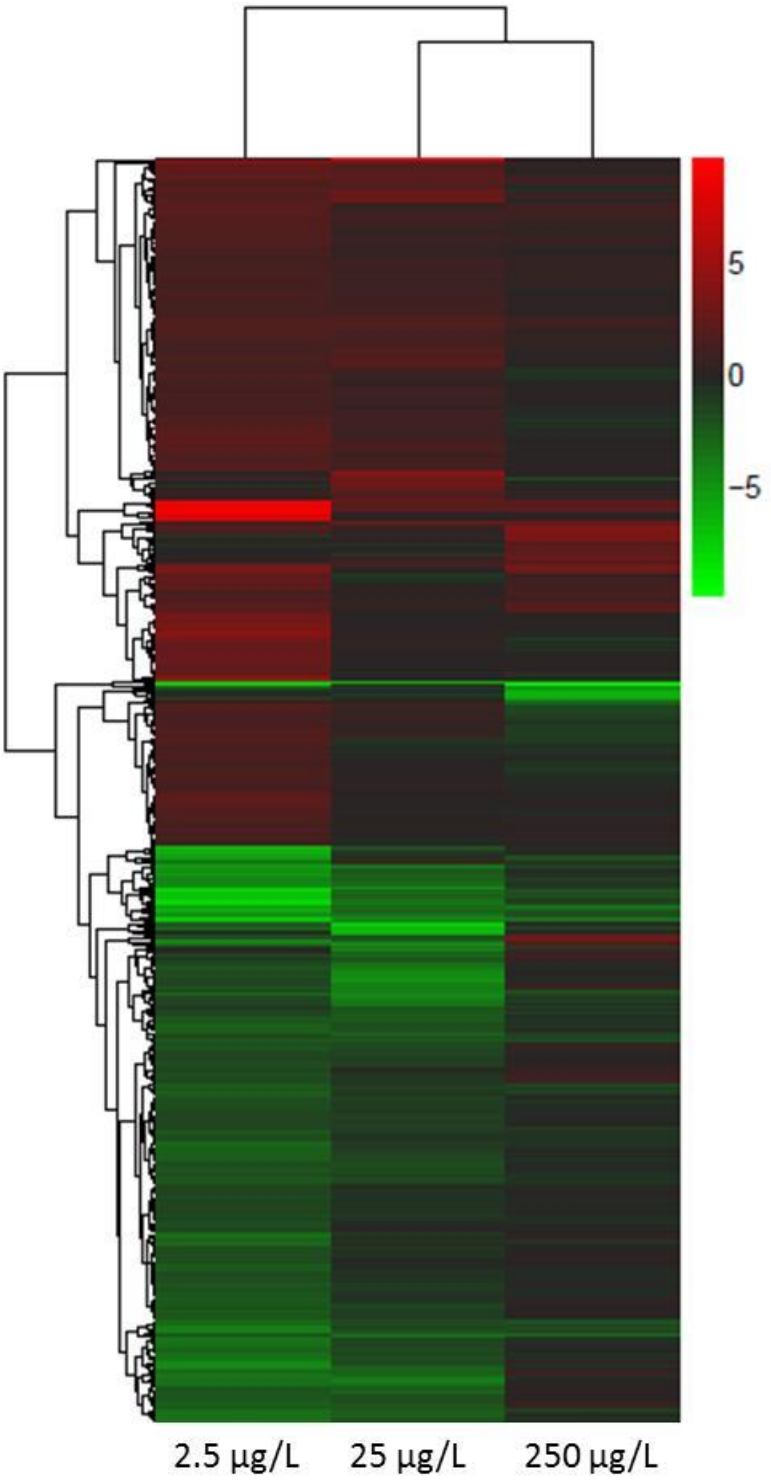


Figure S1. Heatmap illustrating changes in transcript expression for all differentially expressed transcripts across treatments, compared to the control group. Data presented are the mean log2 fold change in expression level in each treatment group compared to the control. Hierarchical trees were generated using an Euclidean distance metric, using the pheatmap package in R.

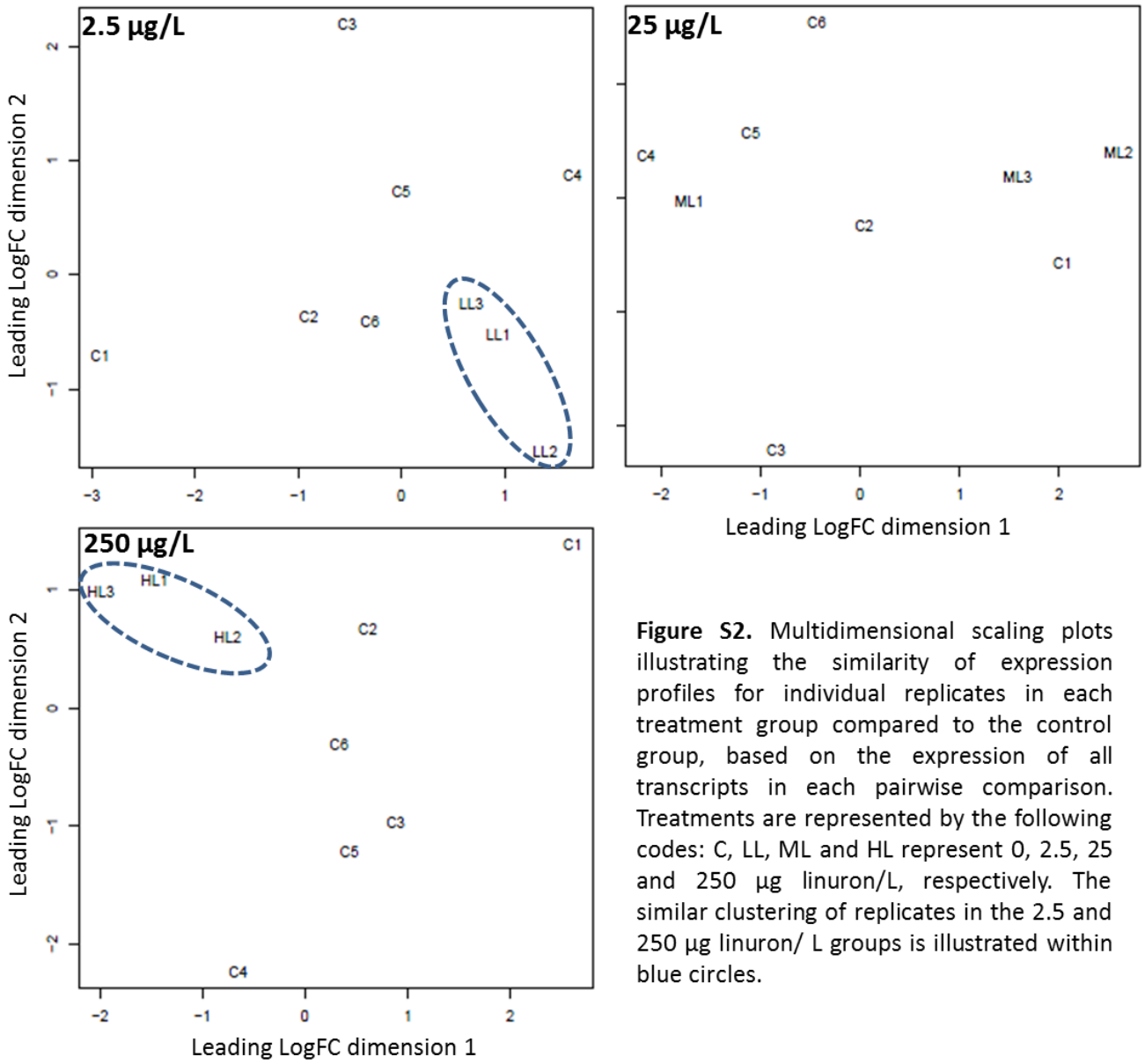


Figure S2. Multidimensional scaling plots illustrating the similarity of expression profiles for individual replicates in each treatment group compared to the control group, based on the expression of all transcripts in each pairwise comparison. Treatments are represented by the following codes: C, LL, ML and HL represent 0, 2.5, 25 and 250 µg linuron/L, respectively. The similar clustering of replicates in the 2.5 and 250 µg linuron/ L groups is illustrated within blue circles.

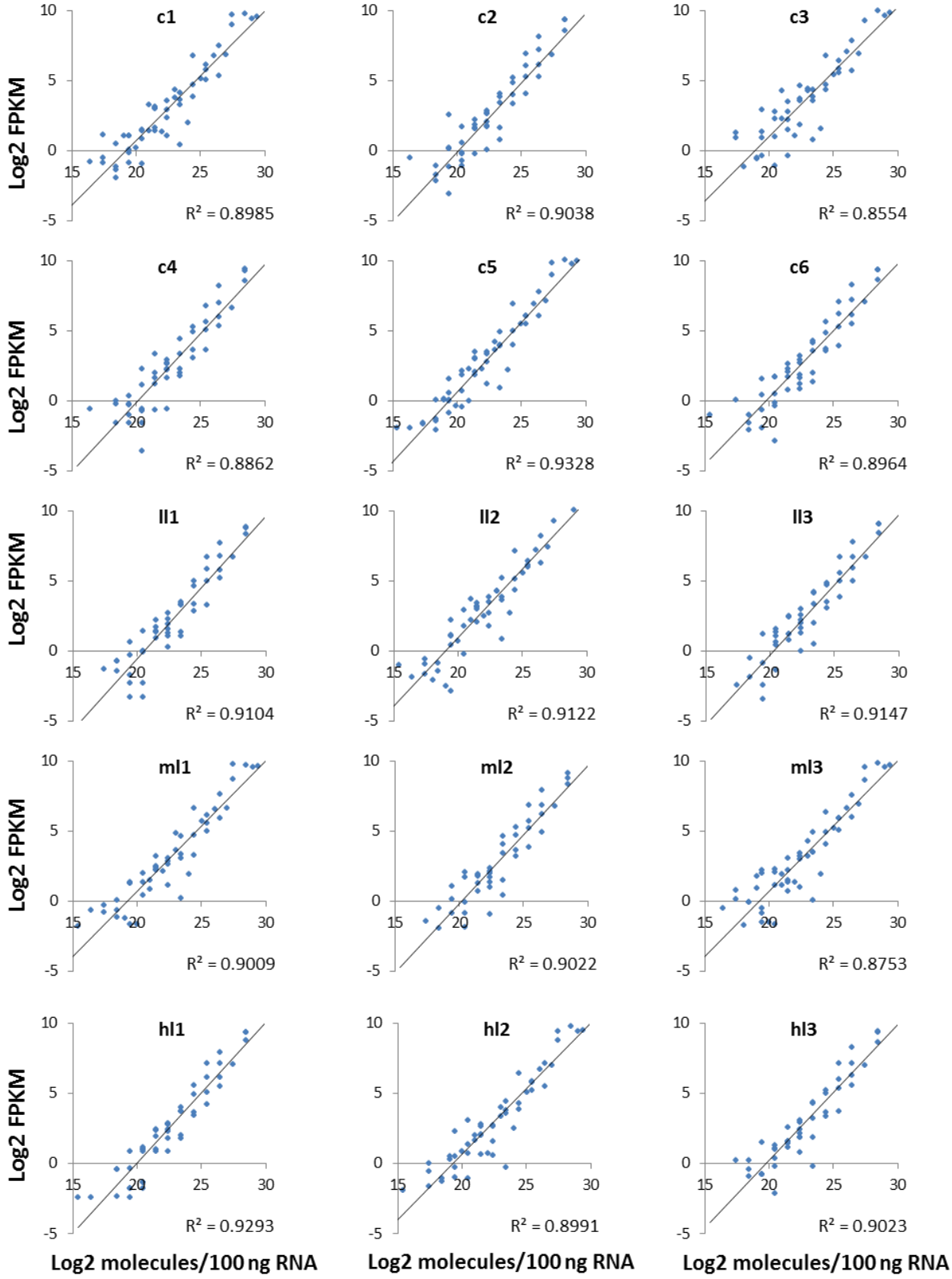


Figure S3. External RNA Controls Consortium **(**ERCC) spike-in control analysis for all individual liver samples sequenced in this project. Graphs show the relationship between the calculated expression level (FPKM) and the expected concentration of each control transcripts.

Table S1. Dynamic range in transcript expression profiles for all individual samples included in this study, calculated based on the measurements for ERCC spike-in controls. Values presented are log2 transformed maximum-minimum FPKM values calculated for ERCC spike-in control transcripts. Only transcripts that had at least 1 mapped read in a minimum of 3 replicate samples were included in the analysis.

Sample	R^2	Log2 dynamic range (FPKM)
c1	0.90	15.00
c2	0.90	14.14
c3	0.86	14.52
c4	0.89	14.58
c5	0.93	15.00
c6	0.90	14.03
ll1	0.91	14.03
ll2	0.91	16.21
ll3	0.91	14.23
ml1	0.90	14.55
ml2	0.90	12.94
ml3	0.88	14.47
hl1	0.93	13.52
hl2	0.90	14.93
hl3	0.90	13.29

Table S2. Gene Ontology Terms and Kegg Pathways over-represented in the lists of differentially expressed transcripts following exposure to 250 µg Linuron/L. Values presented are the number of transcripts associated with each term, the P-values and the adjusted P-values associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.[10] using the *de novo* assembled liver transcriptome generated in this study as a background.

GOTERM_BP_FAT	**Count**	**P-Value**	**FDR**
protein folding	14	1.90E-08	2.70E-05
lipid biosynthetic process	10	3.10E-05	4.30E-02
isoprenoid biosynthetic process	5	6.60E-05	9.10E-02
oxidation reduction	17	1.90E-03	2.60E+00
sterol metabolic process	4	3.00E-03	4.00E+00
fatty acid metabolic process	5	5.40E-03	7.30E+00
fatty acid biosynthetic process	4	7.90E-03	1.00E+01
organic acid biosynthetic process	5	2.10E-02	2.50E+01
carboxylic acid biosynthetic process	5	2.10E-02	2.50E+01
response to abiotic stimulus	5	2.20E-02	2.70E+01
steroid metabolic process	4	3.40E-02	3.80E+01
response to temperature stimulus	3	4.00E-02	4.30E+01
GOTERM_MF_FAT	**Count**	**P-Value**	**FDR**
unfolded protein binding	12	1.40E-09	1.70E-06
adenyl nucleotide binding	29	1.50E-03	1.90E+00
purine nucleoside binding	29	1.60E-03	2.00E+00
nucleoside binding	29	1.70E-03	2.10E+00
cofactor binding	9	1.20E-02	1.40E+01
iron ion binding	9	1.30E-02	1.50E+01
adenyl ribonucleotide binding	25	1.30E-02	1.60E+01
purine nucleotide binding	30	2.00E-02	2.30E+01
ATP binding	24	2.40E-02	2.60E+01
coenzyme binding	7	2.50E-02	2.80E+01
nucleotide binding	34	3.40E-02	3.60E+01
tetrapyrrole binding	5	4.10E-02	4.20E+01
FAD binding	4	4.50E-02	4.40E+01
KEGG_PATHWAY	**Count**	**P-Value**	**FDR**
Steroid biosynthesis	7	2.30E-07	2.40E-04
Terpenoid backbone biosynthesis	6	3.20E-06	3.30E-03
Glutathione metabolism	5	2.20E-03	2.20E+00
Butanoate metabolism	4	1.30E-02	1.30E+01
Metabolism of xenobiotics by cytochrome P450	3	4.00E-02	3.40E+01

Table S3. List of all differentially expressed transcripts calculated in EdgeR (FDR <0.05). Values presented are Log2 transformed fold changes for each treatment group compared to the control group. Red shading indicates significant up-regulation and green shading represents significant down-regulation.

Name	Symbol	Database	Log FC 2.5 µg Linuron/L	Log FC 25 µg Linuron/L	Log FC 250 µg Linuron/L
cyp1a	ENSDARG00000026039	Ensembl	0.00	0.00	9.62
cyp1a	ENSDARG00000026039	Ensembl	2.74	3.09	9.13
NM_001123687.1	NM_001123687.1	refseq	1.86	1.79	9.01
cyp1a	ENSDARG00000026039	Ensembl	2.05	2.57	8.71
cyp1a	ENSDARG00000026039	Ensembl	2.04	2.38	8.66
cyp1a	ENSDARG00000026039	Ensembl	2.28	2.58	8.65
cyp1a	ENSDARG00000026039	Ensembl	2.19	2.21	8.52
cyp1a	ENSDARG00000026039	Ensembl	2.11	2.25	8.42
AF059710.1	AF059710.1	nt	0.00	0.00	7.93
AAD14035.1	AAD14035.1	nt	0.00	0.00	7.87
NM_001123687.1	NM_001123687.1	refseq	0.00	0.00	7.87
AF059710.1	AF059710.1	nt	0.00	0.00	7.59
MR1	ENSG00000153029	Ensembl	0.00	4.22	5.67
hsp90aa1.2	ENSDARG00000024746	Ensembl	0.00	0.00	5.55
XP_706145.2	XP_706145.2	refseq	5.09	0.00	4.96
hsp90aa1.2	ENSDARG00000024746	Ensembl	0.00	0.60	4.83
hsp90aa1.2	ENSDARG00000024746	Ensembl	-1.92	0.55	4.54
NM_001140847.1	NM_001140847.1	refseq	0.00	0.00	4.47
CR356234.11	CR356234.11	nt	0.00	2.74	4.37
NM_001140204.1	NM_001140204.1	refseq	0.00	3.81	4.18
clu	ENSDARG00000010434	Ensembl	0.66	-0.44	4.17
si:dkey-205h13.1	ENSDARG00000079307	Ensembl	0.00	0.00	4.13
si:dkey-251i10.2	ENSDARG00000093957	Ensembl	0.00	0.00	4.13
EU025716.1	EU025716.1	nt	0.00	0.00	3.82
ENSGACG00000006364	ENSGACG00000006364	Ensembl	1.70	3.33	3.80
XP_003201149.1	XP_003201149.1	refseq	0.00	0.00	3.65
si:dkey-251i10.2	ENSDARG00000093957	Ensembl	0.00	0.00	3.64
clu	ENSDARG00000010434	Ensembl	0.31	-0.47	3.62
EU025715.1	EU025715.1	nt	0.00	0.00	3.59
NP_001020682.1	NP_001020682.1	refseq	3.62	0.00	3.57
NM_001173702.1	NM_001173702.1	refseq	0.00	0.00	3.56
CRTC1	ENSDARG00000076068	Ensembl	0.00	2.83	3.49
clu	ENSDARG00000010434	Ensembl	0.34	-0.52	3.41
sb:cb252	ENSDARG00000058206	Ensembl	1.80	0.37	3.40
FBF1	ENSDARG00000090482	Ensembl	0.00	0.00	3.38
mgst3	ENSDARG00000024143	Ensembl	0.71	-1.06	3.34
BT057266.1	BT057266.1	nt	0.71	-0.52	3.34
DNAJA4	ENSDARG00000051762	Ensembl	0.00	0.00	3.34
nckap5l	ENSDARG00000079148	Ensembl	0.00	0.00	3.30
map2	ENSDARG00000055052	Ensembl	0.00	0.00	3.27
AF281332.1	AF281332.1	nt	0.00	0.00	3.26
HM159473.1	HM159473.1	nt	0.00	0.00	3.25
CRTC1	ENSDARG00000076068	Ensembl	0.00	0.00	3.22
sc:d173	ENSDARG00000079175	Ensembl	0.68	-0.35	3.00
GU129140.1	GU129140.1	nt	0.25	0.01	2.93
ep300b	ENSDARG00000061108	Ensembl	0.00	0.00	2.87
gpx1b	ENSDARG00000006207	Ensembl	0.19	1.17	2.81
CU693369.1	ENSDARG00000089534	Ensembl	2.62	0.79	2.78
NM_001146651.1	NM_001146651.1	refseq	0.34	1.21	2.76
NP_001135042.1	NP_001135042.1	refseq	0.43	-0.58	2.75
BT059328.1	BT059328.1	nt	-0.81	1.23	2.70
C1orf50	ENSG00000164008	Ensembl	0.41	-0.15	2.69
si:dkey-205h13.1	ENSDARG00000079307	Ensembl	0.00	0.00	2.68
BT059075.1	BT059075.1	nt	0.00	0.00	2.67
hspa8	ENSDARG00000068992	Ensembl	-0.19	0.82	2.65
GU129139.1	GU129139.1	nt	0.00	0.00	2.61
FJ969488.1	FJ969488.1	nt	0.00	1.36	2.61
C1orf50	ENSG00000164008	Ensembl	-0.44	-0.62	2.61
EU481821.1	EU481821.1	nt	-0.10	-0.45	2.59
ENSORLG00000005110	ENSORLG00000005110	Ensembl	1.96	0.27	2.59
nrf1	ENSDARG00000000018	Ensembl	0.00	0.00	2.58
si:dkey-205h13.1	ENSDARG00000079307	Ensembl	0.00	0.00	2.53
dnaja1l	ENSDARG00000030972	Ensembl	-0.05	1.00	2.52
NP_001135042.1	NP_001135042.1	refseq	0.14	-0.54	2.52
ethe1	ENSDARG00000005713	Ensembl	0.97	-1.80	2.52
scdb	ENSDARG00000030265	Ensembl	-0.63	1.39	2.51
EU621899.1	EU621899.1	nt	0.00	1.95	2.51
cyp7a1a	ENSDARG00000069018	Ensembl	2.46	-0.57	2.50
psph	ENSDARG00000040314	Ensembl	1.72	0.00	2.49
psph	ENSDARG00000040314	Ensembl	1.93	-0.62	2.48
hspa8	ENSDARG00000068992	Ensembl	-0.16	0.96	2.47
PLCE1	ENSDARG00000087921	Ensembl	2.13	0.67	2.45
CU693369.1	ENSDARG00000089534	Ensembl	2.28	0.19	2.44
cactin	ENSDARG00000059866	Ensembl	0.00	0.00	2.43
slc48a1b	ENSDARG00000026109	Ensembl	1.83	0.54	2.41
FJ969489.1	FJ969489.1	nt	1.48	0.90	2.41
rx3	ENSDARG00000052893	Ensembl	1.07	0.93	2.39
pnisr	ENSDARG00000069855	Ensembl	0.00	0.00	2.38
XP_002932246.1	XP_002932246.1	refseq	1.05	-0.24	2.36
BT072073.1	BT072073.1	nt	0.44	2.09	2.33
ddx21	ENSDARG00000063626	Ensembl	1.48	0.23	2.32
gda	ENSDARG00000002986	Ensembl	0.03	0.05	2.31
aldh1l1	ENSDARG00000077004	Ensembl	1.16	0.00	2.30
Gm13150	ENSMUSG00000086147	Ensembl	0.00	0.00	2.30
aldh1l1	ENSDARG00000077004	Ensembl	0.00	0.00	2.29

GU129139.1	GU129139.1	nt	0.74	-0.20	2.27
BT044623.1	BT044623.1	nt	0.57	0.98	2.24
FJ969489.1	FJ969489.1	nt	0.00	0.00	2.23
XP_706145.2	XP_706145.2	refseq	3.06	0.00	2.23
setd6	ENSDARG00000043986	Ensembl	0.63	1.57	2.21
BT045302.1	BT045302.1	nt	-0.79	-0.80	2.20
CR388231.2	ENSDARG00000086979	Ensembl	0.75	0.00	2.20
AY567793.3	AY567793.3	nt	0.00	0.00	2.20
dnaja1l	ENSDARG00000030972	Ensembl	-1.13	0.46	2.20
FJ969489.1	FJ969489.1	nt	1.83	1.41	2.17
EU221180.1	EU221180.1	nt	0.00	0.00	2.16
ZNF518B	ENSDARG00000090832	Ensembl	1.18	0.37	2.15
si:dkey-200l5.2	ENSDARG00000039351	Ensembl	2.59	-0.10	2.15
XP_002938110.1	XP_002938110.1	refseq	0.27	-0.19	2.15
wdr46	ENSDARG00000078396	Ensembl	0.65	1.78	2.14
XP_706145.2	XP_706145.2	refseq	3.49	0.00	2.14
dnajb1b	ENSDARG00000041394	Ensembl	0.00	0.00	2.12
GU129139.1	GU129139.1	nt	1.29	-0.19	2.11
BT059229.1	BT059229.1	nt	1.67	0.00	2.10
uba1	ENSDARG00000037559	Ensembl	0.00	1.60	2.09
gsr	ENSDARG00000019236	Ensembl	0.34	0.35	2.09
akr1b1	ENSDARG00000006215	Ensembl	0.81	-0.37	2.07
BT072394.1	BT072394.1	nt	1.00	0.18	2.06
EU621898.1	EU621898.1	nt	1.13	0.03	2.05
FJ969488.1	FJ969488.1	nt	0.75	-0.40	2.04
NP_001117141.1	NP_001117141.1	refseq	-0.82	0.68	2.03
keap1a	ENSDARG00000016132	Ensembl	0.51	1.16	2.02
fam65a	ENSDARG00000062178	Ensembl	1.52	0.00	2.01
ptplad1	ENSDARG00000016038	Ensembl	0.20	0.11	2.00
ethe1	ENSDARG00000005713	Ensembl	0.64	-1.12	2.00
sema4ab	ENSDARG00000062352	Ensembl	1.44	-0.46	2.00
BT045302.1	BT045302.1	nt	-0.54	-0.90	1.99
BT072108.1	BT072108.1	nt	0.81	0.57	1.99
NP_001133507.1	NP_001133507.1	refseq	1.70	0.70	1.99
pno1	ENSDARG00000008502	Ensembl	1.62	0.87	1.98
gda	ENSDARG00000002986	Ensembl	-0.25	-0.26	1.96
serpinh1b	ENSDARG00000019949	Ensembl	0.94	-0.40	1.95
slc23a1	ENSDARG00000015033	Ensembl	0.00	1.89	1.95
BT047801.1	BT047801.1	nt	-0.79	0.17	1.94
GQ505860.1	GQ505860.1	nt	0.40	4.48	1.93
ENSGACG00000002729	ENSGACG00000002729	Ensembl	0.20	1.91	1.93
sqstm1	ENSDARG00000075014	Ensembl	0.76	0.65	1.92
gtpbp4	ENSDARG00000018961	Ensembl	1.42	0.60	1.92
serpinh1b	ENSDARG00000019949	Ensembl	0.88	-0.80	1.92
ACI33792.1	ACI33792.1	nt	2.14	-0.35	1.91
GIN1	ENSG00000263031	Ensembl	0.58	1.36	1.91
herc3	ENSDARG00000075887	Ensembl	0.75	-0.56	1.91
BT049839.1	BT049839.1	nt	0.36	0.59	1.91
sema4ab	ENSDARG00000062352	Ensembl	1.41	-0.27	1.91
nelfa	ENSDARG00000061411	Ensembl	1.03	0.19	1.91
XM_003446407.1	XM_003446407.1	refseq	0.23	-0.27	1.90
ACN60262.1	ACN60262.1	nt	1.69	0.79	1.89
U58910.1	U58910.1	nt	0.65	2.04	1.89
NP_001180427.1	NP_001180427.1	refseq	-0.48	-1.11	1.88
cct5	ENSDARG00000045399	Ensembl	0.98	0.51	1.88
ARMC3	ENSG00000165309	Ensembl	0.62	-1.19	1.88
pno1	ENSDARG00000008502	Ensembl	1.70	1.00	1.88
gsr	ENSDARG00000019236	Ensembl	0.33	-0.13	1.88
fen1	ENSDARG00000011404	Ensembl	0.24	-0.04	1.87
cct3	ENSDARG00000016173	Ensembl	1.21	1.42	1.87
SLC7A11	ENSG00000151012	Ensembl	2.40	0.87	1.86
uckl1a	ENSDARG00000001686	Ensembl	1.16	0.17	1.86
XP_001922616.3	XP_001922616.3	refseq	1.16	0.13	1.86
WAPAL	ENSDARG00000029768	Ensembl	0.87	-1.60	1.86
uckl1a	ENSDARG00000001686	Ensembl	1.16	0.11	1.85
sgk2a	ENSDARG00000063370	Ensembl	1.08	-0.61	1.85
cct6a	ENSDARG00000021252	Ensembl	0.69	0.59	1.85
BT044047.1	BT044047.1	nt	1.52	-0.51	1.84
BT059080.1	BT059080.1	nt	2.02	0.97	1.82
ankrd12	ENSDARG00000052419	Ensembl	1.95	0.75	1.81
HQ287747.1	HQ287747.1	nt	0.24	-0.28	1.81
aldh1l1	ENSDARG00000077004	Ensembl	0.76	0.05	1.80
BT059080.1	BT059080.1	nt	2.20	1.02	1.80
nmd3	ENSDARG00000015676	Ensembl	1.56	0.82	1.79
cct8	ENSDARG00000008243	Ensembl	0.63	0.75	1.78
SAMD9L	ENSG00000177409	Ensembl	2.85	-2.12	1.78
ENSGACG00000011316	ENSGACG00000011316	Ensembl	1.43	-0.41	1.77
ITIH3	ENSG00000162267	Ensembl	0.94	0.44	1.76
zgc:65997	ENSDARG00000043562	Ensembl	0.77	-1.81	1.76
ENSGACG00000016589	ENSGACG00000016589	Ensembl	0.54	0.17	1.76
ENSGMOG00000014668	ENSGMOG00000014668	Ensembl	-0.09	-0.39	1.76
hsp90ab1	ENSDARG00000029150	Ensembl	1.15	0.32	1.76
XP_706145.2	XP_706145.2	refseq	2.70	0.87	1.75
SMARCA1	ENSDARG00000012776	Ensembl	1.17	-0.90	1.74
ITIH3	ENSG00000162267	Ensembl	0.98	0.60	1.74
gsto1	ENSDARG00000022183	Ensembl	0.45	-0.38	1.73
crp1	ENSDARG00000071454	Ensembl	1.35	0.49	1.73
ENSONIG00000020058	ENSONIG00000020058	Ensembl	1.49	0.33	1.73
bhlhe40	ENSDARG00000004060	Ensembl	1.23	-0.42	1.73
ABLIM2	ENSG00000163995	Ensembl	0.49	0.60	1.71
ENSONIG00000020396	ENSONIG00000020396	Ensembl	1.42	0.58	1.71
ENSONIG00000020746	ENSONIG00000020746	Ensembl	-0.77	-0.71	1.71
WAPAL	ENSDARG00000029768	Ensembl	0.56	-0.72	1.71
ENSORLG00000007209	ENSORLG00000007209	Ensembl	0.47	-0.01	1.70
FJ969490.1	FJ969490.1	nt	-0.91	-1.44	1.70
NM_001146452.1	NM_001146452.1	refseq	-0.36	-0.50	1.70
tp53	ENSDARG00000035559	Ensembl	1.27	0.42	1.70
XP_706145.2	XP_706145.2	refseq	3.02	0.67	1.70

cct3	ENSDARG00000016173	Ensembl	1.12	0.87	1.69
CR792429.5	CR792429.5	nt	0.46	-0.32	1.67
srebf1	ENSDARG00000067607	Ensembl	-0.11	0.01	1.67
dnaja1l	ENSDARG00000030972	Ensembl	0.57	0.61	1.66
BT044711.1	BT044711.1	nt	0.16	0.46	1.66
eif3s10	ENSDARG00000076815	Ensembl	1.36	0.40	1.66
nfe2l2a	ENSDARG00000042824	Ensembl	0.61	0.05	1.65
fam177a1	ENSDARG00000079636	Ensembl	0.66	0.88	1.65
clu	ENSDARG00000010434	Ensembl	-0.27	-0.66	1.65
rel	ENSDARG00000055276	Ensembl	1.83	-0.31	1.64
cyp2j20	ENSDARG00000094057	Ensembl	0.13	-0.79	1.64
eif4a1b	ENSDARG00000003032	Ensembl	1.57	0.98	1.64
aldh1l1	ENSDARG00000077004	Ensembl	0.77	-1.02	1.63
BT057949.1	BT057949.1	nt	0.88	0.78	1.63
BT072610.1	BT072610.1	nt	1.62	0.02	1.63
AY819642.1	AY819642.1	nt	0.88	-2.38	1.62
EU481821.1	EU481821.1	nt	0.19	-0.58	1.62
XM_003458967.1	XM_003458967.1	refseq	0.30	-0.29	1.62
si:ch211-250g4.3	ENSDARG00000092945	Ensembl	-0.16	-1.34	1.61
gamt	ENSDARG00000070844	Ensembl	1.22	-0.48	1.61
ITIH3	ENSG00000162267	Ensembl	0.84	0.63	1.60
fkbp4	ENSDARG00000008447	Ensembl	-0.02	-0.01	1.60
tp53	ENSDARG00000035559	Ensembl	1.00	0.10	1.60
crp1	ENSDARG00000071454	Ensembl	1.45	0.57	1.60
cct5	ENSDARG00000045399	Ensembl	0.63	0.58	1.60
gsto1	ENSDARG00000022183	Ensembl	0.27	-0.38	1.59
ENSGACG00000002729	ENSGACG00000002729	Ensembl	0.45	4.02	1.57
ZNF462	ENSDARG00000063381	Ensembl	0.81	-0.02	1.57
cct3	ENSDARG00000016173	Ensembl	0.75	0.84	1.57
MGST1	ENSDARG00000022165	Ensembl	0.37	-0.80	1.56
MGST1	ENSDARG00000022165	Ensembl	0.62	-0.45	1.56
BT059352.1	BT059352.1	nt	1.00	-1.55	1.56
RTL1	ENSG00000254656	Ensembl	-0.02	0.44	1.56
cct3	ENSDARG00000016173	Ensembl	0.46	1.01	1.55
NM_001139753.1	NM_001139753.1	refseq	0.46	0.41	1.55
BT072448.1	BT072448.1	nt	-0.34	0.36	1.55
Itih3	ENSMUSG00000006522	Ensembl	0.97	0.51	1.55
FTH1	ENSG00000167996	Ensembl	0.31	0.23	1.55
klf11a	ENSDARG00000030844	Ensembl	1.91	0.34	1.55
cox4i2	ENSDARG00000022509	Ensembl	3.07	0.41	1.54
fen1	ENSDARG00000011404	Ensembl	0.02	-0.33	1.54
pir	ENSDARG00000056638	Ensembl	0.91	-0.31	1.54
gclc	ENSDARG00000013095	Ensembl	0.25	0.20	1.53
Itih3	ENSMUSG00000006522	Ensembl	0.74	0.19	1.53
me1	ENSDARG00000053215	Ensembl	0.59	-0.89	1.53
hexim1	ENSDARG00000036482	Ensembl	0.50	0.11	1.53
ITIH3	ENSG00000162267	Ensembl	0.83	0.24	1.52
gemin8	ENSDARG00000053496	Ensembl	0.95	0.21	1.52
BT072448.1	BT072448.1	nt	-0.02	0.47	1.52
Itih3	ENSMUSG00000006522	Ensembl	0.90	0.41	1.52
Itih3	ENSMUSG00000006522	Ensembl	0.97	0.54	1.52
NM_001140424.1	NM_001140424.1	refseq	-0.23	-0.39	1.52
GQ505860.1	GQ505860.1	nt	0.19	3.64	1.51
Itih3	ENSMUSG00000006522	Ensembl	0.87	0.44	1.51
BT060259.1	BT060259.1	nt	0.35	1.18	1.51
DQ246664.1	DQ246664.1	nt	1.22	-0.06	1.51
EU621899.1	EU621899.1	nt	0.43	0.32	1.51
cct2	ENSDARG00000041754	Ensembl	0.42	0.88	1.50
EU621899.1	EU621899.1	nt	-0.51	-0.57	1.50
agmo	ENSDARG00000025595	Ensembl	0.83	-0.66	1.50
GU129140.1	GU129140.1	nt	0.38	-0.23	1.50
agmo	ENSDARG00000025595	Ensembl	0.91	-0.66	1.50
FTH1	ENSG00000167996	Ensembl	0.33	0.19	1.49
tsku	ENSDARG00000040815	Ensembl	0.25	-0.13	1.49
aimp2	ENSDARG00000018903	Ensembl	1.60	-0.07	1.49
GU129139.1	GU129139.1	nt	0.83	-0.84	1.49
eif3s10	ENSDARG00000076815	Ensembl	1.26	0.58	1.49
me1	ENSDARG00000053215	Ensembl	0.74	-1.05	1.49
agxta	ENSDARG00000052099	Ensembl	0.84	-0.41	1.48
ITIH3	ENSG00000162267	Ensembl	0.79	0.21	1.48
eif3s10	ENSDARG00000076815	Ensembl	1.71	0.72	1.48
pabpc4	ENSDARG00000059259	Ensembl	0.41	1.03	1.48
Itih3	ENSMUSG00000006522	Ensembl	0.87	0.50	1.48
eif3s10	ENSDARG00000076815	Ensembl	1.29	0.59	1.47
serpinh1b	ENSDARG00000019949	Ensembl	0.59	-0.88	1.47
gstal	ENSDARG00000090228	Ensembl	-0.11	-0.55	1.47
SLC7A11	ENSG00000151012	Ensembl	1.95	0.08	1.46
BT044807.1	BT044807.1	nt	0.76	0.55	1.46
AC203446.12	AC203446.12	nt	0.53	0.03	1.46
aimp2	ENSDARG00000018903	Ensembl	1.66	0.08	1.46
Itih3	ENSMUSG00000006522	Ensembl	0.79	0.31	1.46
nmd3	ENSDARG00000015676	Ensembl	1.61	0.85	1.45
Itih3	ENSMUSG00000006522	Ensembl	0.75	0.13	1.44
Itih3	ENSMUSG00000006522	Ensembl	0.64	0.09	1.44
crp1	ENSDARG00000071454	Ensembl	1.30	0.52	1.43
atf5a	ENSDARG00000068096	Ensembl	2.14	0.09	1.43
GIN1	ENSG00000263031	Ensembl	0.06	0.26	1.43
BT050044.1	BT050044.1	nt	0.90	0.69	1.43
plod1a	ENSDARG00000059746	Ensembl	-0.39	-0.19	1.43
desi1a	ENSDARG00000033140	Ensembl	0.09	-0.21	1.43
pir	ENSDARG00000056638	Ensembl	0.94	-0.64	1.43
dnajb1a	ENSDARG00000015831	Ensembl	0.10	0.31	1.42
eif3s10	ENSDARG00000076815	Ensembl	1.08	0.57	1.42
nap1l1	ENSDARG00000002400	Ensembl	1.10	0.71	1.42
gstal	ENSDARG00000090228	Ensembl	-0.18	-0.63	1.41
adar	ENSDARG00000012389	Ensembl	0.92	-0.06	1.41
zgc:162356	ENSDARG00000042620	Ensembl	0.28	-0.48	1.41
spag1a	ENSDARG00000004017	Ensembl	1.04	0.25	1.40

sqstm1	ENSDARG00000075014	Ensembl	0.20	0.39	1.40
qdpra	ENSDARG00000040190	Ensembl	0.22	-0.57	1.40
prmt7	ENSDARG00000051902	Ensembl	0.11	1.50	1.39
ENSONIG00000015305	ENSONIG00000015305	Ensembl	-0.01	-0.71	1.39
PDCL3	ENSDARG00000009449	Ensembl	0.96	0.22	1.38
mhc1uba	ENSDARG00000075963	Ensembl	0.80	-0.63	1.36
itm2ba	ENSDARG00000007098	Ensembl	0.40	-0.37	1.36
eif3s10	ENSDARG00000076815	Ensembl	1.14	0.66	1.36
nap1l1	ENSDARG00000002400	Ensembl	0.91	0.46	1.35
cct4	ENSDARG00000013475	Ensembl	0.96	0.45	1.35
eif3s10	ENSDARG00000076815	Ensembl	1.60	0.44	1.35
agxta	ENSDARG00000052099	Ensembl	0.89	-0.64	1.35
eif3s10	ENSDARG00000076815	Ensembl	1.51	0.64	1.34
zgc:162356	ENSDARG00000042620	Ensembl	0.33	-0.44	1.34
nfe2l1b	ENSDARG00000076533	Ensembl	0.65	0.13	1.33
qdprb1	ENSDARG00000037378	Ensembl	0.78	0.15	1.33
sgut1	ENSDARG00000020608	Ensembl	0.23	0.08	1.33
slc51a	ENSDARG00000045306	Ensembl	0.87	-0.33	1.32
EU025708.1	EU025708.1	nt	-0.35	0.38	1.32
cbr1l	ENSDARG00000021149	Ensembl	0.30	-0.61	1.32
slc51a	ENSDARG00000045306	Ensembl	0.82	-0.19	1.31
GU129139.1	GU129139.1	nt	0.78	0.47	1.31
gclc	ENSDARG00000013095	Ensembl	0.07	0.11	1.31
ddx21	ENSDARG00000063626	Ensembl	1.20	0.31	1.31
naca	ENSDARG00000005513	Ensembl	1.08	0.42	1.29
tcp1	ENSDARG00000017891	Ensembl	0.45	0.72	1.29
aldh18a1	ENSDARG00000061123	Ensembl	2.46	0.00	1.29
tap1	ENSDARG00000079766	Ensembl	1.93	0.00	1.29
nmd3	ENSDARG00000015676	Ensembl	1.11	0.68	1.29
uroc1	ENSDARG00000070394	Ensembl	0.90	-0.12	1.29
gclm	ENSDARG00000018953	Ensembl	0.07	0.12	1.28
pebp1	ENSDARG00000042069	Ensembl	0.18	-0.36	1.27
BT072598.1	BT072598.1	nt	0.68	0.19	1.26
hspa8	ENSDARG00000068992	Ensembl	0.70	0.50	1.26
GQ505860.1	GQ505860.1	nt	0.34	3.91	1.26
Arpc1a	ENSMUSG00000029621	Ensembl	0.39	-0.03	1.26
GU129140.1	GU129140.1	nt	0.27	-0.33	1.26
eif3s10	ENSDARG00000076815	Ensembl	1.14	0.64	1.25
BT050401.2	BT050401.2	nt	1.14	0.33	1.25
tbcb	ENSDARG00000068404	Ensembl	-0.14	0.11	1.24
slc51a	ENSDARG00000045306	Ensembl	0.82	-0.56	1.24
lrrc47	ENSDARG00000053138	Ensembl	0.77	0.24	1.24
AC203446.12	AC203446.12	nt	0.31	-0.72	1.24
cct3	ENSDARG00000016173	Ensembl	0.70	0.09	1.23
naca	ENSDARG00000005513	Ensembl	0.97	0.36	1.23
eif3m	ENSDARG00000013931	Ensembl	1.04	0.01	1.22
btf3	ENSDARG00000035400	Ensembl	1.27	0.19	1.22
AHSG	ENSDARG00000069293	Ensembl	1.37	0.16	1.21
AHSG	ENSDARG00000069293	Ensembl	1.32	0.16	1.21
aqp10a	ENSDARG00000007086	Ensembl	0.95	-0.14	1.21
eif3d	ENSDARG00000021257	Ensembl	0.87	0.42	1.20
NAPSA	ENSDARG00000009313	Ensembl	0.78	0.16	1.19
tpt1	ENSDARG00000092693	Ensembl	0.66	0.18	1.19
eif3m	ENSDARG00000013931	Ensembl	0.99	0.14	1.19
NM_001124652.1	NM_001124652.1	refseq	0.43	-1.25	1.19
FM866399.1	FM866399.1	nt	0.93	-0.46	1.18
nap1l1	ENSDARG00000002400	Ensembl	0.88	0.25	1.18
cirbp	ENSDARG00000013351	Ensembl	1.10	0.29	1.17
XP_003457033.1	XP_003457033.1	refseq	0.18	0.17	1.17
AHSG	ENSDARG00000069293	Ensembl	1.32	0.13	1.17
hspa4a	ENSDARG00000004754	Ensembl	-0.01	0.83	1.16
qdprb1	ENSDARG00000037378	Ensembl	0.57	-0.08	1.16
spata5l1	ENSDARG00000061763	Ensembl	0.24	-0.70	1.16
ENSGACG00000002729	ENSGACG00000002729	Ensembl	0.37	3.69	1.15
eif3ba	ENSDARG00000059654	Ensembl	0.97	0.41	1.14
itga6a	ENSDARG00000042282	Ensembl	3.80	3.80	1.14
ddx21	ENSDARG00000063626	Ensembl	1.20	0.44	1.13
cyb5a	ENSDARG00000055643	Ensembl	0.03	0.13	1.06
NM_001139756.1	NM_001139756.1	refseq	0.58	0.44	1.05
GQ505860.1	GQ505860.1	nt	-0.14	3.35	1.02
CCD11006.1	CCD11006.1	nt	2.03	2.21	0.97
Ddit4l	ENSMUSG00000046818	Ensembl	2.20	0.79	0.79
mych	ENSDARG00000077473	Ensembl	2.46	1.83	0.73
TAP2	ENSG00000223481	Ensembl	1.65	-0.09	0.68
NM_001173879.1	NM_001173879.1	refseq	1.55	0.32	0.49
rhof	ENSDARG00000054389	Ensembl	0.86	2.70	0.46
slc1a4	ENSDARG00000000551	Ensembl	2.29	0.40	0.36
mgat4b	ENSDARG00000004115	Ensembl	0.10	2.05	0.30
itpka	ENSDARG00000042856	Ensembl	0.87	3.21	0.30
SAMD9	ENSG00000205413	Ensembl	0.54	-3.11	0.29
BT045925.1	BT045925.1	nt	1.64	0.60	0.26
slc25a38b	ENSDARG00000074533	Ensembl	-0.89	2.38	0.25
nop58	ENSDARG00000058337	Ensembl	0.76	1.80	0.24
GU552297.1	GU552297.1	nt	-3.20	-1.02	0.16
dnaja3b	ENSDARG00000095983	Ensembl	-0.40	1.72	0.07
mmp9	ENSDARG00000042816	Ensembl	-2.74	0.94	0.02
ENSGACG00000002729	ENSGACG00000002729	Ensembl	8.80	0.23	0.01
AB161471.1	AB161471.1	nt	0.00	2.60	0.00
AY100012.1	AY100012.1	nt	0.00	3.76	0.00
BT046750.2	BT046750.2	nt	2.59	0.00	0.00
ccnt2a	ENSDARG00000036685	Ensembl	0.00	2.57	0.00
EU481821.1	EU481821.1	nt	2.06	0.00	0.00
hif1ab	ENSDARG00000034293	Ensembl	0.00	2.61	0.00
hivep2	ENSDARG00000039987	Ensembl	3.88	0.00	0.00
mych	ENSDARG00000077473	Ensembl	0.98	3.31	0.00
NM_001124310.1	NM_001124310.1	refseq	5.37	0.00	0.00
NP_001155125.1	NP_001155125.1	refseq	4.32	0.00	0.00
slc1a5	ENSDARG00000090706	Ensembl	0.00	3.56	0.00

URGCP	ENSDARG00000078731	Ensembl	3.66	0.00	0.00
XP_706145.2	XP_706145.2	refseq	3.42	0.00	0.00
Nxpe3	ENSMUSG00000075033	Ensembl	-0.74	-6.26	-0.06
NR_030020.1	NR_030020.1	refseq	-0.57	2.85	-0.11
TDRD7B	ENSDARG00000077523	Ensembl	-0.34	-5.97	-0.14
MYLK4	ENSDARG00000091260	Ensembl	-2.61	-4.51	-0.28
hmox1	ENSDARG00000027529	Ensembl	0.92	4.19	-0.35
NM_001140457.1	NM_001140457.1	refseq	-4.49	-0.54	-0.36
IFI44L	ENSG00000137959	Ensembl	0.25	-2.98	-0.45
si:ch73-233k15.2	ENSDARG00000073718	Ensembl	0.95	2.20	-0.50
XM_003437881.1	XM_003437881.1	refseq	-6.63	-3.11	-0.67
BT072498.1	BT072498.1	nt	-0.48	2.32	-0.68
ENSGACG00000006364	ENSGACG00000006364	Ensembl	2.72	-0.17	-0.70
EU221180.1	EU221180.1	nt	-2.01	-1.46	-0.71
BT059271.1	BT059271.1	nt	-2.19	0.63	-0.73
ankrd16	ENSDARG00000003822	Ensembl	0.06	3.53	-1.00
lamb1b	ENSDARG00000045524	Ensembl	-2.91	-0.88	-1.19
nfil3-5	ENSDARG00000094965	Ensembl	-2.03	-0.26	-1.20
aldh7a1	ENSDARG00000018426	Ensembl	-1.15	0.24	-1.21
zp2.2	ENSDARG00000091737	Ensembl	-2.87	0.80	-1.22
FJ969490.1	FJ969490.1	nt	-1.45	-0.11	-1.22
KRTCAP2	ENSDARG00000070386	Ensembl	-0.81	-0.28	-1.24
ACI66788.1	ACI66788.1	nt	0.33	3.33	-1.24
pptc7a	ENSDARG00000011122	Ensembl	-0.51	-0.19	-1.24
bhmt	ENSDARG00000013430	Ensembl	-2.30	0.52	-1.25
hspg2	ENSDARG00000076564	Ensembl	-0.34	-6.01	-1.27
GQ505858.1	GQ505858.1	nt	-0.82	-0.48	-1.28
HM159472.1	HM159472.1	nt	-0.49	-4.83	-1.28
odc1	ENSDARG00000007377	Ensembl	-1.72	3.70	-1.30
NM_001139827.1	NM_001139827.1	refseq	-0.38	-0.13	-1.30
nqo1	ENSDARG00000010250	Ensembl	-0.80	-6.65	-1.32
cox6b1	ENSDARG00000045230	Ensembl	0.16	-0.01	-1.32
acat2	ENSDARG00000007127	Ensembl	-0.66	0.18	-1.33
mpc1	ENSDARG00000093448	Ensembl	-0.72	-0.28	-1.34
pnp5a	ENSDARG00000078619	Ensembl	-4.41	0.04	-1.34
cox6b1	ENSDARG00000045230	Ensembl	-0.08	-0.01	-1.34
sult2st2	ENSDARG00000053331	Ensembl	-0.78	-0.22	-1.36
errfi1	ENSDARG00000070171	Ensembl	-0.85	0.28	-1.37
apoa4	ENSDARG00000040298	Ensembl	-0.47	0.27	-1.38
EU481821.1	EU481821.1	nt	-7.01	-1.36	-1.39
NP_001134572.1	NP_001134572.1	refseq	-0.76	-0.15	-1.39
adck5	ENSDARG00000075405	Ensembl	-1.08	-0.99	-1.40
fkbp5	ENSDARG00000028396	Ensembl	-2.64	0.80	-1.41
srebf2	ENSDARG00000063438	Ensembl	-0.62	-0.09	-1.41
mib	ENSDARG00000087895	Ensembl	-0.86	-0.06	-1.41
bhmt	ENSDARG00000013430	Ensembl	-2.50	0.54	-1.43
GQ505860.1	GQ505860.1	nt	-0.99	0.21	-1.43
errfi1	ENSDARG00000070171	Ensembl	-0.81	0.27	-1.43
AJ313031.1	AJ313031.1	nt	-4.20	-0.81	-1.44
ostc	ENSDARG00000035838	Ensembl	-0.76	0.13	-1.44
AAX24812.2	AAX24812.2	nt	-1.07	-0.11	-1.45
gadd45gb	ENSDARG00000019417	Ensembl	-2.47	1.13	-1.46
tmed10	ENSDARG00000041391	Ensembl	-0.66	0.08	-1.46
EF427382.1	EF427382.1	nt	-1.09	0.09	-1.47
hsd17b7	ENSDARG00000091751	Ensembl	-0.66	-0.25	-1.48
zp2.2	ENSDARG00000091737	Ensembl	-3.79	0.67	-1.49
BT071857.1	BT071857.1	nt	-0.73	-0.13	-1.49
adssl1	ENSDARG00000009867	Ensembl	-2.30	-0.90	-1.50
msmo1	ENSDARG00000055876	Ensembl	-1.30	-0.35	-1.50
pnp5a	ENSDARG00000078619	Ensembl	-4.70	0.01	-1.51
BT058978.1	BT058978.1	nt	-0.79	0.95	-1.51
DQ246664.1	DQ246664.1	nt	-4.80	-0.30	-1.51
ENSONIG00000020746	ENSONIG00000020746	Ensembl	-1.12	-0.33	-1.51
BT071857.1	BT071857.1	nt	-0.65	-0.03	-1.52
hmgcra	ENSDARG00000052734	Ensembl	-1.12	0.00	-1.52
GABRB1	ENSDARG00000076127	Ensembl	-3.77	-0.42	-1.53
ACI66376.1	ACI66376.1	nt	-0.31	0.76	-1.54
lman1	ENSDARG00000069980	Ensembl	-0.74	-0.22	-1.55
errfi1	ENSDARG00000070171	Ensembl	-0.70	0.13	-1.55
pnp5a	ENSDARG00000078619	Ensembl	-4.80	-0.09	-1.56
GLDC	ENSDARG00000035120	Ensembl	-1.49	-0.11	-1.57
uba5	ENSDARG00000063588	Ensembl	-0.56	-0.13	-1.57
SBK1	ENSG00000188322	Ensembl	-3.47	-1.10	-1.58
ENSONIG00000014676	ENSONIG00000014676	Ensembl	-1.77	-1.38	-1.58
epb41l3b	ENSDARG00000019917	Ensembl	-1.26	-0.16	-1.58
pla2g12b	ENSDARG00000015662	Ensembl	0.11	0.38	-1.59
aifm1	ENSDARG00000058088	Ensembl	-1.05	-0.33	-1.59
BT056591.1	BT056591.1	nt	-2.27	-0.29	-1.59
rab1ba	ENSDARG00000058044	Ensembl	-0.37	0.43	-1.59
apoa4	ENSDARG00000040298	Ensembl	-0.78	-0.04	-1.60
GABRB1	ENSDARG00000076127	Ensembl	-3.19	-0.26	-1.60
HM159473.1	HM159473.1	nt	-3.92	-1.07	-1.60
BT072526.1	BT072526.1	nt	-0.88	-0.12	-1.61
pnp5a	ENSDARG00000078619	Ensembl	-4.58	-0.05	-1.62
HM159472.1	HM159472.1	nt	-4.23	1.04	-1.62
aifm1	ENSDARG00000058088	Ensembl	-0.73	-0.20	-1.63
GABRB1	ENSDARG00000076127	Ensembl	-4.00	-0.10	-1.64
NM_001173711.1	NM_001173711.1	refseq	-1.02	0.21	-1.64
GU817336.1	GU817336.1	nt	-0.15	-0.29	-1.64
actr3b	ENSDARG00000008790	Ensembl	-0.80	-0.30	-1.66
BT059277.1	BT059277.1	nt	-0.05	-0.37	-1.68
TYMP	ENSG00000025708	Ensembl	-0.70	0.08	-1.69
EU025709.1	EU025709.1	nt	-1.27	0.00	-1.70
HM066869.1	HM066869.1	nt	-1.09	-0.56	-1.70
TYMP	ENSG00000025708	Ensembl	-0.78	-0.47	-1.72
ddt	ENSDARG00000044751	Ensembl	-1.68	-0.39	-1.73
BT075324.1	BT075324.1	nt	-0.77	-0.61	-1.74
ZNF91	ENSG00000167232	Ensembl	-0.42	-0.31	-1.75

ddt	ENSDARG00000044751	Ensembl	-1.73	-0.38	-1.76
adam11	ENSDARG00000079204	Ensembl	-1.81	-1.57	-1.76
GPR83	ENSDARG00000058120	Ensembl	-0.38	-0.64	-1.76
aifm1	ENSDARG00000058088	Ensembl	-0.86	-1.51	-1.78
ENSONIG00000014676	ENSONIG00000014676	Ensembl	-0.46	-0.28	-1.79
odc1	ENSDARG00000007377	Ensembl	-1.67	3.67	-1.79
ACI66375.1	ACI66375.1	nt	-0.97	-0.38	-1.79
BT057913.1	BT057913.1	nt	-3.04	-0.15	-1.79
ENSONIG00000020396	ENSONIG00000020396	Ensembl	-0.80	0.58	-1.79
tm7sf2	ENSDARG00000032816	Ensembl	-1.07	-0.23	-1.79
WDR7	ENSG00000091157	Ensembl	-2.58	0.12	-1.80
cldn11a	ENSDARG00000020031	Ensembl	-2.34	0.24	-1.80
SLC16A1	ENSDARG00000016963	Ensembl	-0.94	0.12	-1.81
hmgcra	ENSDARG00000052734	Ensembl	-1.11	0.18	-1.81
EU025714.1	EU025714.1	nt	-1.12	-0.55	-1.81
ppa2	ENSDARG00000009685	Ensembl	-0.25	-0.43	-1.81
apoa4	ENSDARG00000040298	Ensembl	-0.78	0.07	-1.82
apoa4	ENSDARG00000040298	Ensembl	-0.67	0.20	-1.82
epb41l3b	ENSDARG00000019917	Ensembl	-1.41	0.30	-1.82
pnp5a	ENSDARG00000078619	Ensembl	-4.61	-0.13	-1.83
pnp5a	ENSDARG00000078619	Ensembl	-4.51	-0.10	-1.84
HM159473.1	HM159473.1	nt	-3.48	-0.52	-1.84
GABRB1	ENSDARG00000076127	Ensembl	-3.21	-0.21	-1.84
ip6k2	ENSDARG00000008310	Ensembl	-1.39	-0.20	-1.84
prdx5	ENSDARG00000055064	Ensembl	-1.37	0.62	-1.84
GQ505858.1	GQ505858.1	nt	-2.95	-0.92	-1.84
apoa4	ENSDARG00000040298	Ensembl	-0.77	0.04	-1.85
slc25a29	ENSDARG00000057352	Ensembl	-1.76	0.37	-1.86
GQ505859.1	GQ505859.1	nt	-0.71	-0.79	-1.86
prdx5	ENSDARG00000055064	Ensembl	-1.67	0.53	-1.86
GQ505858.1	GQ505858.1	nt	-4.10	-0.90	-1.86
nfil3-5	ENSDARG00000094965	Ensembl	-1.63	-0.26	-1.86
errfi1	ENSDARG00000070171	Ensembl	-0.54	-0.21	-1.87
idua	ENSDARG00000062904	Ensembl	-0.86	-0.59	-1.87
tspan13b	ENSDARG00000070479	Ensembl	-0.62	-0.17	-1.87
cdkn1bb	ENSDARG00000088081	Ensembl	-1.20	-0.78	-1.87
AC203446.12	AC203446.12	nt	-1.06	-0.53	-1.88
lss	ENSDARG00000061274	Ensembl	-1.27	-0.39	-1.88
idua	ENSDARG00000062904	Ensembl	-0.98	-0.77	-1.89
GPR83	ENSDARG00000058120	Ensembl	-0.60	-0.34	-1.90
BT058843.1	BT058843.1	nt	-0.94	-0.06	-1.90
idua	ENSDARG00000062904	Ensembl	-0.72	-0.37	-1.90
4833439L19Rik	ENSMUSG00000025871	Ensembl	-2.16	0.43	-1.92
epb41l3b	ENSDARG00000019917	Ensembl	-1.16	-0.11	-1.92
HM159473.1	HM159473.1	nt	-2.83	-1.41	-1.92
ebp	ENSDARG00000046098	Ensembl	-1.01	-0.13	-1.93
NM_001173613.1	NM_001173613.1	refseq	-0.58	-0.20	-1.94
ENSONIG00000015305	ENSONIG00000015305	Ensembl	-0.61	-0.23	-1.95
zp2.5	ENSDARG00000086522	Ensembl	-3.93	0.09	-1.95
idua	ENSDARG00000062904	Ensembl	-1.37	-0.82	-1.95
tcnl	ENSDARG00000068088	Ensembl	-2.42	-0.64	-1.95
hmgcra	ENSDARG00000052734	Ensembl	-1.29	0.10	-1.95
ENSORLG00000005110	ENSORLG00000005110	Ensembl	-0.43	0.02	-1.96
MVD	ENSDARG00000074035	Ensembl	-1.67	-0.34	-1.97
GU129139.1	GU129139.1	nt	-1.06	-0.33	-1.97
ENSORLG00000007209	ENSORLG00000007209	Ensembl	-0.34	-0.04	-1.98
apoa4	ENSDARG00000040298	Ensembl	-0.65	0.18	-1.98
fkbp11	ENSDARG00000037000	Ensembl	-0.61	0.44	-1.99
IRS2	ENSDARG00000037099	Ensembl	-1.23	0.10	-2.00
slc6a9	ENSDARG00000018534	Ensembl	-2.01	0.09	-2.00
tm7sf2	ENSDARG00000032816	Ensembl	-1.17	-0.20	-2.00
EU621899.1	EU621899.1	nt	-2.76	0.35	-2.02
cbln8	ENSDARG00000019294	Ensembl	-0.87	-0.01	-2.02
larp4b	ENSDARG00000062146	Ensembl	-0.49	0.20	-2.03
pgp	ENSDARG00000029695	Ensembl	-1.02	-0.53	-2.05
idua	ENSDARG00000062904	Ensembl	-0.98	-0.71	-2.05
epb41l3b	ENSDARG00000019917	Ensembl	-0.97	0.19	-2.05
cbln8	ENSDARG00000019294	Ensembl	-0.90	-0.03	-2.06
EU025718.1	EU025718.1	nt	-1.91	0.08	-2.06
TYMP	ENSG00000025708	Ensembl	-0.95	0.14	-2.07
NM_001140492.1	NM_001140492.1	refseq	-2.10	-1.60	-2.08
errfi1	ENSDARG00000070171	Ensembl	-1.34	-0.04	-2.08
pptc7a	ENSDARG00000011122	Ensembl	-1.58	-0.31	-2.10
errfi1	ENSDARG00000070171	Ensembl	-0.63	-0.39	-2.10
slc27a1a	ENSDARG00000006240	Ensembl	-0.16	0.05	-2.11
aacs	ENSDARG00000012468	Ensembl	-1.70	-0.08	-2.13
pptc7a	ENSDARG00000011122	Ensembl	-1.63	-0.18	-2.13
tpi1a	ENSDARG00000025012	Ensembl	-0.61	-0.25	-2.14
errfi1	ENSDARG00000070171	Ensembl	-0.88	-0.21	-2.14
nabp1a	ENSDARG00000004692	Ensembl	-1.69	0.19	-2.17
EU025715.1	EU025715.1	nt	-4.39	-1.38	-2.18
pptc7a	ENSDARG00000011122	Ensembl	-1.57	-0.44	-2.18
lss	ENSDARG00000061274	Ensembl	-1.15	0.18	-2.18
deptor	ENSDARG00000040930	Ensembl	-0.82	0.58	-2.19
ulk1a	ENSDARG00000062518	Ensembl	-2.49	0.33	-2.19
4833439L19Rik	ENSMUSG00000025871	Ensembl	-1.76	0.20	-2.19
idi1	ENSDARG00000019976	Ensembl	-0.62	-0.13	-2.20
fgb	ENSDARG00000008969	Ensembl	-0.71	1.34	-2.21
lss	ENSDARG00000061274	Ensembl	-1.35	-0.59	-2.22
pptc7a	ENSDARG00000011122	Ensembl	-1.66	-0.57	-2.23
chst12a	ENSDARG00000028786	Ensembl	-0.55	-0.26	-2.23
XM_695908.4	XM_695908.4	refseq	-2.57	-0.77	-2.24
rbp4l	ENSDARG00000044684	Ensembl	-1.37	-0.63	-2.24
NM_001173711.1	NM_001173711.1	refseq	-0.69	-0.42	-2.25
nabp1a	ENSDARG00000004692	Ensembl	-0.80	0.63	-2.26
NM_001140025.1	NM_001140025.1	refseq	-2.37	0.69	-2.28
lss	ENSDARG00000061274	Ensembl	-1.30	-0.75	-2.29
fam213aa	ENSDARG00000057378	Ensembl	-3.82	-0.03	-2.29

EU621899.1	EU621899.1	nt	-3.38	-2.10	-2.29
4833439L19Rik	ENSMUSG00000025871	Ensembl	-1.42	0.46	-2.30
cyp51	ENSDARG00000042641	Ensembl	-1.16	-0.40	-2.30
ficd	ENSDARG00000035595	Ensembl	-1.03	0.31	-2.31
Col5a2	ENSMUSG00000026042	Ensembl	-3.03	-2.98	-2.32
BT047240.1	BT047240.1	nt	-0.40	-0.29	-2.32
EU221180.1	EU221180.1	nt	-0.54	-1.62	-2.34
lss	ENSDARG00000061274	Ensembl	-2.00	0.63	-2.36
gpt2	ENSDARG00000012199	Ensembl	-0.97	-1.25	-2.39
EF427381.1	EF427381.1	nt	-0.68	-1.05	-2.39
rbp4l	ENSDARG00000044684	Ensembl	-1.19	-0.45	-2.40
idua	ENSDARG00000062904	Ensembl	-1.16	-0.42	-2.40
fdps	ENSDARG00000040890	Ensembl	-1.50	-0.38	-2.41
BT059360.1	BT059360.1	nt	-1.11	0.02	-2.41
FDFT1	ENSDARG00000060260	Ensembl	-1.10	-0.06	-2.41
CR944667.2	ENSDARG00000088972	Ensembl	-0.41	0.13	-2.42
MYOCD	ENSDARG00000076267	Ensembl	-6.58	-1.31	-2.42
GU817335.1	GU817335.1	nt	-1.03	0.22	-2.42
slc27a1a	ENSDARG00000006240	Ensembl	-0.98	-0.32	-2.43
BT059181.1	BT059181.1	nt	-1.75	-1.00	-2.46
FBLN7	ENSDARG00000089519	Ensembl	-2.72	0.19	-2.46
slc26a4	ENSDARG00000069431	Ensembl	-4.68	2.46	-2.46
DQ156150.1	DQ156150.1	nt	-1.89	-0.71	-2.48
lss	ENSDARG00000061274	Ensembl	-2.35	-0.53	-2.50
fdps	ENSDARG00000040890	Ensembl	-1.47	-0.45	-2.50
pptc7a	ENSDARG00000011122	Ensembl	-1.96	-0.42	-2.51
sqlea	ENSDARG00000079946	Ensembl	-1.33	-0.43	-2.51
XP_003453558.1	XP_003453558.1	refseq	-1.16	-0.31	-2.54
NM_001014308.1	NM_001014308.1	refseq	-2.47	0.01	-2.54
cbln8	ENSDARG00000019294	Ensembl	-1.31	-0.44	-2.58
tm7sf2	ENSDARG00000032816	Ensembl	-1.70	-0.63	-2.60
elovl2	ENSDARG00000045414	Ensembl	-0.60	-0.80	-2.62
gpt2	ENSDARG00000012199	Ensembl	-1.31	-1.18	-2.62
pptc7a	ENSDARG00000011122	Ensembl	-2.03	-0.57	-2.62
rogdi	ENSDARG00000096253	Ensembl	-0.23	-0.56	-2.64
lss	ENSDARG00000061274	Ensembl	-1.92	0.58	-2.64
AC203456.8	AC203456.8	nt	-1.17	-0.70	-2.65
FQ310506.3	FQ310506.3	nt	-1.64	-1.67	-2.69
lss	ENSDARG00000061274	Ensembl	-1.74	-0.04	-2.75
NP_001167307.1	NP_001167307.1	refseq	-1.03	0.24	-2.75
NM_001140378.1	NM_001140378.1	refseq	-1.71	-0.59	-2.75
sqlea	ENSDARG00000079946	Ensembl	-1.42	-0.43	-2.76
elovl2	ENSDARG00000045414	Ensembl	-0.61	-0.81	-2.78
NM_001141140.1	NM_001141140.1	refseq	-2.42	0.98	-2.80
NM_001140385.1	NM_001140385.1	refseq	-1.08	-0.44	-2.81
slc43a2b	ENSDARG00000061120	Ensembl	-3.11	-0.30	-2.81
GU129139.1	GU129139.1	nt	-0.36	0.43	-2.85
slc26a4	ENSDARG00000069431	Ensembl	-4.36	2.14	-2.86
NM_001173711.1	NM_001173711.1	refseq	-0.75	-0.55	-2.87
pnp5a	ENSDARG00000078619	Ensembl	-6.35	-1.13	-2.88
cables2a	ENSDARG00000076964	Ensembl	-1.11	0.06	-2.89
igfbp1a	ENSDARG00000014947	Ensembl	-4.22	-1.25	-2.89
hmgcs1	ENSDARG00000052738	Ensembl	-1.37	-0.12	-2.94
ADJ94947.2	ADJ94947.2	nt	-2.10	-1.23	-2.95
SPINK4	ENSDARG00000091609	Ensembl	-1.86	-0.06	-3.01
nipal3	ENSDARG00000021147	Ensembl	-1.44	0.15	-3.01
ect2	ENSDARG00000007278	Ensembl	-0.78	-4.98	-3.04
GU129140.1	GU129140.1	nt	-1.74	-1.90	-3.05
FQ310506.3	FQ310506.3	nt	-1.59	-2.03	-3.05
FBLN7	ENSDARG00000089519	Ensembl	-2.67	0.64	-3.09
g6pca.2	ENSDARG00000013721	Ensembl	-1.77	-0.53	-3.15
errfi1	ENSDARG00000070171	Ensembl	-0.72	0.36	-3.16
FBLN7	ENSDARG00000089519	Ensembl	-3.91	0.33	-3.17
ENSGMOG00000014668	ENSGMOG00000014668	Ensembl	0.50	-0.78	-3.18
ctrl	ENSDARG00000068680	Ensembl	-3.94	-0.33	-3.20
lss	ENSDARG00000061274	Ensembl	-1.39	-0.79	-3.24
XP_003453558.1	XP_003453558.1	refseq	-1.26	-0.26	-3.25
BT044692.1	BT044692.1	nt	-1.93	-0.50	-3.27
EU025716.1	EU025716.1	nt	-1.36	-0.16	-3.29
EU025717.1	EU025717.1	nt	-2.45	-0.42	-3.31
si:dkey-56d12.4	ENSDARG00000070845	Ensembl	-2.41	-0.11	-3.33
mhc1uba	ENSDARG00000075963	Ensembl	1.01	-0.93	-3.34
g6pca.2	ENSDARG00000013721	Ensembl	-2.16	-0.61	-3.36
EU025717.1	EU025717.1	nt	-3.22	-0.63	-3.44
BT074345.1	BT074345.1	nt	-1.62	-0.78	-3.46
efr3bb	ENSDARG00000069318	Ensembl	-0.78	-1.76	-3.54
g6pca.2	ENSDARG00000013721	Ensembl	-2.26	-0.58	-3.54
GU552297.1	GU552297.1	nt	-2.73	1.31	-3.55
GULP1	ENSG00000144366	Ensembl	-1.77	-1.82	-3.57
mmp9	ENSDARG00000042816	Ensembl	-4.33	1.33	-3.58
bhlha15	ENSDARG00000045166	Ensembl	-1.22	-0.22	-3.58
ddit4	ENSDARG00000037618	Ensembl	-5.71	-0.64	-3.76
g6pca.2	ENSDARG00000013721	Ensembl	-2.39	-0.66	-3.79
tk1	ENSDARG00000086561	Ensembl	-2.31	0.34	-3.92
ACI67710.1	ACI67710.1	nt	-3.11	-0.52	-3.96
NP_001134919.1	NP_001134919.1	refseq	-1.63	-1.18	-3.96
g6pca.2	ENSDARG00000013721	Ensembl	-2.75	-0.94	-4.04
COQ10B	ENSG00000115520	Ensembl	-2.84	0.03	-4.05
Peg10	ENSMUSG00000092035	Ensembl	-2.73	-1.21	-4.06
cry-dash	ENSDARG00000002396	Ensembl	-1.04	-1.97	-4.06
epha8	ENSDARG00000023609	Ensembl	-2.79	-2.16	-4.11
ENSGACG00000011316	ENSGACG00000011316	Ensembl	-2.43	-1.16	-4.18
diabloa	ENSDARG00000035323	Ensembl	-3.31	-1.19	-4.24
Vmo1	ENSMUSG00000020830	Ensembl	-1.95	-0.27	-4.24
CGREF1	ENSDARG00000075444	Ensembl	-3.28	-0.65	-4.37
g6pca.2	ENSDARG00000013721	Ensembl	-2.73	-0.74	-4.40
ugt5c1	ENSDARG00000061444	Ensembl	-2.06	-0.52	-4.47
EU221178.1	EU221178.1	nt	-1.65	0.74	-4.47

GQ505858.1	GQ505858.1	nt	-3.24	-1.78	-4.56
SYCN	ENSDARG00000090469	Ensembl	-3.79	-1.15	-4.63
BT072255.1	BT072255.1	nt	-2.18	-0.17	-4.67
BT125319.1	BT125319.1	nt	-2.02	2.92	-4.77
GU129140.1	GU129140.1	nt	0.99	0.25	-4.85
tgm2l	ENSDARG00000093381	Ensembl	-3.75	-1.38	-4.88
si:dkey-56d12.4	ENSDARG00000070845	Ensembl	-2.30	-0.22	-4.95
guca1b	ENSDARG00000013393	Ensembl	-3.17	-1.92	-5.05
EU025716.1	EU025716.1	nt	-2.54	-0.67	-5.09
ENSONIG00000020058	ENSONIG00000020058	Ensembl	-0.11	-1.88	-5.18
gabarapl2	ENSDARG00000027200	Ensembl	-2.53	-3.58	-5.22
EU008541.1	EU008541.1	nt	-4.05	-0.58	-5.27
HQ447060.1	HQ447060.1	nt	-3.29	-1.17	-5.28
CU459095.1	ENSDARG00000086495	Ensembl	-3.48	-2.87	-5.62
EU621901.1	EU621901.1	nt	-3.51	0.71	-5.66
ENSGACG00000016589	ENSGACG00000016589	Ensembl	-1.54	-2.79	-5.69
tspan13b	ENSDARG00000070479	Ensembl	-1.00	-0.81	-5.92
C2H10orf67	ENSDARG00000076896	Ensembl	-0.55	0.29	-5.96
EU221180.1	EU221180.1	nt	-1.78	-0.17	-6.03
HM159469.1	HM159469.1	nt	-2.68	-2.35	-6.04
NM_001140496.1	NM_001140496.1	refseq	-2.11	0.53	-6.24
C2H10orf67	ENSDARG00000076896	Ensembl	-2.17	-0.29	-6.26
CU459095.1	ENSDARG00000086495	Ensembl	-2.55	-1.78	-6.47
CRHR2	ENSDARG00000062377	Ensembl	-3.40	-1.72	-6.47
EU025719.1	EU025719.1	nt	-3.79	-4.36	-6.52
guca1b	ENSDARG00000013393	Ensembl	-6.70	-6.42	-6.59
CU459095.1	ENSDARG00000086495	Ensembl	-2.62	-1.44	-6.80
guca1b	ENSDARG00000013393	Ensembl	-4.73	-2.81	-6.91
guca1b	ENSDARG00000013393	Ensembl	-2.59	-1.62	-7.31
guca1b	ENSDARG00000013393	Ensembl	-4.41	-1.39	-7.41
CELA1	ENSDARG00000043173	Ensembl	-3.36	-3.78	-7.50
guca1b	ENSDARG00000013393	Ensembl	-3.58	-1.96	-7.66
GU129139.1	GU129139.1	nt	-3.28	-0.94	-7.95
GU129139.1	GU129139.1	nt	-2.41	-2.00	-8.37
uncharacterised	uncharacterised	uncharacterised	0.00	3.88	3.86
uncharacterised	uncharacterised	uncharacterised	1.98	1.25	1.89
uncharacterised	uncharacterised	uncharacterised	0.60	-0.61	2.68
uncharacterised	uncharacterised	uncharacterised	0.96	-0.06	2.40
uncharacterised	uncharacterised	uncharacterised	0.82	1.11	2.11
uncharacterised	uncharacterised	uncharacterised	0.69	0.81	2.06
uncharacterised	uncharacterised	uncharacterised	0.72	-0.39	2.03
uncharacterised	uncharacterised	uncharacterised	1.80	-0.71	1.97
uncharacterised	uncharacterised	uncharacterised	0.64	0.06	1.96
uncharacterised	uncharacterised	uncharacterised	0.78	0.46	1.95
uncharacterised	uncharacterised	uncharacterised	0.69	0.12	1.92
uncharacterised	uncharacterised	uncharacterised	0.63	-0.91	1.83
uncharacterised	uncharacterised	uncharacterised	0.90	-0.06	1.81
uncharacterised	uncharacterised	uncharacterised	0.72	-0.94	1.78
uncharacterised	uncharacterised	uncharacterised	1.61	-0.03	1.68
uncharacterised	uncharacterised	uncharacterised	1.23	0.58	1.66
uncharacterised	uncharacterised	uncharacterised	-0.23	0.93	1.65
uncharacterised	uncharacterised	uncharacterised	0.43	-1.08	1.49
uncharacterised	uncharacterised	uncharacterised	0.04	-0.86	1.47
uncharacterised	uncharacterised	uncharacterised	1.37	0.42	1.40
uncharacterised	uncharacterised	uncharacterised	0.79	-0.98	1.40
uncharacterised	uncharacterised	uncharacterised	0.56	-0.06	1.24
uncharacterised	uncharacterised	uncharacterised	2.11	0.00	2.64
uncharacterised	uncharacterised	uncharacterised	1.87	0.00	2.54
uncharacterised	uncharacterised	uncharacterised	2.16	0.00	2.48
uncharacterised	uncharacterised	uncharacterised	1.18	0.00	2.35
uncharacterised	uncharacterised	uncharacterised	1.26	0.00	2.28
uncharacterised	uncharacterised	uncharacterised	1.39	0.00	2.21
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	6.84
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	3.01
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	2.98
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	2.88
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	2.76
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	2.68
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	2.27
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	2.24
uncharacterised	uncharacterised	uncharacterised	0.00	0.00	1.93
uncharacterised	uncharacterised	uncharacterised	-1.48	0.30	-1.33
uncharacterised	uncharacterised	uncharacterised	-2.95	-3.11	-3.54
uncharacterised	uncharacterised	uncharacterised	-0.51	-0.50	-1.37
uncharacterised	uncharacterised	uncharacterised	0.10	-0.39	-1.46
uncharacterised	uncharacterised	uncharacterised	-1.19	-0.14	-1.49
uncharacterised	uncharacterised	uncharacterised	-1.15	-0.42	-1.60
uncharacterised	uncharacterised	uncharacterised	-0.52	0.51	-1.62
uncharacterised	uncharacterised	uncharacterised	-1.09	0.38	-1.64
uncharacterised	uncharacterised	uncharacterised	-0.89	0.76	-1.70
uncharacterised	uncharacterised	uncharacterised	-1.23	0.25	-1.75
uncharacterised	uncharacterised	uncharacterised	-1.13	1.02	-1.76
uncharacterised	uncharacterised	uncharacterised	-0.74	1.41	-1.83
uncharacterised	uncharacterised	uncharacterised	-1.44	0.38	-1.85
uncharacterised	uncharacterised	uncharacterised	-1.02	-0.29	-1.92
uncharacterised	uncharacterised	uncharacterised	-0.17	0.01	-1.93
uncharacterised	uncharacterised	uncharacterised	-0.96	-1.03	-1.97
uncharacterised	uncharacterised	uncharacterised	-1.20	0.80	-2.02
uncharacterised	uncharacterised	uncharacterised	-1.50	-0.26	-2.05
uncharacterised	uncharacterised	uncharacterised	-2.12	-1.94	-2.08
uncharacterised	uncharacterised	uncharacterised	-0.93	-1.03	-2.14
uncharacterised	uncharacterised	uncharacterised	-1.86	-0.82	-2.15
uncharacterised	uncharacterised	uncharacterised	-1.85	2.05	-2.22
uncharacterised	uncharacterised	uncharacterised	-1.45	0.05	-2.26
uncharacterised	uncharacterised	uncharacterised	-2.02	0.93	-2.33
uncharacterised	uncharacterised	uncharacterised	-0.53	-0.17	-2.40
uncharacterised	uncharacterised	uncharacterised	-0.31	-0.13	-2.40
uncharacterised	uncharacterised	uncharacterised	-2.33	0.73	-2.57

uncharacterised	uncharacterised	uncharacterised	-0.80	0.52	-2.63
uncharacterised	uncharacterised	uncharacterised	-1.38	-0.28	-2.66
uncharacterised	uncharacterised	uncharacterised	-0.03	-0.24	-2.90
uncharacterised	uncharacterised	uncharacterised	-1.51	-0.52	-2.92
uncharacterised	uncharacterised	uncharacterised	-1.80	-1.56	-2.96
uncharacterised	uncharacterised	uncharacterised	-2.23	-0.34	-3.04
uncharacterised	uncharacterised	uncharacterised	-1.60	-0.07	-3.08
uncharacterised	uncharacterised	uncharacterised	-2.53	-1.11	-3.23
uncharacterised	uncharacterised	uncharacterised	-0.98	-0.26	-3.35
uncharacterised	uncharacterised	uncharacterised	-1.99	-0.33	-3.37
uncharacterised	uncharacterised	uncharacterised	-3.43	0.40	-3.44
uncharacterised	uncharacterised	uncharacterised	-1.31	-0.53	-3.50
uncharacterised	uncharacterised	uncharacterised	-2.94	0.66	-3.57
uncharacterised	uncharacterised	uncharacterised	-0.80	0.08	-3.62
uncharacterised	uncharacterised	uncharacterised	-1.48	0.19	-4.20
uncharacterised	uncharacterised	uncharacterised	-2.76	-0.46	-4.88
uncharacterised	uncharacterised	uncharacterised	-4.55	-4.34	-5.53
uncharacterised	uncharacterised	uncharacterised	-0.57	-2.31	-5.80
uncharacterised	uncharacterised	uncharacterised	-0.88	0.22	-5.94
uncharacterised	uncharacterised	uncharacterised	-1.19	-0.06	-6.10
uncharacterised	uncharacterised	uncharacterised	-4.92	-1.01	-6.38
uncharacterised	uncharacterised	uncharacterised	-6.87	-6.60	-6.76
uncharacterised	uncharacterised	uncharacterised	-3.36	-1.62	-6.76
uncharacterised	uncharacterised	uncharacterised	-1.12	-9.73	-7.44
uncharacterised	uncharacterised	uncharacterised	-3.40	-1.11	-7.72
uncharacterised	uncharacterised	uncharacterised	-1.08	-8.73	-8.90
uncharacterised	uncharacterised	uncharacterised	-0.05	2.25	-0.13
uncharacterised	uncharacterised	uncharacterised	-1.92	3.32	-2.07
uncharacterised	uncharacterised	uncharacterised	-0.29	-3.16	1.30
uncharacterised	uncharacterised	uncharacterised	-1.12	-5.94	-0.06
uncharacterised	uncharacterised	uncharacterised	1.59	-0.16	1.26
uncharacterised	uncharacterised	uncharacterised	-4.21	-0.57	0.37
uncharacterised	uncharacterised	uncharacterised	-6.32	0.07	0.16
uncharacterised	uncharacterised	uncharacterised	-5.98	-0.56	-0.19
uncharacterised	uncharacterised	uncharacterised	-4.22	-2.04	-1.04
uncharacterised	uncharacterised	uncharacterised	-2.58	1.36	-1.11
uncharacterised	uncharacterised	uncharacterised	-2.19	-0.85	-1.30
uncharacterised	uncharacterised	uncharacterised	-2.73	-0.38	-1.37
uncharacterised	uncharacterised	uncharacterised	-3.65	0.17	-1.40
uncharacterised	uncharacterised	uncharacterised	-4.35	-1.40	-1.52
uncharacterised	uncharacterised	uncharacterised	-7.80	0.03	-1.83
uncharacterised	uncharacterised	uncharacterised	-3.87	0.65	-2.08
uncharacterised	uncharacterised	uncharacterised	-4.31	-0.54	-2.39
uncharacterised	uncharacterised	uncharacterised	3.45	0.00	1.93
uncharacterised	uncharacterised	uncharacterised	2.22	0.00	1.39
uncharacterised	uncharacterised	uncharacterised	3.40	0.00	0.00
uncharacterised	uncharacterised	uncharacterised	4.26	0.00	0.00
uncharacterised	uncharacterised	uncharacterised	3.03	0.00	0.00
uncharacterised	uncharacterised	uncharacterised	3.26	0.00	0.00
uncharacterised	uncharacterised	uncharacterised	2.92	0.00	0.00
uncharacterised	uncharacterised	uncharacterised	3.13	0.00	0.00

CHAPTER 5

Global transcriptomic profiling demonstrates induction of oxidative stress and compensatory stress responses in brown trout exposed to glyphosate and Roundup

Manuscript in preparation for BMC Genomics

Global transcriptomic profiling demonstrates induction of oxidative stress and compensatory stress responses in brown trout exposed to glyphosate and Roundup.

Tamsyn M. Uren Webster and Eduarda M. Santos

Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

Abstract

Glyphosate, the active ingredient in Roundup formulations, is the most widely used herbicide worldwide. Glyphosate may contaminate surface waters and has been detected in food residues, drinking water and human urine, raising concerns for potential environmental and human health impacts. Research has shown that glyphosate and Roundup can induce a broad range of biological effects in fish, mammals and other species, particularly via generation of oxidative stress. However, there has been no comprehensive investigation of the global molecular mechanisms of toxicity of glyphosate and Roundup. We aimed to characterise and compare the global mechanisms of toxicity of glyphosate and Roundup in the liver of brown trout (*Salmo trutta*), an ecologically and economically important European species, using RNA-seq on an Illumina HiSeq 2500 platform. To do this, we exposed juvenile female brown trout to 0, 0.01, 0.5 and 10 mg/L of glyphosate and Roundup (glyphosate acid equivalent) for 14 days, and sequenced 6 replicate liver samples from each treatment. We assembled the brown trout transcriptome using an optimised *de novo* approach, and subsequent differential expression analysis identified a total of 1020 differentially-regulated transcripts across all treatments. Functional analysis revealed a strong over-representation of compensatory cellular stress response pathways, including promotion of apoptosis, which are consistent with induction of oxidative stress. The mechanisms of toxicity identified were similar across both glyphosate and Roundup treatments, including for environmentally relevant concentrations. The significant alterations in transcript expression observed at the lowest concentrations tested raises concerns for the toxicity of glyphosate and Roundup to fish populations inhabiting contaminated rivers.

Introduction

Glyphosate is a broad-spectrum, post emergence herbicide that acts by inhibiting plant aromatic amino acid synthesis via the shikimate pathway [1, 2]. In recent years, glyphosate has been the most widely used agricultural herbicide worldwide [3, 4], and it is also used extensively in urban and domestic environments [4, 5]. Glyphosate can be used alone, but it is more commonly applied as part of a formulated product and the most widely used of these are Roundup herbicides. Roundup formulations vary with application purpose, but contain a number of adjuvants that enhance the herbicidal properties of glyphosate. One of the most important is polyethoxylated tallow amine (POEA), a surfactant that enhances glyphosate cellular uptake in plants [6, 7]. Concentrations of glyphosate entering surface waters are not routinely monitored, but values in the range of 10-15 µg/L have been reported in rivers [e.g.8, 9], while measurements in the range of 500-800 µg/L have been recorded only occasionally and are generally associated with direct application to wetland environments [7, 10]. Glyphosate residues have also been found in food and in drinking water [7], and a recent study reports traces of glyphosate in 44 % of human urine samples collected throughout Europe [11]. The widespread use and measured concentrations in humans and in the environment has raised concerns about its toxicity and the risk that it may pose for human and wildlife health.

Although the target mechanism of action of glyphosate is specific to plants, a range of toxicological effects in a number of vertebrate and invertebrate species have been demonstrated. Both glyphosate and Roundup have been widely shown to induce cellular oxidative stress through generation of ROS and/or interference with the antioxidant system. In fish, short-term exposures to high concentrations (1-20 mg/L) of Roundup altered levels of cellular antioxidants and induced oxidative damage of DNA, lipids and proteins [e.g. 12, 13-16], while environmentally relevant concentrations of Roundup, glyphosate and POEA induced DNA damage in blood and liver cells of eel and catfish [17-21]. Similarly in rats, treatment with > 100 mg/kg glyphosate and Roundup generated oxidative stress and induced lipid peroxidation [22], and 10 mg/kg glyphosate induced oxidative stress, DNA damage and an increase in apoptosis [23]. In human cell lines both glyphosate (from 50 mg/L) and

Roundup (from 18 mg/L) induced apoptosis [24-26], and Roundup also increased necrosis [27, 28]. Roundup, and to a lesser extent glyphosate, caused endocrine disruption in cell lines [24, 25, 29-31]. Additionally, disruption of steroidogenic enzymes and reproductive health have been demonstrated following Roundup exposure in rats [32-34]. In fish, recent work from our laboratory demonstrated that high concentrations of glyphosate and Roundup affect reproduction in zebrafish in a process mediated via disruption of steroid hormone synthesis and induction of oxidative stress [35]. Other demonstrated biological effects of glyphosate and/or its commercial formulations include immunotoxity, neurotoxicity and developmental toxicity [e.g. 36, 37-40]. Generally, Roundup has been found to be more toxic than pure glyphosate. This has been attributed to the inherent toxicity of POEA [18, 39], and potentially other formulation products. Additionally, formulation products may enhance the toxicity of glyphosate by facilitating cellular entry [22].

To date, despite the high rate of glyphosate usage and the concerns about its potential to cause human and environmental health impacts, no comprehensive studies investigating the global mechanisms of toxicity of glyphosate and its commercial formulation have been performed. This study aimed to investigate and compare transcriptional response of brown trout (*Salmo trutta*) exposed to glyphosate and Roundup. Brown trout are an ecologically and economically important European species, known to be sensitive to environmental stressors. Due to their ecological niche, brown trout are likely to be affected by these compounds, particularly as a result of agricultural runoff. We conducted an exposure of juvenile female brown trout to three concentrations of both glyphosate and Roundup, including environmentally relevant concentrations, and investigated the toxicological effects of these compounds in the liver using RNA-seq on an Illumina HiSeq 2500 platform.

Materials and methods

Fish maintenance

Juvenile brown trout (six months old; originating from a local aquaculture facility) were maintained in 35 L glass tanks, and acclimated to laboratory conditions for three weeks prior to the start of the exposure. Each tank was aerated and supplied

with a water flow rate of 140 L / day. The aquarium water supply was reverse-osmosis treated tap water reconstituted with analar-grade salts to produce a standardized synthetic freshwater according to OECD guidelines (final concentrations to give a conductivity of 300 mS: 122 mg/L $CaCl{\cdot}22H_2O$, 9.4 mg/L $NaHCO_3$, 50 mg/L $MgSO_4{\cdot}7H_2O$, 2.5 mg/L KCl, 50 mg/L NaCl), as described in Paull et al. [41], and maintained at 12 ± 0.2 °C and pH 7.2-7.8. Fish were kept under a 16:8 h light:dark cycle (with 30 minute dawn/dusk transitional periods) and fed with 0.5 mm trout pellets (Biomar, Grangemouth, UK) at a rate of 2% body weight per day.

Chemical exposure and sampling

Chemical exposure was conducted via a flow through system for a period of 14 days. The treatment groups consisted of three concentrations of glyphosate; 0.01, 0.5 and 10 mg/L (analytical grade; Molekula, Wimborne, UK), three concentrations of Roundup; 0.01, 0.5 and 10 mg/L glyphosate acid equivalent (using Roundup GC liquid glyphosate concentrate containing 120 g/L glyphosate acid; Monsanto, Cambridge, UK); and a control group. These treatment groups will be referred to throughout as LG, MG, HG and LR, MR, HR for the 0.01, 0.5 and 10 mg/L Glyphosate and Roundup treatments, respectively. The two lower concentrations were chosen to represent concentrations that may occur in the environment frequently (0.01 mg/L) or during occasional peak contamination events (0.5 mg/L). The highest concentration tested (10 mg/L) was included to facilitate the analysis of the mechanisms of toxicity of glyphosate and Roundup but is unlikely to occur in surface waters. Each treatment group was comprised of two replicate 35 L tanks containing 12 fish. Water samples were collected from each tank on days 0, 7 and 14 of the exposure period and stored at -20 °C prior to chemical analysis of glyphosate and Roundup which was conducted by an external company (South West Water, Exeter laboratories).

Fish were humanely sacrificed on day 14 of the exposure period by a lethal dose of benzocaine (0.5 g/L; Sigma-Aldrich) followed by destruction of the brain, in accordance with UK Home Office regulations. Wet weight and fork length were recorded, and the condition factor (k= (weight (g) x 100)/ (fork length $(cm)^3$)) was calculated for individual fish. Sex was determined by visual observation of the

gonads. Livers were dissected and weighed, and the hepatosomatic index (HSI) (liver weight (mg)/ total weight (mg)) x 100)) was determined for individual fish. Portions of the liver from female fish were snap frozen in liquid nitrogen and stored at -80°C prior to transcript profiling.

RNA extraction, library preparation and sequencing

Transcript profiling was conducted in the liver of 6 females per treatment group. For the MG treatment group, only 3 individuals were analysed because there were only three females in both replicate tanks. RNA was extracted from female livers using an RNeasy Mini extraction kit (Qiagen), incorporating on-column DNase treatment, according to the manufacturer's instructions. The concentration, purity and integrity of RNA was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). All RNA input to library construction was of high quality with 260/280 and 260/230 ratios > 1.8 and RIN scores > 8. ERCC spike-in control mixes (Ambion) were added to all individual RNA samples, according to the manufacturer's instructions to allow for analysis of the accuracy of the transcript quantification and dynamic range. cDNA libraries from all samples were then prepared using the Illumina TruSeq RNA Sample Preparation kit, multiplexed with 24 samples per lane and sequenced using an Illumina HiSeq 2500, to generate 100 bp paired-end reads.

Transcriptome Assembly and Annotation

All analyses were carried out on a local server running under the NEBC Bio-Linux 7 environment [23]. Remaining Illumina adaptor sequences were removed and the first 12 bp of all raw sequence reads were trimmed to remove 5' bias caused by random hexamer priming [42] using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). 3' sliding window quality trimming was performed using (http://wiki.bioinformatics.ucdavis. edu/index.php/Trim.sliding Window.pl) and all reads where < 90% bases had a Phred quality score >20, and those shorter than 15 bp, were discarded. Digital normalisation was performed to remove highly duplicated reads using the normalize-by-median.py script part of the khmer package described by Brown et al. [43], with the recommended k-mer value of 20 and a coverage threshold of 20. This process reduces the computer memory requirements of transcriptome assembly, and also reduces the risk of potential

sequencing error accumulation in abundant transcripts [44]. All retained reads were then paired, separated into forward and reverse fastq files for Trinity assembly and shuffled into a single interleaved fastq file for the Velvet assembly using a custom script.

In order to obtain the most appropriate transcriptome assembly for downstream expression analysis, we conducted *de novo* transcriptome assemblies using Velvet (version 1.2.08; [45]) followed by Oases (version 0.2.08; [46]), and Trinity (version r2013-02-25; [47]), and compared them. The Trinity assembly was conducted using the default parameters, specifying a minimum contig length of 200 bp. Separate Velvet-Oases assemblies were created specifying ins_length 161 -ins_length_sd 150 and using k-mers ranging from 33 to 69 (with steps of 6), then these were merged using the Oases-Merge function (K=27) specifying -min_trans_lgth 200. All transcripts in the final assemblies were annotated using Blastx against Ensembl peptide databases (Release 71; April 2013) using an e-value cut off < $1e^{-15}$ and assigned in the following preferential order; zebrafish; human and mouse; stickleback, medaka, nile tilapia and cod. For transcripts found to be differentially expressed (see below) that were not annotated in this way, additional annotation was performed using Blast (< $1e^{-15}$) against refseq, nr and nt databases.

Transcriptomic Analysis

Raw sequence reads from individual samples were mapped back against both Trinity and Oases transcriptome assemblies using Bowtie2 (version 2.1.0, [48]), using the -k 1 parameter to report a single best hit for each read and limit ambiguous mapping to redundant transcripts. Raw count data for each transcript was extracted using idxstats in samtools (version 0.1.18, [49]) and input into edgeR [50] for differential expression analysis. In edgeR, the recommended criteria of having at least 1 mapped read from a minimum of 6 samples for each transcript was imposed. Tagwise dispersion was applied with the recommended prior.df =10. Initial comparison of transcript expression between the two control treatment groups showed strong similarities, and only 3 differentially expressed transcripts, therefore pairwise tests were conducted between the 12 replicates from the combined control groups and six replicates from each of the other treatment groups. Transcripts were considered differentially expressed with a FDR < 0.1 (Benjamini-Hochberg

correction). Hierarchical clustering was performed on all differentially expressed transcripts using an Euclidean distance metric, in the Pheatmap package for R. Functional analysis was then performed for differentially expressed genes from each treatment using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.7; [51]), with the final brown trout liver transcriptome as a background. Kegg pathways and Gene Ontology (GO) terms for Biological Process, Cellular Component and Molecular Function were considered significantly over-represented when $P < 0.05$.

Results and Discussion

Water chemistry

Water samples taken from each tank at three separate timepoints (day 0, day 7 and day 14) were combined into a single sample for each replicate tank and analysed for the concentrations of glyphosate in an external accredited laboratory. The mean measured concentrations for each treatment (2 replicate tanks) were 0.0097 ± 0.0003; 0.46 ± 0.01 and 9.9 ± 0.41 mg glyphosate/ L for the 0.01, 0.5 and 10 mg glyphosate/L treatment groups respectively, and 0.011 ± 0.0003; 0.50 ± 0.007; 9.31 ± 0.17 mg glyphosate/L for the 0.01, 0.5 and 10 mg Roundup/L treatment groups, respectively. The measured concentrations for all treatments are therefore within 88.7-110.7 % of the nominal concentrations.

Morphometric parameters

The mean mass and length of all female fish was 13.1 ± 0.3 g and 9.97 ± 0.09 cm. There were no significant differences in size and condition factor (mean 1.31) or HSI (mean 1.66) between treatment groups. There were no mortalities and we observed no alteration of behaviour during the course of the exposure, suggesting that these concentrations of glyphosate and Roundup had no overt toxic effects on general health.

Sequencing and de novo transcriptome assembly

Sequencing of female liver samples generated a total of 969.4 million paired 100 bp reads, averaging 20.2 million reads per library, 92.5 % (897 million) of which were retained following processing and quality filtering. Following digital normalisation, a

total of 101.5 million paired reads, originating from all libraries, were retained and input into the *de novo* transcriptome assemblies. Statistics for the Trinity and Velvet-Oases transcriptome assemblies are shown in Table S1. The final Velvet-Oases assembly produced a considerably more redundant assembly consisting of 893,904 transcripts compared to 258,702 in the Trinity assembly, while the number of putative gene loci was more similar (146,233 and 109,301 respectively). Previously, Velvet-Oases transcriptome assemblies were found to be more redundant than Trinity assemblies [52]. The percentage of transcripts annotated using Blastx against Ensembl peptide databases was similar in both assemblies (45 % of Trinity transcripts and 47 % of Oases transcripts), as were the transcript length parameters. However, the Oases assembly included representation of over 2000 more unique transcripts (based on the annotation against the Ensembl zebrafish database), potentially indicating better coverage of the brown trout liver transcriptome. For species without a good quality reference genome, like the brown trout, the quality and reliability of transcript expression analysis using RNA-seq is dependent on the quality of the *de novo* transcriptome assembly. This can be assessed using a number of parameters, which may vary based on the dataset and study objectives. Transcript redundancy is an important measure of assembly quality because high levels of redundancy tend to increase levels of ambiguous read mapping and reduce statistical power in differential expression analysis. However, inclusion of the greatest number of unique gene isoforms, including rare transcripts, is important for subsequent expression analysis and biological interpretation. Both the Trinity and Oases assemblies, therefore, have advantageous characteristics, so we performed the differential expression analysis for both assemblies independently, followed by a comparison of the resulting gene lists.

Transcript expression analysis

A greater percentage of reads were mapped to the Oases assembly compared to the Trinity assembly (mean 94 % and 90 % per sample, respectively) using Bowtie2 [53]. However, for the Trinity assembly, a greater percentage of transcripts met the criteria of having at least one mapped read in six replicate samples and were retained for differential expression analysis in EdgeR. The retained transcripts from the Trinity assembly also included representation of 32 % more of the transcripts in the Ensembl zebrafish database. Additionally, calculated values of biological coefficient

of variation (BCV) for pairwise comparisons across all treatments were consistently lower using the Trinity assembly (average 34.6 %) compared to the Oases assembly (average 37.4 %). Furthermore, there was a lower degree of annotation redundancy in the list of differentially expressed transcripts identified using the Trinity assembly. These differences likely reflect the greater degree of transcript redundancy in the Oases transcriptome assembly, and together, indicate that the Trinity assembly, for the present dataset, was of higher quality for transcript expression analysis. Therefore, we used the results obtained using the Trinity assembly for further biological interpretation and functional analysis.

The MG treatment group, which had only three replicates, had the highest BCV value of all treatment groups, and multidimensional scaling (MDS) plots show that there was one individual in this group with a very different transcript expression profile compared to the other replicates (Figure S1). Transcript expression analysis revealed an unrealistically high number of differentially expressed transcripts in this group compared to the control (>5000), presumably because of the strong influence of this individual in a group with few replicates, which increased biological variation and potentially false positive discovery. This treatment group was therefore removed from the analysis.

The numbers of up- and down-regulated transcripts in each treatment group, including overlaps between treatment groups, using the Trinity assembly are shown in Figure 1. The transcript level expression plots for each treatment are shown in Figure S2 and the full list of differentially expressed transcripts are presented in Table S4. The total number of transcripts differentially expressed in one or more Roundup treatment groups compared to the controls was 923 (656 of which were up-regulated; 266 were down-regulated; and 1 was up and down regulated in the LR and HR groups, respectively) and in the two glyphosate-treated groups was 303 (258 of which were up-regulated and 45 were down-regulated). Of these, 143 transcripts were differentially regulated following exposure to both glyphosate and Roundup (135 transcripts were up-regulated and 8 transcripts were down-regulated). The results of the differential expression analysis using the Oases assembly show a similar pattern in the number of differentially expressed transcripts in each treatment group, including a predominance of up-regulation, and are presented in the

supporting information (S Fig 3). Clustering analysis of all 1020 differentially expressed transcripts showed that there were strong similarities in the expression profiles of all Roundup treatment groups and the LG group (Fig 2). In contrast, the transcript profile for fish exposed to HG was clearly different from that of the other groups, and there were also considerably fewer differentially expressed transcripts in this group (Fig 1,2).

The ERCC spike-in control analysis for all individual samples is presented in Figures S4, Figure S5 and Table S2. For all samples there was a strong correlation between calculated FPKM value and expected concentration of spiked-transcripts (mean R^2=0.918±0.002), and the mean calculated dynamic range in expression level was 25,722 FPKM. There was also a strong correlation between calculated and expected fold changes in transcript expression between samples spiked with ERCC mix 1 and ERCC mix 2 ($p = 1.5E-18$, $R^2 = 0.6223$). Together these results provide strong technical validation for the quantitative transcript profile analysis presented here.

Functional analysis and biological interpretation

Functional analysis of differentially expressed transcripts identified 68 over-represented GO-All terms (Biological Process, Molecular Function and Cellular Compartment), and 11 over-represented Kegg pathways ($p<0.05$), across all treatments (Table S3).

Oxidative stress, cellular stress response and apoptosis

There was some evidence of an increase in oxidative stress, characterised by an up-regulation of the antioxidant system, following both Roundup and glyphosate exposure. Two transcripts encoding glutathione reductase (*gsr*) were significantly up-regulated by MR, and there were increasing trends in expression of these transcripts in the other treatment groups. This key enzyme is responsible for the restoration of reduced glutathione (GSH), a major cellular antioxidant which neutralises ROS, and in the process is itself oxidised [54]. Additionally, three transcripts encoding heme oxygenase 1 (*hmox1*) were up-regulated by MR, and were differentially-regulated by LG and MR. These proteins have roles as cellular anti-oxidants and have important roles in maintenance of cellular redox balance [55]. In particular, heme oxygenase has previously been shown to be amongst the most responsive markers of cellular

oxidative stress, compared to the sometimes inconsistent responses of some of the more classical antioxidants [56]. Our data supports previously published reports that demonstrated that glyphosate and Roundup induce oxidative stress in fish and other species, including at concentrations measured in the environment, resulting in damage of DNA, lipids and proteins, and modulation of the cellular antioxidant system [e.g. 12, 13-21].

Cellular stress response is mediated by a diverse array of interacting signalling pathways which respond to a range of environmental stressors. Depending on the degree and duration of the stress, cellular response can vary. For example, low concentrations of ROS tend to induce pro-survival mechanisms, while a greater degree of oxidative stress, and cellular damage, can promote apoptosis as a protective mechanism. Regulation of apoptosis is therefore an essential component of the cellular stress response, and this is discussed below.

A number of transcripts belonging to families of stress-response proteins were differentially regulated. Transcripts encoding hypoxia induced gene 1 (*hig1*) and hypoxia up-regulated protein 1 (*hyou1*) were significantly up-regulated in the MR and LG treatments, respectively, with increasing trends in the other treatment groups. These proteins are known to play an important role as general stress factors, including in response to oxidative stress, and regulation of apoptosis [57]. Additionally, heat shock proteins (*hsp5, hsp13, hspb2, dnajb11, dnajb9, dnajc3*), a gene family encoding ubiquitous stress-response proteins that can bind, stabilise and remove damaged proteins [54, 57], were differentially regulated across various treatment groups. We observed an up-regulation of the tumour-suppressor protein p53 (*tp53*) in the LG treatment. This is a transcription factor that mediates several cellular stress responses including arrest in the cell cycle, which prevents propagation of mutations, initiation of DNA repair mechanisms and initiation of apoptosis when DNA damage is extensive [54, 58]. Genotoxicity induced by oxidative stress has previously been associated with an increase in p53 transcription [54, 59]. Furthermore, we also found evidence of differential regulation of transcripts involved in DNA-repair; DNA damage-inducible transcripts (*ddit4, ddit4l*) were up-regulated by HG and LG, respectively, and DNA damage-inducible protein 4a (*gadd45a*) was down-regulated in MR, HR and LG.

Mitogen-activated protein kinase (MAPK) signalling was over-represented in both the MR and LG treatments (Table S3). MAPK cascades transmit signals from cell surface receptors through a series of activated proteins to the nucleus, inducing transcriptional responses. MAPK signalling plays an essential role in cellular stress response, including positive and inhibitory regulation of apoptosis, particularly through the p38 and JNK groups of MAPK pathways [54, 60, 61]. Transcriptional profiling revealed that treatment with MR induced several of the major p38-activated MAPKs (*mapk14a, mapk14b*), and *mapk3k6,* a known activator of *mapk14* and JNK-activated MAPKs, was up-regulated by LG. Additionally, a number of transcripts encoding MAPK-interacting serine/threonine kinases (*mknk1* and *mknk2b*), which are activated by p38-MAPKs, were up-regulated across LR, MR, HR and LG treatments. Together, this provides some evidence that both glyphosate and Roundup induced an up-regulation of several key components of MAPK signalling.

AP-1 is an important transcription factor involved in regulating apoptosis, as well as cell proliferation and growth, in response to various stimuli including ROS, cytokines and growth factors [61]. Key components of AP-1, Fos-like antigen 2 (*fosl2*) and activating transcription factor 3 (*atf3*), were some of the most up-regulated transcripts across treatments. Nuclear factor kappa N (NF-κB) is another transcription factor involved in the regulation of a large number of genes involved in cellular stress response, including pro-survival and pro-apoptosis mechanisms [54] and transcription of NF-κB components are known to be induced by ROS [56]. We found that three key members of the NF-κB family (*nfkb2, rela, relb*) were up-regulated in fish exposed to LR, MR, HR and LG treatments. Furthermore, a number of transcripts involved in TNF signalling, which interacts with, and activates NF-κB, were also up-regulated including *tnfr14* (LR, MR, HR, LG), *tnip3, optn, sqstm1* (MR, HR, LG), *tnip2* (MR, HR) and *tnfr2, cd40*, *traf3, traf2b* (MR). TNF signalling responds to numerous cellular stressors and is also involved in regulation of apoptosis [62], and has previously been reported to be modulated in rats exposed to both glyphosate and Roundup, in association with oxidative stress [22]. Together, this provides evidence of up-regulation of several of the major regulatory pathways responsible for inducing cellular stress response.

Calcium signalling was over-represented in LG, and a number of transcripts with roles in maintaining calcium homeostasis were differentially expressed in both

Roundup and glyphosate treatment groups (Table S3). Calcium signalling has a crucial regulatory role in an extensive range of cellular processes, including regulation of apoptosis and cellular stress response [63]. In particular, mobilisation of Ca^{2+} is important in the upstream activation of transcription factors including AP-1, p53 and NF-κB, by ROS [56]. Ca^{2+} transporting ATPases (*atp2a2a, atp2a2b*), known to be strongly affected by oxidative stress and have a role in regulating apoptosis [63], were significantly up-regulated in LG and MR, and there were increasing trends in the other treatment groups. Calsynterin (*clstn1*), calcitonin receptor (*calcrla*) and tescalcin (*tescb*) were all up-regulated by LG and calcineurin-like (*chp1*) was up-regulated by MR. Calcium channel (*cacng6b)* was down-regulated by LR, and together with hippocalcin (*hpcl1*), was down-regulated by MR and HR.

Overall, our data suggests a widespread induction of a cellular stress response mechanism during the exposures, and we hypothesise this was likely to have been due to generation of oxidative stress. We found evidence that this response was induced by all treatment concentrations of Roundup, as well as LG, although there was differential modulation of these regulatory signalling pathways between groups. This may reflect dose-specific effects, including a differential balance between pro-survival and pro-apoptotic pathways.

The *apoptosis* Kegg pathway displaying a summary of differentially regulated genes and processes across treatment groups is shown in Figure 3. The signalling pathways discussed above can regulate apoptosis in a positive or negative way. In addition to this, transcript profiling revealed up-regulation of a number of factors that specifically promote apoptosis. Briefly, apoptosis is controlled by initiator and effector caspases, which cleave numerous cellular targets, and are activated in response to regulation by intrinsic and extrinsic signalling pathways [58, 59, 64]. Intrinsic signalling involves the release of apoptotic factors in response to intracellular stress. These target the mitochondria, causing the release of cytochrome c and other caspase-activating factors, a process which is under the control of the apoptosis-regulator Bcl-2 family [59]. Transcript analysis revealed mitochondrion-associated apoptosis-inducing factor (*aifm2*) was significantly up-regulated by MR treatment and there were increasing trends in the expression of this transcript in all other treatment groups. Additionally, caspase recruitment domain-containing protein 14 (*card14*),

which interacts with members of the Bcl family, and is a positive regulator of apoptosis, was up-regulated by MR and HR. Extrinsic signalling control of apoptosis involves binding of extracellular factors to cell surface death receptors from the TNF receptor superfamily, which initiates signalling pathways leading to caspase activation [59, 64]. Two transcripts encoding lymphocyte G0/G1 switch protein 2 (*G0S2*) were amongst the most strongly up-regulated transcripts (7-25 fold by MR, HR and LG treatments). This gene has been found to strongly promote apoptosis by binding Bcl-2 and inhibiting its antiapoptotic activity, through induction by TNF signalling and NF-kB activity [64, 65]. Programmed cell death protein 6 (*pcdp6*) and cytotoxic granule-associated RNA binding protein (*tia1*), which are also pro-apoptotic factors and interact with the TNF family Fas-receptor, were up-regulated by MR. Transcripts encoding sphingomyelin synthase 2 (*sgms2*) and sphingomyelin phosphodiesterase 5 (*smpd5*), which are both important in the generation of ceramide, another pro-apoptotic factor known to be induced by TNF and Fas ligands [66], were both also up-regulated by MR, and MR and HR, respectively. We additionally found some evidence of an increase in autophagy, another form of programmed cell death, which is regulated by a number of the same pathways as apoptosis [64]. Autophagy related homolog 5 (*atg5*) was another of the most up-regulated transcripts (5-35 fold) by MR, HR and LG treatment.

These transcriptional changes suggest a shift towards pro-apoptotic cellular stress response pathways in fish exposed to MR and HR, and to a lesser extent, LG. These results align strongly with previous research, where various Roundup formulations and glyphosate alone have been shown to cause an increase in the rate of apoptosis in various human cell lines, characterised by elevated caspase activity [24, 25, 27, 28], altered Bcl protein activity and loss of mitochondrial integrity [26]. Although apoptosis-regulating pathways were affected by LR exposure, we found no changes in expression of specific pro-apoptosis factors. This suggests a dominance of pro-survival stress response mechanisms at lower treatment concentrations of Roundup, which is likely to generate lower levels of oxidative stress.

Cell proliferation and turnover

A number of the signalling pathways which regulate apoptosis, are also involved in regulating cell proliferation and growth. Additionally, up-regulator of cell proliferation

(*urgcp*) expression was increased in LG, while early growth response 1 (*erg1*) and connective tissue growth factor (*ctgfa*) were increased in MR and LG. Syndecan (*sdc4*), a G-protein cell surface co-receptor that interacts with various growth factors was up-regulated by up to 10-fold in LR, MR, HR and LG treatments, while various transcripts encoding the guanine nucleotide exchange factor *mcfl2* were down-regulated by MR, HR and LG treatments. The insulin signalling pathway, which is important in the promotion of cellular growth, as well as lipid and energy metabolism and homeostasis, was also over-represented. Within these pathways, insulin-induced gene 1 (*insig1*), insulin receptor substrates (*IRS2, IRS4*) and insulin-like growth factor binding protein (*igfbp1a*) were up-regulated in MR, HR, MR and LG respectively, providing evidence for regulation of cell proliferation and growth. A balance between programmed cell death and cell proliferation is essential in the maintenance of tissue homeostasis, and both processes are regulated by integrated, complex, signalling pathways with a considerable degree of crosstalk. It is also known that an increased rate of cell proliferation can accompany increased apoptosis to maintain tissue homeostasis [58, 59], therefore we hypothesise that cell proliferation may be up-regulated by Roundup and glyphosate treatment to compensate increased cellular loss through apoptosis.

Various transcripts associated with the cytoskeleton were also differentially regulated, including several involved in regulating actin filament dynamics and reorganisation; *nav2* (down-regulated in MR group), *synpo2* (up-regulated in MR), *syncrip* (up-regulated in MR and LG), *ssh2b* (up-regulated in LR, MR, HR and LG), *fnbp* (down-regulated in MR and HR), *wasb* (down-regulated in HG) and *antxr1* (up-regulated in MR and MG). Intermediate filament related transcript (*evpla*) and microtubule associated transcript (*mapre1b*) were also up-regulated in MR and HR, and MR, respectively. Additionally, GO terms related to the extracellular matrix (ECM) were amongst the most significantly enriched GO terms, especially in the MR group. In particular, a number of transcripts encoding collagens (*col1a1a, col1a1b, col1a2, col6a2, col6a3, col8a1a, col16a1*) were down-regulated by MR, while *col13a1* was up-regulated by HR and collagen binding protein (*col4a3bp*) was up-regulated by MR. Furthermore, a number of transcripts encoding matrix metalloproteinase collagenases (*mmp9, mmp13a, mmp13*) were up-regulated across LR, MR, HR and LG treatments by 3-5 fold. In addition to collagen

degradation, collagenases are important in the cleavage and activation of molecules involved in regulation of apoptosis and immune response. Other differentially regulated, interacting, ECM components include laminins (*lama4, lamb1b*; down MR), integrin (*itgax;* up-regulated by LR, MR and HR) and fibronectin (*fndc3a*; up-regulated by LR). These changes in the regulation of ECM components, which have important roles in cellular proliferation and growth, cell signalling and regulation of the cell cycle, may be associated with increased rate of cell turnover. In addition, collagen metabolism is also a specific target of oxidative stress and ROS are known to both inhibit collagen transcription and increase collagenase activity [67]. Collagenases are also suspected to contain antioxidant-response elements (AREs) [56].

Metabolic processes

There was an enrichment of GO terms related to a number of metabolic processes, especially lipid metabolism in the LR and MR treatment groups (Table S3). In particular, several transcripts with roles in cholesterol biosynthesis were amongst the most consistently up-regulated across treatment groups. Six transcripts encoding the sterol regulatory element binding transcription factor (*srebf2*) were up-regulated across the LR, MR, HR and LG groups. This regulatory protein plays a key role in controlling cholesterol biosynthesis. Additionally cholesterol 25-hydrolase (*ch25h*) was significantly up-regulated by 5-13 fold in by MR, HR and LG, with increasing trends in the other treatment groups, and lathosterol oxidase (*sc5d*) was up-regulated by LR, MR, HR and LG treatment. This corresponds with previous research where treatment with both glyphosate and Roundup elevated the serum concentration of cholesterol, and triglycerides, in rats [22]. Additionally, a number of transcripts involved in wider lipid metabolism were altered following exposure to environmentally relevant concentrations of glyphosate in flounder [68, 69]. Given the role of lipids as key structural components of cellular membranes, and also in signalling and intracellular transport processes, we hypothesise that this observed increase in lipid metabolism was induced in association with an increased rate of cell proliferation and turnover, and/or to replace lipids damaged by oxidative stress. Additionally, there is some evidence that suggests cholesterol may interact with ROS, and enhance antioxidant and immune response in rainbow trout [70]. Our results also suggest that Roundup may have a more pronounced effect on wider lipid

metabolism than glyphosate, possibly reflecting the toxicity of the surfactants present in Roundup formulation.

A number of transcripts from the solute carrier family of membrane-bound transporters were up-regulated. *slc43*, which transports large, neutral amino acids, was amongst the most up-regulated transcripts (> 10 fold) in LR, MR, HR and LG groups, and amino acid transporters *slc3a2* (MR, HR) and *slc6a16* (LG) were also up-regulated. Additionally, citrate transporter *slc13a5* (HR, MG), monocarboxylic acid transporter *slc16a6b* (HR, LG), fatty acid transporter *slc27a4* (MR) and acetyl-coA transporter *slc33a1* (MR) were all up-regulated. Secondary transport of molecules including glucose and amino acids are among the diverse functions of Na^+/K^+ ATPase (*atp1a2a*), which was up-regulated by 6-8 fold by LR, MR, HR and LG. The associated ion transport regulator (*fxyd5b*), which has a role in regulating Na^+/K^+ ATPase, was also up-regulated by MR and LG, and the potassium channel (*irk11*) was up-regulated in LR, MR, HR and LG. Furthermore, transporters of zinc (*slc39a8*) and iron (*slc25a28*) were up-regulated by MR, and the copper transporter (*slc31a1*) and iron-binding protein ferritin (*fth1b*) were up-regulated by HR. These essential metals are key cofactors in numerous metabolic enzymes. This dominant up-regulation may be associated with an increase in metabolism and cellular turnover.

Energy metabolism

Several transcripts encoding riboflavin transporters (*slc52a3* and *rft2*) were up-regulated by the greatest extent of all differentially expressed transcripts, ranging from 5-30 fold increases across all five treatment groups. Riboflavin (vitamin B2) is the central component of flavoproteins; flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)[71]. Additionally, four transcripts encoding riboflavin kinase (*rfk*), which is a key enzyme in FMN and FAD synthesis, were all up-regulated by a minimum of 2.5 fold by LR, MR, HR and LG. These strong and consistent changes in transcript expression therefore suggest that increased synthesis of FAD and/or FMN was a key response to Roundup and glyphosate exposure. The redox activity of the flavin group makes FAD and FMN essential components of many cellular enzymes, including several found to be up-regulated in this study, for example lathersterol oxidase and apoptosis inducing factors [71, 72], and therefore potentially contributing to the up-regulation of their synthesis. FAD is also essential in the restoration of GSH

from GSSG by glutathione reductase [56]. One of the principal roles of FAD is as the electron carrying cofactor in succinate dehydrogenase, which is a key enzymatic component of both oxidative phosphorylation complex II and the citric acid cycle. FAD is also a coenzyme in the oxidative decarboxylation of pyruvate. FMN is a key component of complex I NADH ubiquitinone dehydrogenase; the first enzyme in the mitochondrial electron transfer chain. Two transcripts encoding somatic cytochrome c (*cycsb*), that transfers electrons between complexes in the electron transfer chain, were also significantly up-regulated by MR and LG, and there were increasing trends in the other groups. Additionally, transcripts encoding leptin (*lep*) were up-regulated by 3-14-fold across MR, HR and LG treatments. This hormone has a key role in regulating energy balance and expenditure, and is also associated with cellular stress response. Overall, this might suggest some up-regulation of aerobic respiration, perhaps to meet the energetic demands of increased cellular turnover, metabolism and other aspects of cellular stress response to glyphosate and Roundup toxicity. This corresponds with existing evidence of an up-regulation of transcripts involved in energy metabolism following exposure to environmentally relevant concentrations of glyphosate in flounder [68] and oyster [73], although very high concentrations of > 84.5 mg/L Roundup were found to impair respiration in isolated rat liver cells [74].

Innate immune system

Functional analysis also revealed enrichment of processes involved in innate immune response. In particular, toll-like receptor (TLR) signalling, RIG-1-like (RLR) receptor signalling and Nod-like receptor (NLR) signalling were enriched Kegg pathways. These pathways involve specific recognition of pathogen-associated molecular patterns and trigger a number of interacting downstream signalling pathways which culminate in cytokine production, recruitment of immune cells and transcriptional changes that constitute immune response [75, 76]. Transcripts encoding toll-like receptor 5b (*tlr5b*), which specifically recognises bacterial flagella, were amongst the most up-regulated transcripts (between 5 and 24 fold increases) by LR, MR, HR and LG, and *tlr21* was also up-regulated in MR. Transcripts encoding NLRC2 and NOD2 were up-regulated in HR and MR respectively, and other transcripts linked to pathogen recognition and response through these signalling pathways were also up-regulated including *unc93a* and *cylda* (MR), *pglyrp6* and

rsad2 (LG). In addition to this, several components of the complement system, *itgax* (LR, MR, HR, MG) and *c7* (MR, HR, LG), were amongst the most consistently up-regulated transcripts across treatment groups. A number of transcripts involved in the regulation of cytokine signalling were also up-regulated. These included transcripts related to interleukins (*il4r, il10r, il17r* (MR and HR), *irak3, nfil3* (MR)), interferons (*irf7, ifit5, mx2* (HR and LG)) and chemokines (*ccl5* (LG) and *ccr4* (HG)) and also suppressors of cytokine signalling *socs2* (HR) and *socs3b* (LG). Additionally, the immune system shares a number of regulatory signalling pathways with those involved in cellular stress response discussed above, including TNF signalling and NF-κB activation [62, 75]. Overall, this reveals up-regulation of signalling pathways involved in innate immune response induced by treatment with both Roundup and glyphosate. This may indicate that the immune system is a target of toxicity and/or that these chemicals increase susceptibility of the fish to pathogen infection. This is supported by previous research which showed differential expression of immune-related transcripts by glyphosate [68, 69, 73] and some evidence that glyphosate formulations modulate the immune system of fish and caimen [77-79], and may alter fish susceptibility to infection and disease [38, 78].

Overall, transcriptional profiling reveals that glyphosate and Roundup exposure induces an alteration of many of the complex, interacting signalling pathways that control cellular stress response, in particular those involved in regulating apoptosis. Cluster analysis and examination of individual transcripts revealed there was a considerable degree of similarity between the transcript expression profiles of fish exposed to all three concentrations of Roundup and the lowest concentration of glyphosate, suggesting common mechanisms of toxicity and cellular response. However, up-regulation of signalling specifically promoting apoptosis was more pronounced in the MR, HR and LG groups, potentially indicating greater cellular toxicity was induced by these treatments. These results are broadly consistent with a cellular response to oxidative stress, and we hypothesise that this mechanism has a central role in the toxicity of both Roundup and glyphosate. We also found evidence indicating an associated increase in cell proliferation and cellular turnover, and an up-regulation of metabolic processes. A strong up-regulation of these compensatory processes associated with cellular stress response is consistent with the observed dominance of up-regulation in the list of differentially expressed transcripts.

Importantly, we found evidence of considerable transcriptional changes in fish exposed to low, environmentally relevant, concentrations of both glyphosate and Roundup, and these were broadly similar to those occurring at higher treatment concentrations which have previously been more widely associated with adverse health effects.

Acknowledgements

We wish to thank Jan Shears for help with the fish husbandry, Ronny van Aerle for advice on the bioinformatics, and Audrey Farbos, Karen Moore and Konrad Paszkiewicz for facilitating the sequencing experiments. This work was funded by a Natural Environment Research Council CASE PhD studentship (Grant number NE/I528326/1) and the Salmon & Trout Association.

Supporting Information

The supporting information contains: comparative statistics for Trinity and Velvet-Oases assemblies (Table S1); multidimensional scaling plots illustrating expression profiles for all treatments (Figure S1); differential expression plots for each treatment (Figure S2); venn diagrams illustrating regulated transcripts using Velvet-Oases assembly (Figure S3); results of ERCC spike control analysis (Figure S4, Table S2, Figure S5); and enriched GO terms and Kegg pathways in each treatment group (Table S3).

References

1. Schonbrunn, E.; Eschenburg, S.; Shuttleworth, W. A.; Schloss, J. V.; Amrhein, N.; Evans, J. N. S.; Kabsch, W., Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proceedings of the National Academy of Sciences* **2001,** *98*, (4), 1376-1380.

2. Steinrucken, H. C.; Amrhein, N., The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochemical and Biophysical Research Communications* **1980,** *94*, (4), 1207-1212.

3. European_Commission, The use of plant protection products in the European Union. E*urostat* **2007**.

4. US_EPA, Pesticides Industry Sales and Usage: 2006 and 2007 Market Estimates. **2011**.

5. Botta, F.; Lavison, G. l.; Couturier, G.; Alliot, F.; Moreau-Guigon, E.; Fauchon, N.; Guery, B. n. d.; Chevreuil, M.; Blanchoud, H. l. Transfer of glyphosate and its degradate AMPA to surface waters through urban sewerage systems. *Chemosphere* **2009,** *77*, (1), 133-139.

6. Brausch, J. M.; Smith, P. N., Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, Thamnocephalus platyurus. *Archives of Environmental Contamination and Toxicology* **2007,** *52*, (2), 217-221.

7. Giesy, J. P.; Dobson, S.; Solomon, K. R., Ecotoxicological risk assessment for Roundup herbicide. *Reviews of environmental contamination & toxicology* **2000,** *167*, 35-120.

8. Byer, J. D.; Struger, J.; Klawunn, P.; Todd, A.; Sverko, E., Low cost monitoring of glyphosate in surface waters using the ELISA method: An evaluation. *Environmental Science & Technology* **2008,** *42*, (16), 6052-6057.

9. Struger, J.; Thompson, D.; Staznik, B.; Martin, P.; McDaniel, T.; Marvin, C., Occurrence of glyphosate in surface waters of southern Ontario. *Bulletin of Environmental Contamination and Toxicology* **2008,** *80*, (4), 378-384.

10. Battaglin, W. A.; Rice, K. C.; Focazio, M. J.; Salmons, S.; Barry, R. X., The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005-2006. *Environ. Monit. Assess.* **2009,** *155*, (1), 281-307.

11. MLHB, Determination of Glyphosate residues in human urine samples from 18 European countries. *Report Glyphosate MLHB-2013-06-06* **2013**.

12. Cavalcante, D.; Martinez, C.; Sofia, S., Genotoxic effects of Roundup on the fish *Prochilodus lineatus*. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **2008,** *655*, (1), 41-46.

13. Modesto, K. A.; Martinez, C. u. B., Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus.* *Chemosphere* **2010,** *78*, (3), 294-299.

14. Cavas, T.; Konen, S., Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay. *Mutagenesis* **2007,** *22*, (4), 263-268.

15. Lushchak, O. V.; Kubrak, O. I.; Storey, J. M.; Storey, K. B.; Lushchak, V. I., Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. *Chemosphere* **2009,** *76*, (7), 932-937.

16. Ferreira, D.; Costa da Motta, A.; Kreutz, L. C.; Toni, C.; Loro, V.; Barcellos, L., Assessment of oxidative stress in *Rhamdia quelen* exposed to agrichemicals. *Chemosphere* **2010,** *79*, (9), 914-921.

17. Guilherme, S.; Gaivao, I.; Santos, M.; Pacheco, M., European eel (Anguilla anguilla) genotoxic and pro-oxidant responses following short-term exposure to Roundup, a glyphosate-based herbicide. *Mutagenesis* **2010,** *25*, (5), 523-530.

18. Guilherme, S.; Santos, M.; Barroso, C.; Gaivao, I.; Pacheco, M., Differential genotoxicity of Roundup formulation and its constituents in blood cells of fish (Anguilla anguilla): considerations on chemical interactions and DNA damaging mechanisms. *Ecotoxicology* **2012**, 1-10.

19. Guilherme, S.; Gaivao, I.; Santos, M.; Pacheco, M., DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide “Elucidation of organ-specificity and the role of oxidative stress. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **2012**.

20. de Castilhos Ghisi, N.; Cestari, M. M., Genotoxic effects of the herbicide Roundup in the fish Corydoras paleatus (Jenyns 1842) after short-term, environmentally low concentration exposure. *Environ. Monit. Assess.* **2012**, 1-7.

21. de Castilhos Ghisi, N.; Cestari, M. M., Genotoxic effects of the herbicide Roundup in the fish Corydoras paleatus (Jenyns 1842) after short-term,

environmentally low concentration exposure. *Environ. Monit. Assess.* **2013,** *185*, (4), 1-7.

22. El-Shenawy, N. S., Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. *Environmental Toxicology and Pharmacology* **2009,** *28*, (3), 379-385.

23. Astiz, M.; Alaniz, M. a. J. d.; Marra, C. A., Effect of pesticides on cell survival in liver and brain rat tissues. *Ecotoxicology and Environmental Safety* **2009,** *72*, (7), 2025-2032.

24. Gasnier, C.; Dumont, C.; Benachour, N.; Clair, E.; Chagnon, M. C.; Seralini, G. E., Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology* **2009,** *262*, 184-191.

25. Clair, A.; Mesnage, R.; Travert, C.; Seralini, G. E., A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. *Toxicology in Vitro* **2012,** *26*, (2), 269-279.

26. Kim, Y.; Hong, J.; Gil, H.; Song, H.; Hong, S., Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. *Toxicology in Vitro* **2013,** *1*, 191-197.

27. Mesnage, R.; Bernay, B.; Seralini, G.-E., Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. *Toxicology* **2012**.

28. Benachour, N.; Seralini, G.-E., Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chemical Research in Toxicology* **2008,** *22*, (1), 97-105.

29. Walsh, L. P.; McCormick, C.; Martin, C.; Stocco, D. M., Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. *Environmental Health Perspectives* **2000,** *108*, (8), 769.

30. Richard, S.; Moslemi, S.; Sipahutar, H.; Benachour, N.; Seralini, G. Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environmental Health Perspectives* **2005,** *113*, (6), 716.

31. Benachour, N.; Sipahutar, H.; Moslemi, S.; Gasnier, C.; Travert, C.; Seralini, G., Time-and dose-dependent effects of roundup on human embryonic and placental cells. *Archives of Environmental Contamination and Toxicology* **2007,** *53*, (1), 126-133.

32. Romano, M. A.; Romano, R. M.; Santos, L. D.; Wisniewski, P.; Campos, D. A.; de Souza, P. B.; Viau, P.; Bernardi, M. M.; Nunes, M. T.; de Oliveira, C. A.,

Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. *Archives of Toxicology* **2012,** *86*, (4), 663-673.

33. Romano, R. M.; Romano, M. A.; Bernardi, M. M.; Furtado, P. V.; Oliveira, C. A., Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. *Archives of Toxicology* **2010,** *84*, 309-317.

34. Dallegrave, E.; Mantese, F. D.; Oliveira, R. T.; Andrade, A. J.; Dalsenter, P. R.; Langeloh, A., Pre-and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. *Archives of Toxicology* **2007,** *81*, (9), 665-673.

35. Uren Webster, T. M.; Laing, L. V.; Florance, H.; Santos, E. M., Effects of glyphosate and its formulation, Roundup, on reproduction in zebrafish (*Danio rerio*). **Submitted**.

36. Glusczak, L.; Miron, D. d. S.; Moraes, B. S.; Simaues, R. l. R.; Schetinger, M. R. C.; Morsch, V. M.; Loro, V. n. L., Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish *Rhamdia quelen*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **2007,** *146*, (4), 519-524.

37. Kreutz, L. C.; Gil Barcellos, L. J.; de Faria Valle, S.; de Oliveira Silva, T. l.; Anziliero, D.; Davi dos Santos, E.; Pivato, M.; Zanatta, R., Altered hematological and immunological parameters in silver catfish *Rhamdia quelen* following short term exposure to sublethal concentration of glyphosate. *Fish & Shellfish Immunology* **2011,** *30*, (1), 51-57.

38. Kelly, D. W.; Poulin, R.; Tompkins, D. M.; Townsend, C. R., Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival. *Journal of Applied Ecology* **2010,** *47*, (2), 498-504.

39. Howe, C. M.; Berrill, M.; Pauli, B. D.; Helbing, C. C.; Werry, K.; Veldhoen, N., Toxicity of glyphosate based pesticides to four North American frog species. *Environmental Toxicology and Chemistry* **2004,** *23*, (8), 1928-1938.

40. Relyea, R. A., New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecol. Appl.* **2012,** *22*, (2), 634-647.

41. Paull, G. C.; Van Look, K. J. W.; Santos, E. M.; Filby, A. L.; Gray, D. M.; Nash, J. P.; Tyler, C. R., Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing

population-level effects of environmental chemicals. *Aquat. Toxicol.* **2008,** *87*, 115-126.

42. Hansen, K. D.; Brenner, S. E.; Dudoit, S., Biases in Illumina transcriptome sequencing caused by random hexamer priming. *Nucleic Acids Research* **2010,** *38*, (12), e131.

43. Brown, C. T.; Howe, A.; Zhang, Q.; Pyrkosz, A. B.; Brom, T. H., A reference-free algorithm for computational normalization of shotgun sequencing data. *arXiv:1203.4802 [q-bio.GN]* **2012**.

44. Francis, W. R.; Christianson, L. M.; Kiko, R.; Powers, M. L.; Shaner, N. C.; Haddock, S. H., A comparison across non-model animals suggests an optimal sequencing depth for de novo transcriptome assembly. *BMC Genomics* **2013,** *14*, (1), 167.

45. Zerbino, D. R.; Birney, E., Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. *Genome Research* **2008,** *18*, (5), 821-829.

46. Schulz, M. H.; Zerbino, D. R.; Vingron, M.; Birney, E., Oases: Robust de novo RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics* **2012**.

47. Grabherr, M. G.; Haas, B. J.; Yassour, M.; Levin, J. Z.; Thompson, D. A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q., Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **2011,** *29*, (7), 644-652.

48. Langmead, B.; Salzberg, S. L., Fast gapped-read alignment with Bowtie 2. *Nature Methods* **2012,** *9*, 357-359.

49. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R., The sequence alignment/map format and SAMtools. *Bioinformatics* **2009,** *25*, (16), 2078-2079.

50. Robinson, M. D.; McCarthy, D. J.; Smyth, G. K., edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010,** *26*, (1), 139-140.

51. Huang, D. W.; Sherman, B. T.; Lempicki, R. A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* **2008,** *4*, (1), 44-57.

52. Lu, B.; Zeng, Z.; Shi, T., Comparative study of de novo assembly and genome-guided assembly strategies for transcriptome reconstruction based on RNA-Seq. *Science China Life Sciences* **2013,** *56*, (2), 143-155.

53. Langmead, B.; Salzberg, S. L., Fast gapped-read alignment with Bowtie 2. *Nat Meth* **2012,** *9*, (4), 357-359.

54. Martindale, J. L.; Holbrook, N. J., Cellular response to oxidative stress: Signaling for suicide and survival*. *Journal of cellular physiology* **2002,** *192*, (1), 1-15.

55. Bauer, M.; Bauer, I., Heme oxygenase-1: redox regulation and role in the hepatic response to oxidative stress. *Antioxidants and Redox Signaling* **2002,** *4*, (5), 749-758.

56. Di Giulio, R. T.; Meyer, J. N., *Reactive Oxygen Species and Oxidative Stress*. Taylor and Francis: 2008.

57. Kalmar, B.; Greensmith, L., Induction of heat shock proteins for protection against oxidative stress. *Advanced drug delivery reviews* **2009,** *61*, (4), 310-318.

58. Andreef, M.; Goodrich, D. W.; Pardee, A. B., Cell Proliferation, Differentiation, and Apoptosis. In *Holland-Frei Cancer Medicine*, 5th ed.; Bast, R. C.; Pollock, R. E., Eds. Hamilton (ON): BC Decker: 2000.

59. Mates, J.; Segura, J. A.; Alonso, F. J.; Marquez, J., Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. *Archives of Toxicology* **2008,** *82*, (5), 273-299.

60. Chang, L.; Karin, M., Mammalian MAP kinase signalling cascades. *Nature* **2001,** *410*, (6824), 37-40.

61. Tanos, T.; Marinissen, M. J.; Leskow, F. C.; Hochbaum, D.; Martinetto, H.; Gutkind, J. S.; Coso, O. A., Phosphorylation of c-Fos by Members of the p38 MAPK Family ROLE IN THE AP-1 RESPONSE TO UV LIGHT. *Journal of Biological Chemistry* **2005,** *280*, (19), 18842-18852.

62. Wajant, H.; Pfizenmaier, K.; Scheurich, P., Tumor necrosis factor signaling. *Cell Death & Differentiation* **2003,** *10*, (1), 45-65.

63. Ermak, G.; Davies, K. J., Calcium and oxidative stress: from cell signaling to cell death. *Molecular immunology* **2002,** *38*, (10), 713-721.

64. Ouyang, L.; Shi, Z.; Zhao, S.; Wang, F., T; Zhou, T., T; Liu, B.; Bao, J., K, Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation* **2012,** *45*, (6), 487-498.

65. Welch, C.; Santra, M. K.; El-Assaad, W.; Zhu, X.; Huber, W. E.; Keys, R. A.; Teodoro, J. G.; Green, M. R., Identification of a protein, G0S2, that lacks Bcl-2 homology domains and interacts with and antagonizes Bcl-2. *Cancer Research* **2009,** *69*, (17), 6782-6789.

66. Dbaibo, G. S.; El-Assaad, W.; Krikorian, A.; Liu, B.; Diab, K.; Idriss, N. Z.; El-Sabban, M.; Driscoll, T. A.; Perry, D. K.; Hannun, Y. A., Ceramide generation by two distinct pathways in tumor necrosis factor Î±-induced cell death. *Febs Letters* **2001,** *503*, (1), 7-12.

67. Siwik, D. A.; Pagano, P. J.; Colucci, W. S., Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. *American Journal of Physiology-Cell Physiology* **2001,** *280*, (1), C53-C60.

68. Marchand, J.; Tanguy, A.; Charrier, G.; Quiniou, L.; Plee-Gauthier, E.; Laroche, J., Molecular identification and expression of differentially regulated genes of the European flounder, Platichthys flesus, submitted to pesticide exposure. *Marine Biotechnology* **2006,** *8*, (3), 275-294.

69. Evrard, E.; Devaux, A.; Bony, S.; Burgeot, T.; Riso, R.; Budzinski, H.; Du, M. L.; Quiniou, L.; Laroche, J., Responses of the European flounder Platichthys flesus to the chemical stress in estuaries: Load of contaminants, gene expression, cellular impact and growth rate. *Biomarkers* **2010,** *15*, (2), 111-127.

70. Deng, J.; Kang, B.; Tao, L.; Rong, H.; Zhang, X., Effects of dietary cholesterol on antioxidant capacity, non-specific immune response, and resistance to Aeromonas hydrophila in rainbow trout (Oncorhynchus mykiss) fed soybean meal-based diets. *Fish & Shellfish Immunology* **2013,** *34*, (1), 324-331.

71. Van Berkel, W.; Kamerbeek, N.; Fraaije, M., Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. *Journal of biotechnology* **2006,** *124*, (4), 670-689.

72. Vahsen, N.; Cande, C.; Briere, J.-J.; Benit, P.; Joza, N.; Larochette, N.; Mastroberardino, P. G.; Pequignot, M. O.; Casares, N.; Lazar, V., AIF deficiency compromises oxidative phosphorylation. *The EMBO journal* **2004,** *23*, (23), 4679-4689.

73. Tanguy, A.; Boutet, I.; Laroche, J.; Moraga, D., Molecular identification and expression study of differentially regulated genes in the Pacific oyster Crassostrea gigas in response to pesticide exposure. *FEBS Journal* **2005,** *272*, (2), 390-403.

74. Peixoto, F., Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. *Chemosphere* **2005,** *61*, (8), 1115-1122.

75. Kawai, T.; Akira, S., Toll-like Receptor and RIG-1-like Receptor Signaling. *Annals of the New York Academy of Sciences* **2008,** *1143*, (1), 1-20.

76. Janeway Jr, C. A.; Medzhitov, R., Innate immune recognition. *Annu. Rev. Immunol.* **2002,** *20*, (1), 197-216.

77. El-Gendy, K.; Aly, N.; El-Sebae, A., Effects of edifenphos and glyphosate on the immune response and protein biosynthesis of bolti fish (Tilapia nilotica). *Journal of Environmental Science & Health Part B* **1998,** *33*, (2), 135-149.

78. Kreutz, L. C.; Gil Barcellos, L. J.; Marteninghe, A.; Davi dos Santos, E.; Zanatta, R., Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (Rhamdia quelen) to Aeromonas hydrophila challenge. *Fish & Shellfish Immunology* **2010,** *29*, (4), 694-697.

79. Latorre, M. A.; Lopez Gonzallez, E. C.; Larriera, A.; Poletta, G. L.; Siroski, P. A., Effects of in vivo exposure to Roundup on immune system of Caiman latirostris. *Journal of immunotoxicology* **2012**, 1-6.

Figure Legends

Figure 1. Venn diagrams displaying the numbers of differentially expressed transcripts (FDR<0.1) in each treatment group obtained from EdgeR using the Trinity assembly. Red and green numbers represent up- and down-regulated transcripts, respectively. Treatments are represented by the following codes: LR, MR and HR represent 0.01, 0.5 and 10 mg/L Roundup, and LG and HG represent 0.01 and 10 mg/L glyphosate.

Figure 2. Heatmap illustrating changes in expression level of all differentially expressed transcripts across treatment groups. Data presented are the mean log2 fold change in expression level in each treatment group compared to the control. Hierarchical trees were generated using an Euclidean distance metric. Treatments are represented by the following codes: LR, MR and HR represent 0.01, 0.5 and 10 mg/L Roundup, and LG and HG represent 0.01 and 10 mg/L glyphosate.

Figure 3. Kegg pathway representing *Apoptosis*, which was found to be over-represented in the list of differentially-regulated transcripts in all treatment groups. Individual differentially expressed transcripts and enriched related processes are highlighted in red.

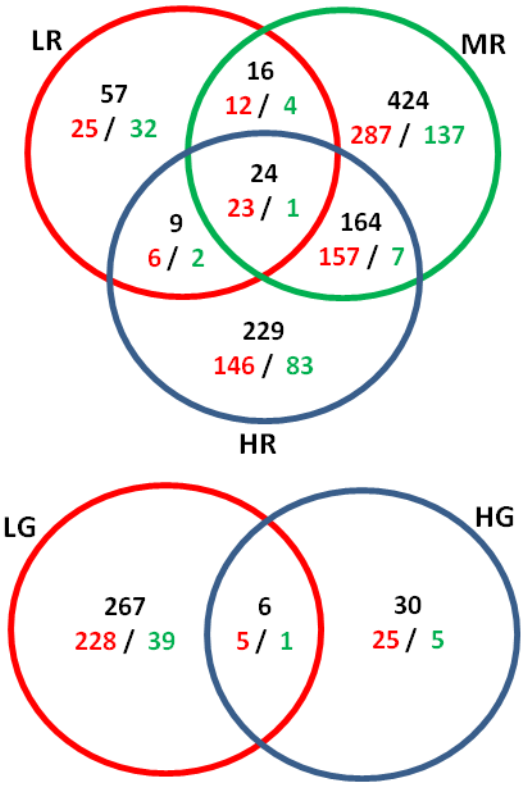

LR
MR
HR
57
25 / 32
16
12 / 4
424
287 / 137
24
23 / 1
9
6 / 2
164
157 / 7
229
146 / 83
LG
HG
267
228 / 39
6
5 / 1
30
25 / 5

Figure 1

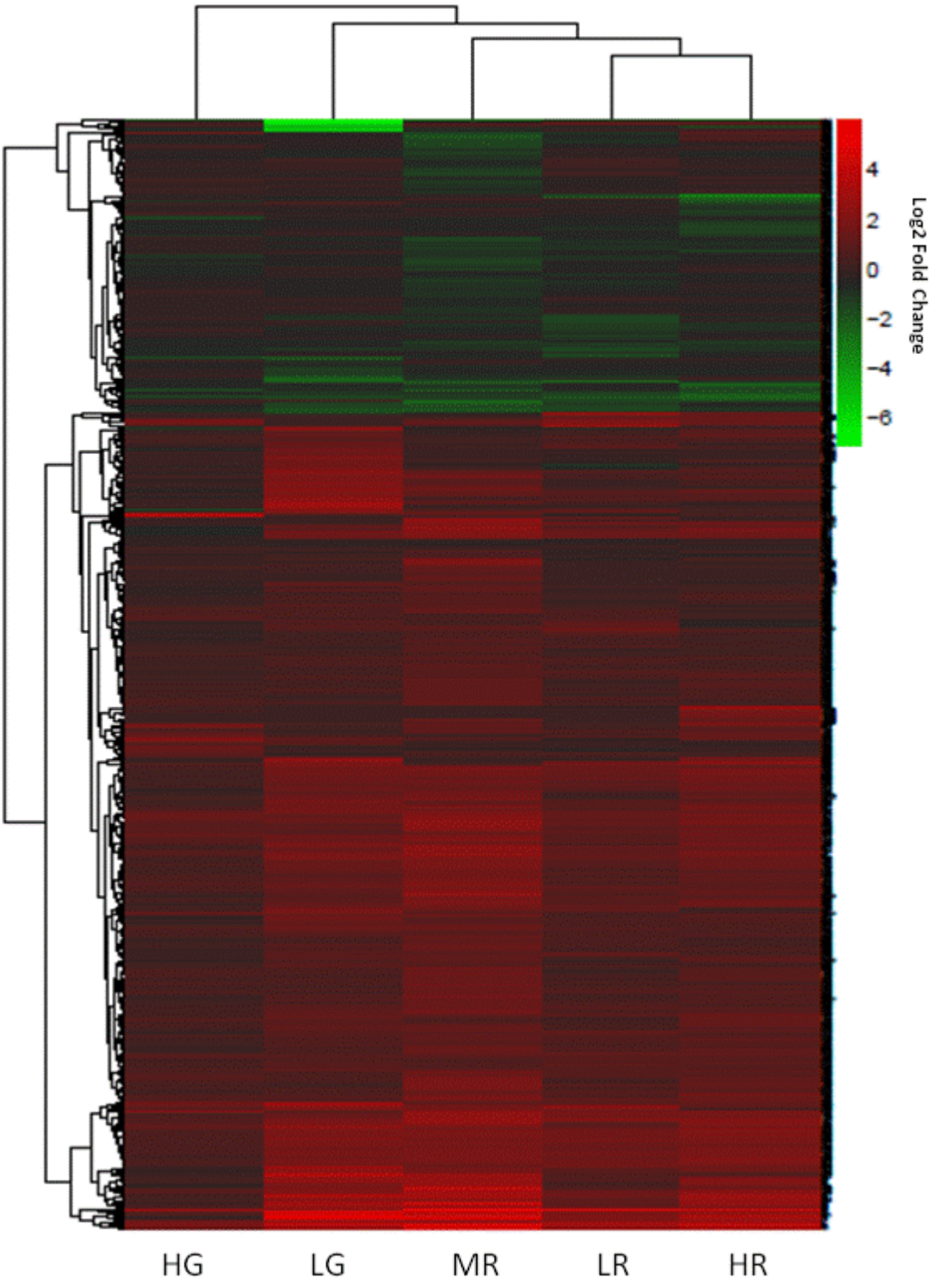


Figure 2

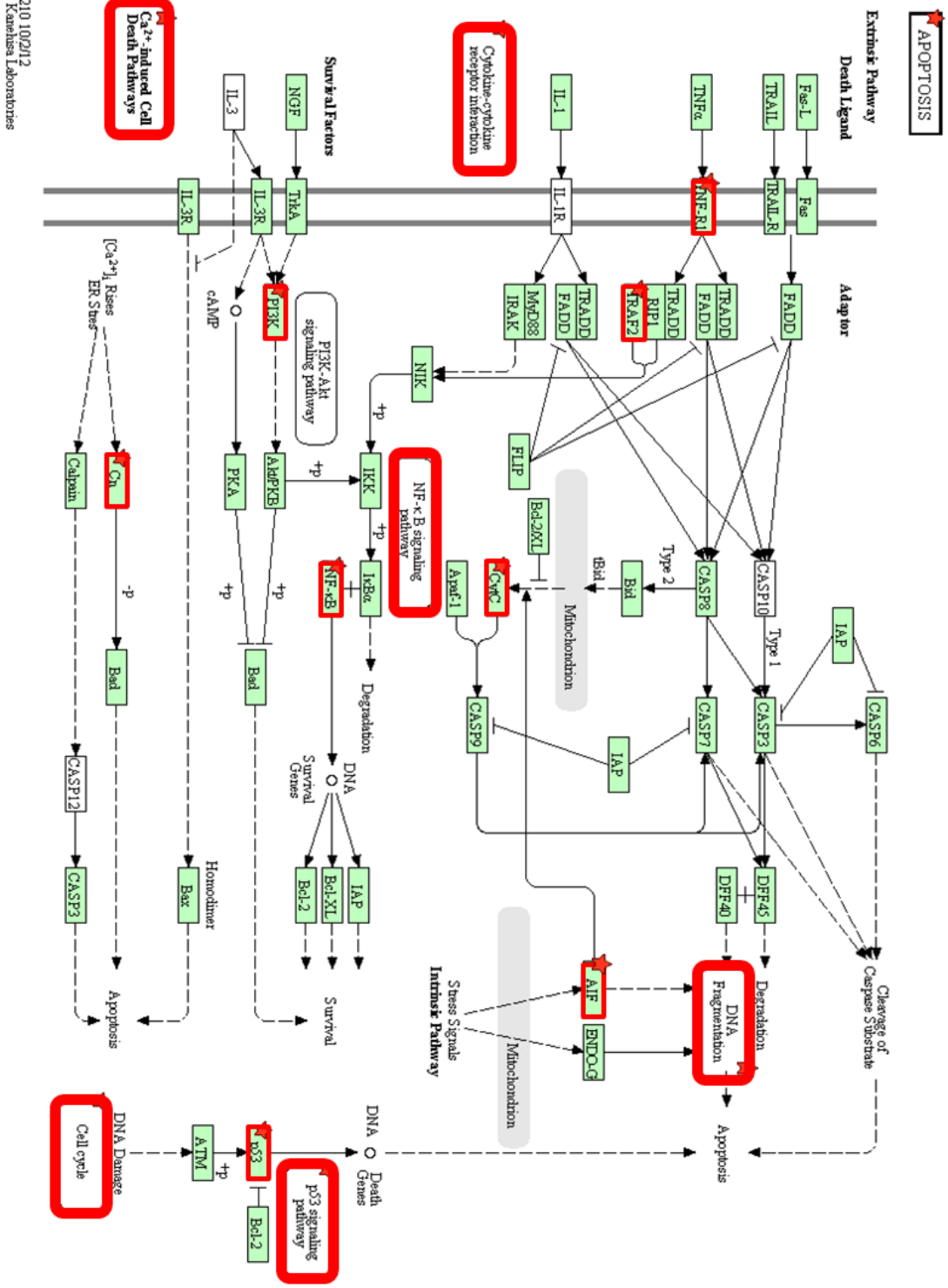

APOPTOSIS
Extrinsic Pathway
Death Ligand
Adaptor
Fas-L
TRAIL
TNFα
IL-1
Fas
TRAIL-R
TNF-R1
IL-1R
FADD
TRADD
RIP1
TRAF2
MyD88
IRAK
NIK
FLIP
Bcl-2/XL
Bid
tBid
Type 2
CASP8
CASP10
Type 1
CASP3
CASP6
CASP7
CASP9
IAP
Mitochondrion
CytC
Apaf-1
Cytokine-cytokine receptor interaction
Ca2+ induced Cell Death Pathways
Survival Factors
IL-3
NGF
IL-3R
TrkA
PI3K
PI3K-Akt signaling pathway
AMPK
PKA
cAMP
IKK
NF-κB signaling pathway
NF-κB
IκBα
Degradation
DNA
Survival Genes
Bcl-2
Bcl-XL
IAP
Survival
[Ca2+]i Rises
ER Stres
Calpain
Cn
-p
Bad
CASP12
CASP3
Apoptosis
Homodimer
Bax
Cleavage of Caspase Substrate
DFF40
DFF45
DNA Fragmentation
Degradation
Stress Signals
Intrinsic Pathway
AIF
ENDO-G
Mitochondrion
Apoptosis
DNA
Death Genes
p53
p53 signaling pathway
Bcl-2
ATM
DNA Damage
Cell cycle
04210 10/2/12
(c) Kanehisa Laboratories

Figure 3

Supporting Information

Global transcriptomic profiling demonstrates induction of oxidative stress and compensatory stress responses in brown trout exposed to glyphosate and Roundup.

Tamsyn. M. Uren Webster[1] and Eduarda M. Santos[1]

1. Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

This Supporting Information contains:

Assembly	No. transcripts	No. Loci	Mean length (bp)	N50 (bp)	% annotated	No. zf transcript annotations	% reads re-mapped	No. transcripts retained by EdgeR	No. retained zf transcript annotations	Mean BCV
Velvet-Oases	893,904	146,233	1198.4	2012	47 %	19,893	94 %	115,217	8,966	37.4 %
Trinity	258,702	109,301	1065.6	2107	45 %	17,852	89 %	67,954	11,886	34.6 %

Table S1. Comparative summary statistics describing the *de novo* transcriptome assemblies constructed using the Velvet-Oases and Trinity pipelines. Data presented include the number and length of transcripts built for each assembly and the percentage of transcripts annotated using Blastx against Ensembl peptide databases using an e-value cut off < $1e^{-15}$; the percentage of raw reads that re-mapped against each assembly using Bowtie2; the number of transcripts that passed the imposed criteria for inclusion in EdgeR analysis (at least 1 mapped read in at least 6 replicate libraries) and the number of unique zebrafish transcript annotations included within these; and the mean biological coefficient of variation (BCV) calculated using EdgeR for each treatment group.

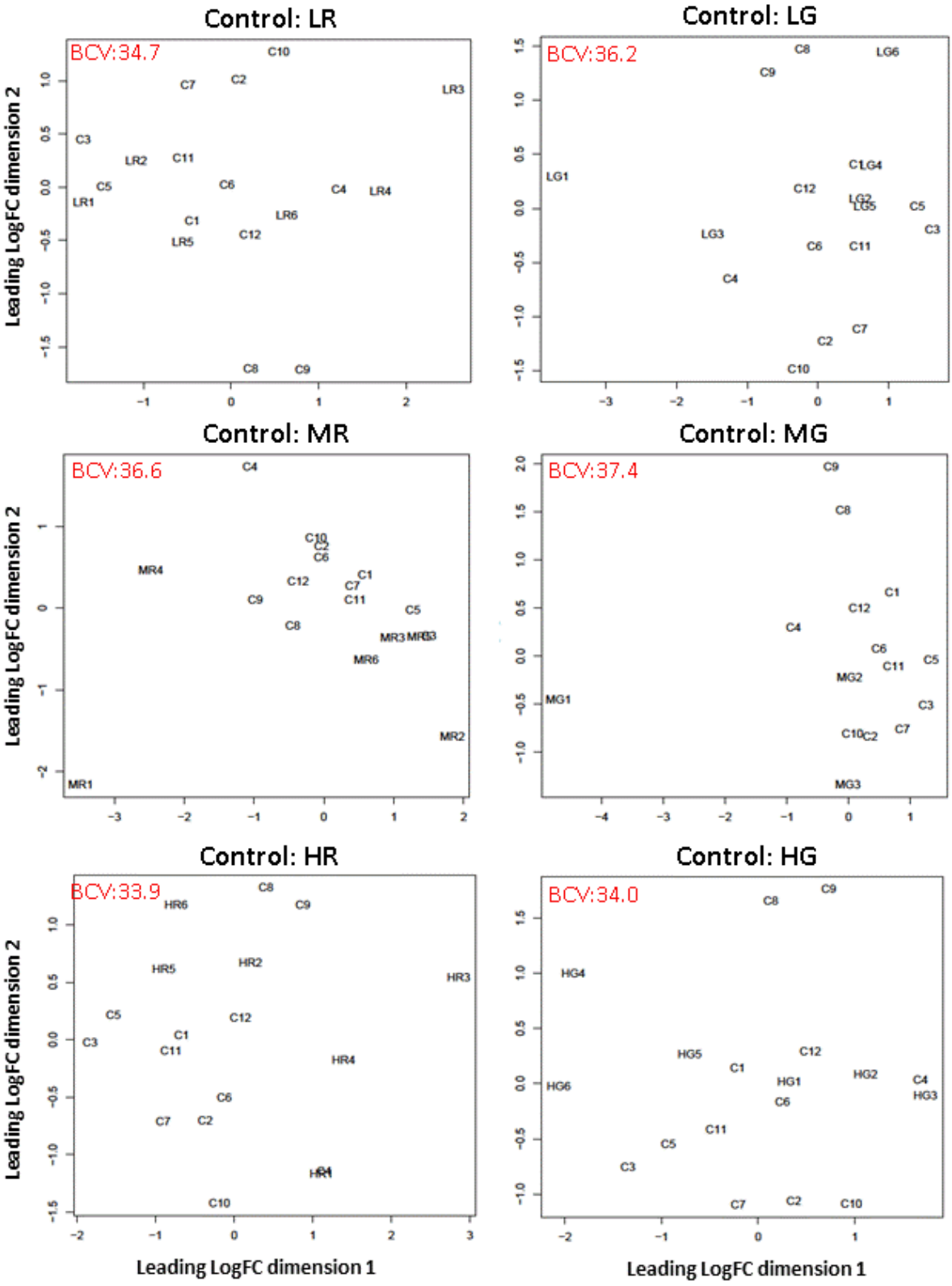


Figure S1. Multidimensional scaling plots illustrating the similarity of expression profiles for individual replicates in each treatment group compared to the control group, based on the expression of all transcripts in each pairwise comparison. Treatments are represented by the following codes: LR, MR and HR represent 0.01, 0.5 and 10 mg Roundup/L, and LG, MG and HG represent 0.01, 0.5 and 10 mg glyphosate /L.

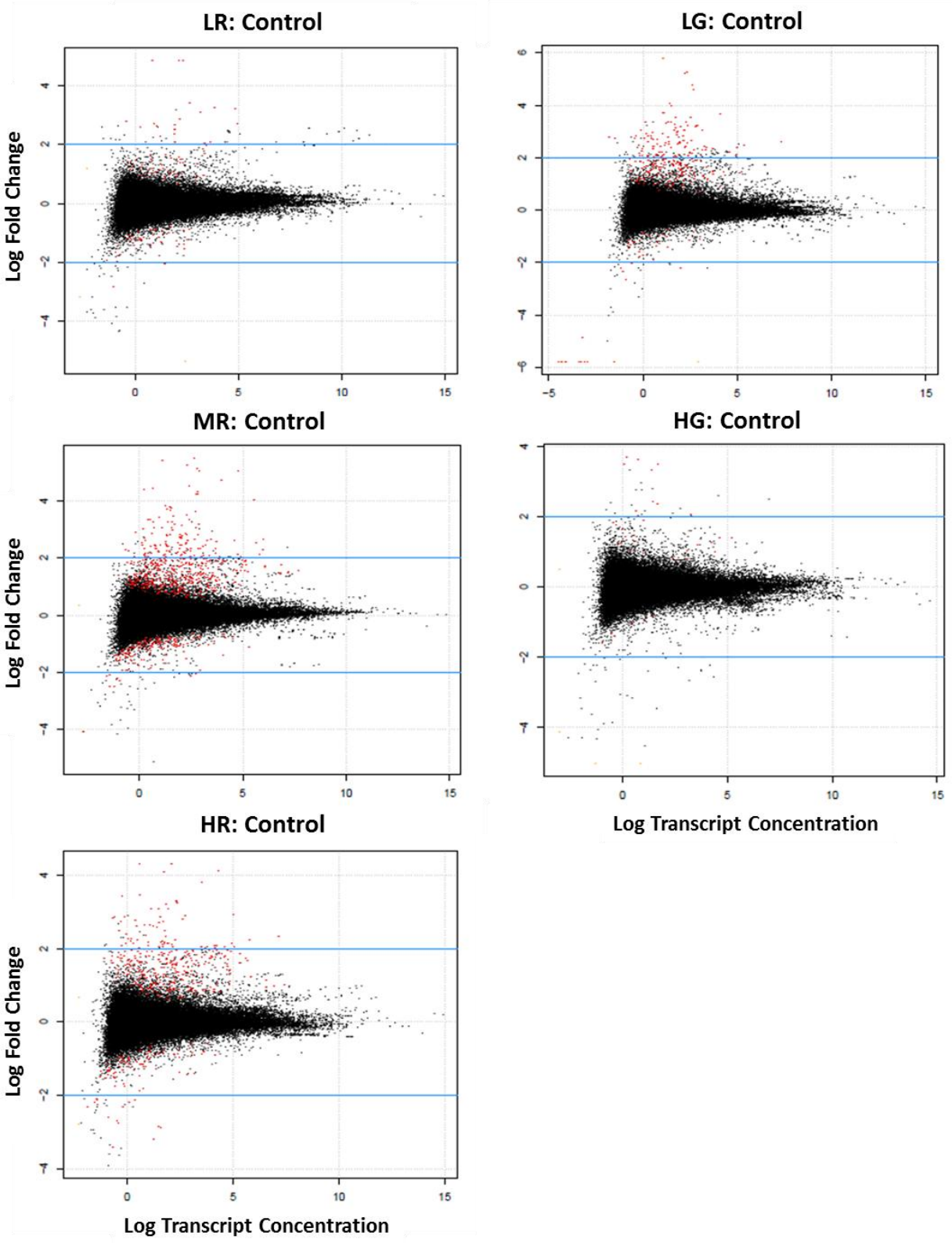


Figure S2. Smear plots illustrating differential expressed transcripts for each treatment group. Values plotted represent the concentration and fold change compared to the control group for all transcripts included in each pairwise test. Red dots represent differentially expressed transcripts. Treatments are represented by the following codes: LR, MR and HR represent 0.01, 0.5 and 10 mg Roundup/L and LG and HG represent 0.01 and 10 mg glyphosate/L.

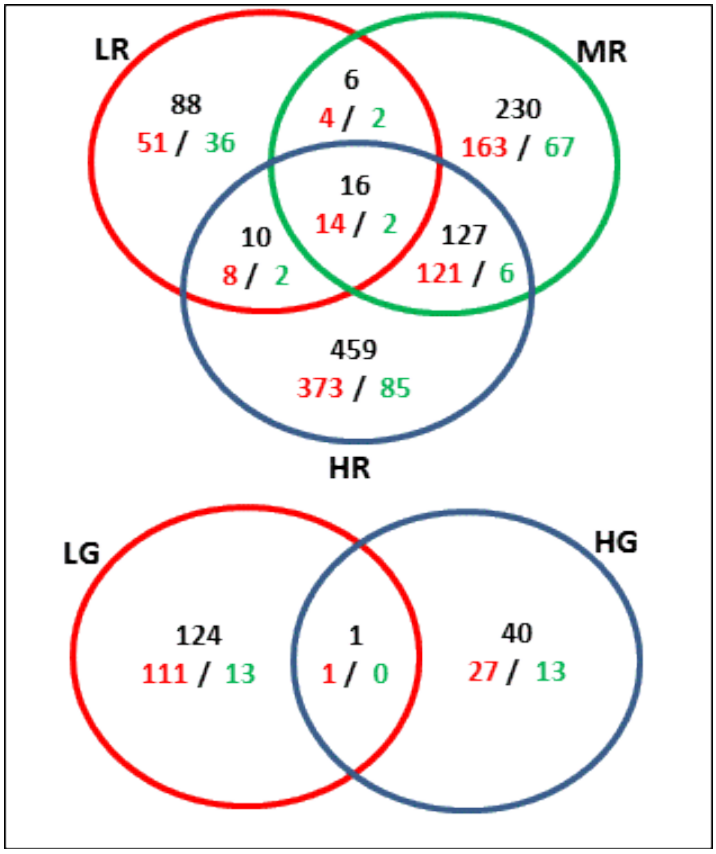
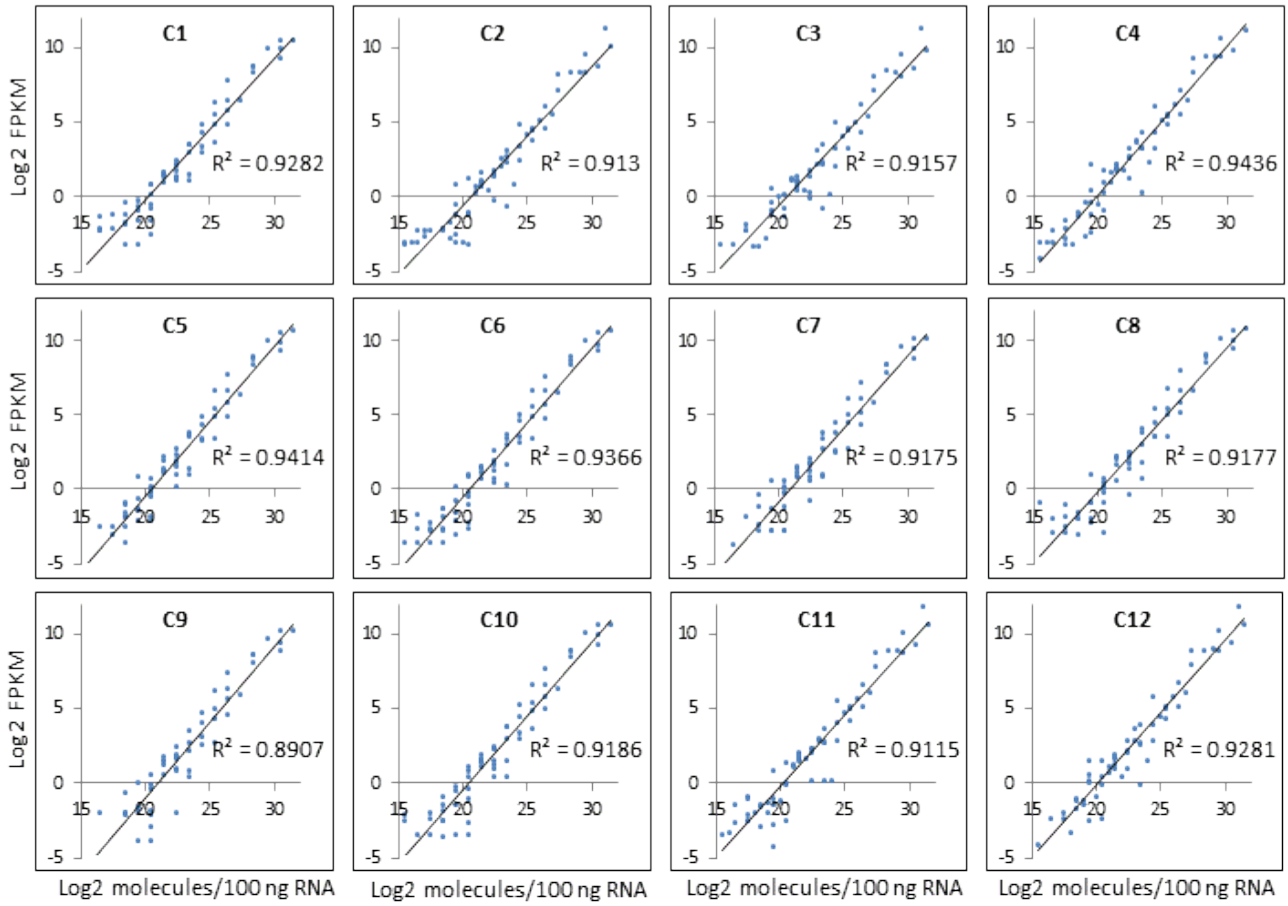
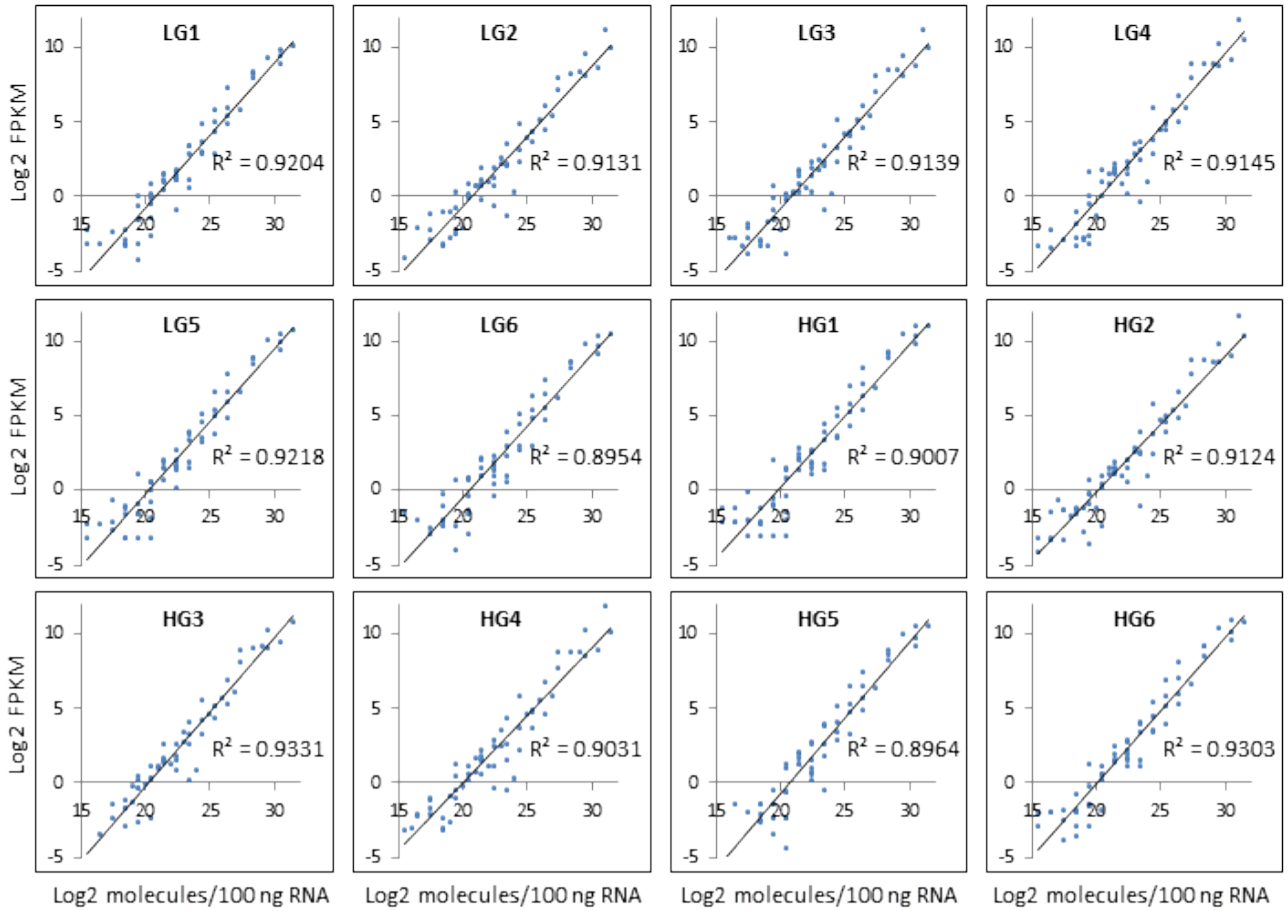


Figure S3. Venn diagrams illustrating the numbers of differentially expressed transcripts (FDR<0.1) and overlaps between differentially expressed transcripts in each treatment group, obtained from EdgeR, based on the Velvet-Oases assembly. Red and green numbers represent up- and down-regulated transcripts, respectively. Treatments are represented by the following codes: LR, MR and HR represent 0.01, 0.5 and 10 mg Roundup/L and LG and HG represent 0.01 and 10 mg glyphosate/L.


C1
R² = 0.9282
C2
R² = 0.913
C3
R² = 0.9157
C4
R² = 0.9436
C5
R² = 0.9414
C6
R² = 0.9366
C7
R² = 0.9175
C8
R² = 0.9177
C9
R² = 0.8907
C10
R² = 0.9186
C11
R² = 0.9115
C12
R² = 0.9281
Log2 FPKM
Log2 molecules/100 ng RNA


LG1
R² = 0.9204
LG2
R² = 0.9131
LG3
R² = 0.9139
LG4
R² = 0.9145
LG5
R² = 0.9218
LG6
R² = 0.8954
HG1
R² = 0.9007
HG2
R² = 0.9124
HG3
R² = 0.9331
HG4
R² = 0.9031
HG5
R² = 0.8964
HG6
R² = 0.9303
Log2 FPKM
Log2 molecules/100 ng RNA

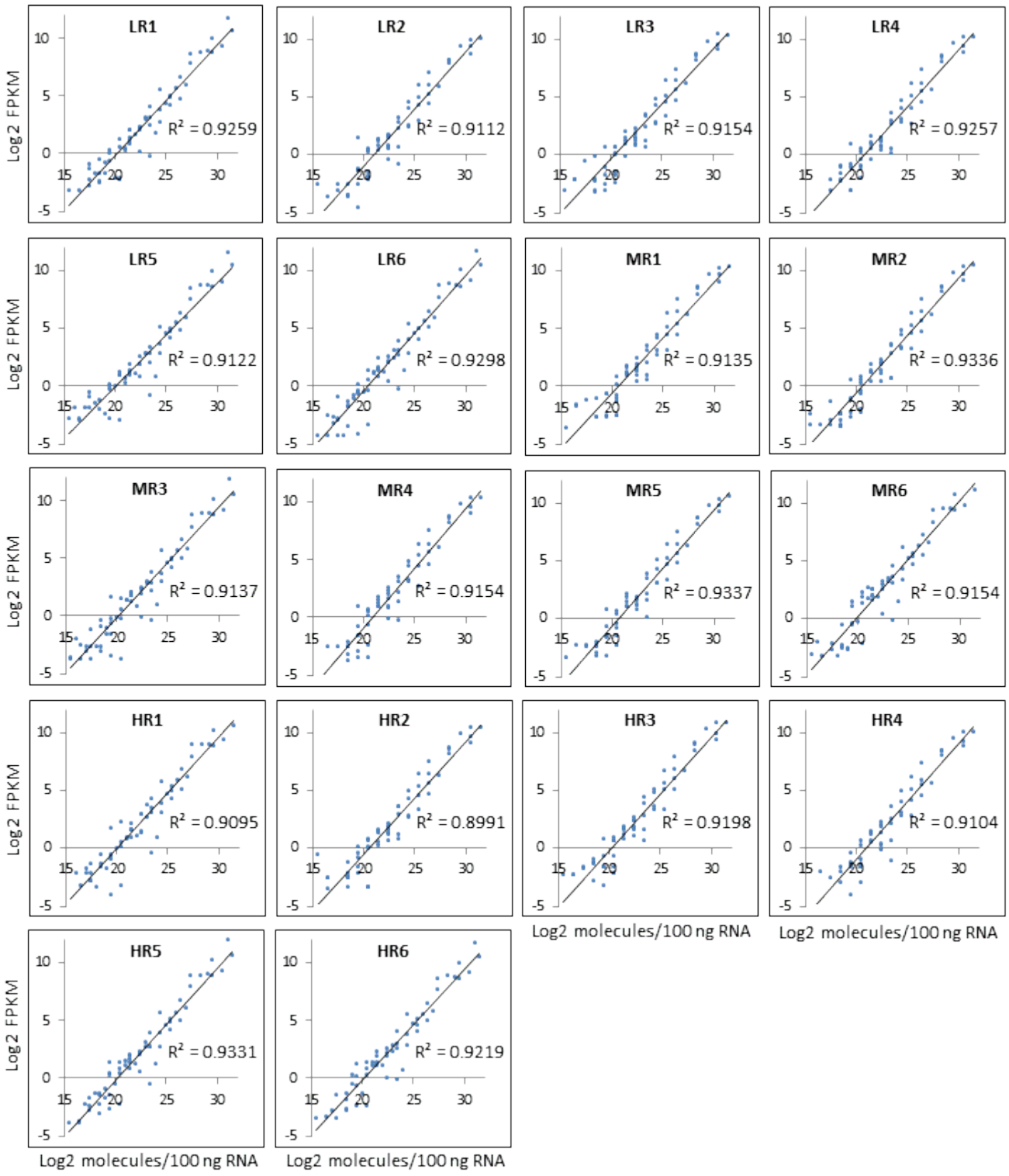


Figure S4. External RNA Controls Consortium **(**ERCC) spike-in control analysis for all individual liver samples sequenced in this project. Graphs show the relationship between the calculated expression level (FPKM) and the expected concentration of each control transcripts. Individual fish are represented by the following codes: C1-C12 represent the control individuals; LR1-LR6 represent individuals exposed to 0.01 mg Roundup/L; MR1-MR6 represent individuals exposed to 0.5 mg Roundup/L; HR1-HR6 represent individuals exposed to 10 mg Roundup/L; LG1-LG6 represent individuals exposed to 0.01 mg glyphosate/L; and HG1-HG6 represent individuals exposed to 10 mg glyphosate/L.

Table S2. Dynamic range calculated based on the measurements for ERCC spike-in control transcripts for all individual libraries sequenced. Values presented are log2 transformed maximum-minimum FPKM values calculated for ERCC spike-in control transcripts. Only transcripts that had at least 1 mapped read in a minimum of 6 replicate samples were included in the analysis. Individual fish are represented by the following codes: C1-C12 represent the control individuals; LR1-LR6 represent individuals exposed to 0.01 mg Roundup/L; MR1-MR6 represent individuals exposed to 0.5 mg Roundup/L; HR1-HR6 represent individuals exposed to 10 mg Roundup/L; LG1-LG6 represent individuals exposed to 0.01 mg glyphosate/L; and HG1-HG6 represent individuals exposed to 10 mg glyphosate/L.

Sample	Log2 dynamic range (FPKM)	Sample	Log2 dynamic range (FPKM)	Sample	Log2 dynamic range (FPKM)
C1	13.74	LG1	14.36	LR1	14.91
C2	14.52	LG2	14.52	LR2	14.64
C3	14.54	LG3	15.16	LR3	13.71
C4	15.61	LG4	15.32	LR4	13.32
C5	14.21	LG5	13.92	LR5	14.53
C6	14.29	LG6	14.49	LR6	16.05
C7	13.88	HG1	14.18	MR1	14.00
C8	13.83	HG2	15.26	MR2	13.97
C9	14.11	HG3	15.51	MR3	15.60
C10	14.28	HG4	14.99	MR4	14.07
C11	16.19	HG5	14.92	MR5	13.86
C12	15.26	HG6	14.76	MR6	15.70
				HR1	16.08
				HR2	13.95
				HR3	14.08
				HR4	14.08
				HR5	15.75
				HR6	15.19

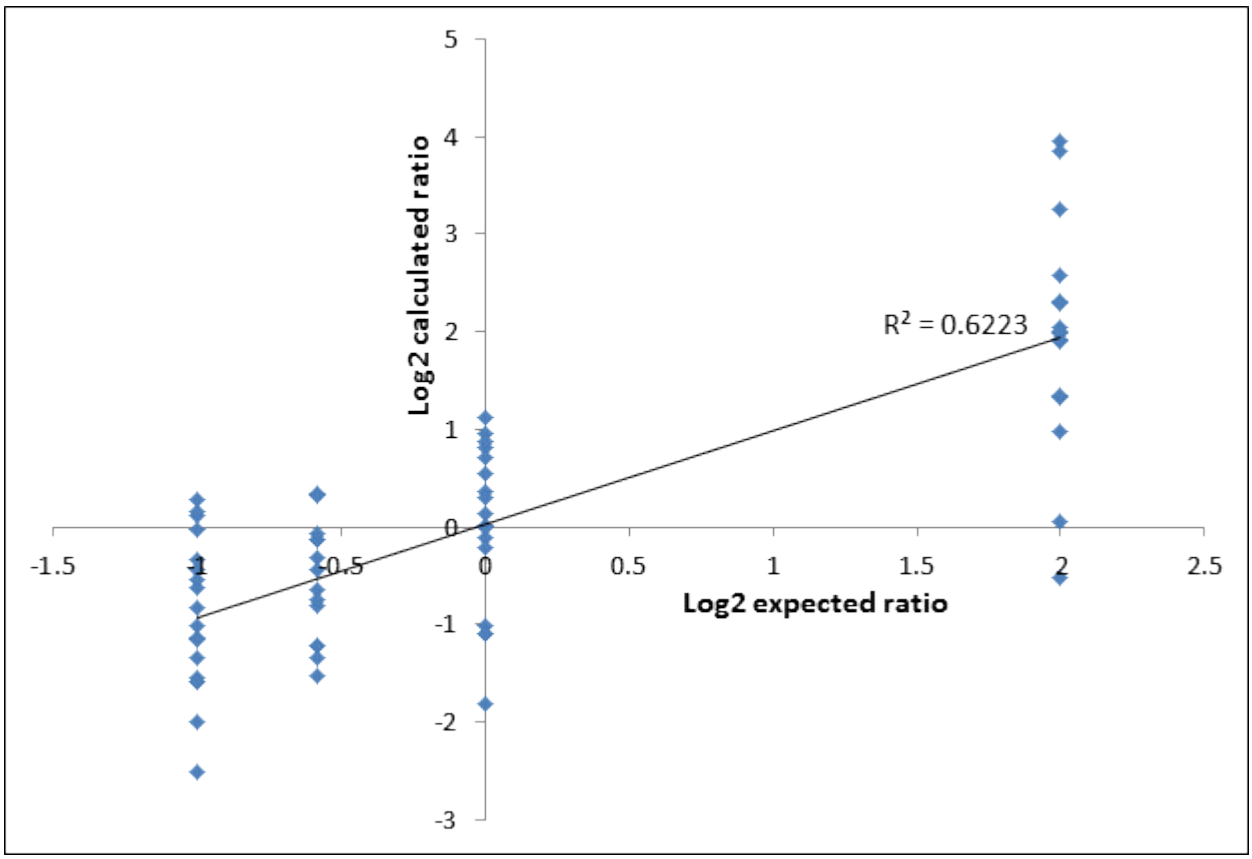


Figure S5. The relationship between the expected and calculated fold change in expression of ERCC spike-in control transcripts. Values plotted are log2 transformed ratios of transcript expression (FPKM) in samples spiked with ERCC mix 1 and mix 2.

Table S3. Gene Ontology Terms and Kegg Pathways over-represented in the list of differentially expressed transcripts for each treatment group. Values presented are the P-values and adjusted P-values associated with this over-representation. Analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6 .7 (Huang et al. 2008, *Nature Protocols*, 4:44-57), using the *de novo* liver transcriptome assembly generated in this study as a background.

BIOLOGICAL PROCESS (BP ALL)	0.01 mg Roundup/ L		0.5 mg Roundup/ L		10 mg Roundup/ L		0.01 mg glyphosate/ L		10 mg glyphosate/ L	
	P Value	FDR	P Value	FDR	P Value	FDR	P Value	FDR	P Value	FDR
biogenic amine metabolic process			4.80E-02	5.50E+01						
biological regulation			1.30E-02	1.80E+01						
cellular amino acid derivative metabolic process			2.40E-02	3.00E+01						
defense response							4.50E-02	5.50E+01		
diencephalon development	4.30E-02	4.30E+01								
lipid metabolic process			4.10E-02	4.70E+01						
mesoderm development					2.00E-02	2.30E+01				
multi-organism process							3.30E-05	4.70E-02		
positive regulation of apoptosis							4.70E-02	5.70E+01		
positive regulation of cell death							4.70E-02	5.70E+01		
positive regulation of programmed cell death							4.70E-02	5.70E+01		
regulation of biological process	5.00E-02	4.90E+01	3.70E-03	5.40E+00						
regulation of biosynthetic process	7.90E-03	9.60E+00	3.70E-03	5.30E+00						
regulation of cell growth							4.40E-02	4.90E+01		
regulation of cellular biosynthetic process	7.80E-03	9.50E+00	3.50E-03	5.10E+00						
regulation of cellular metabolic process	1.10E-02	1.40E+01	8.50E-03	1.20E+01						
regulation of cellular process	3.70E-02	3.80E+01	4.10E-03	6.00E+00						
regulation of gene expression	9.10E-03	1.10E+01	5.00E-03	7.20E+00			4.10E-02	4.00E+00		
regulation of growth							4.50E-02	5.50E+01		
regulation of macromolecule biosynthetic process	7.60E-03	9.20E+00	3.30E-03	4.80E+00						
regulation of macromolecule metabolic process	1.40E-02	1.60E+01	1.30E-02	1.70E+01						
regulation of metabolic process	1.70E-02	1.90E+01	2.00E-02	2.60E+01						
regulation of nitrogen compound metabolic process	2.70E-02	2.90E+01	1.10E-02	1.50E+01						
regulation of primary metabolic process	1.20E-02	1.40E+01	9.80E-03	1.40E+01						
regulation of RNA metabolic process			2.30E-03	3.40E+00						
regulation of transcription	2.30E-02	2.50E+01	7.40E-03	1.00E+01						
regulation of transcription, DNA-dependent			1.90E-03	1.00E+01						
response to bacterium							3.40E-02	3.80E+01		
response to biotic stimulus							3.80E-06	5.40E-03		
response to other organism							1.80E-05	2.50E-02		
response to stimulus			2.30E-02	2.90E+01	2.30E-02	2.70E+01	4.60E-04	6.40E-01		
response to stress					4.80E-02	5.50E+01	5.20E-03	7.00E+00		
response to virus							2.20E-03	3.00E+00		
sterol metabolic process				4.80E+01						
			4.30E-02							

MOLECULAR FUNCTION (MF ALL)	0.01 mg Roundup/ L		0.5 mg Roundup/ L		10 mg Roundup/ L		0.01 mg glyphosate/ L		10 mg glyphosate/ L	
	P Value	FDR	P Value	FDR	P Value	FDR	P Value	FDR	P Value	FDR
adenyl ribonucleotide binding							4.90E-02	4.00E+01		
calcium-transporting ATPase activity							5.00E-02	5.80E+01		
carboxy-lyase activity			4.10E-02	4.80E+01						
DNA binding			9.60E-03	1.20E+01	2.50E-02	2.50E+01				
extracellular matrix structural constituent			2.60E-02	2.80E+01						
heat shock protein binding							4.30E-02	4.10E+01		
ligand-dependent nuclear receptor activity			4.60E-02	5.20E+01						
metalloendopeptidase activity					2.90E-02	2.90E+01				
nucleotide binding			3.50E-02	3.60E+01			4.30E-02	4.10E+01		
purine nucleotide binding							3.00E-02	4.00E+01		
purine ribonucleotide binding							4.30E-02	4.10E+01		
SAP kinase activity			4.40E-02	5.00E+01						
sequence-specific DNA binding			3.40E-02	3.60E+01						
steroid hormone receptor activity			4.60E-02	5.20E+01						
transcription factor activity			1.10E-03	1.40E+00	3.90E-02	5.70E+01	4.90E-02	6.30E+01		
transcription regulator activity	5.90E-03	5.80E+00	1.60E-03	2.00E+00			4.60E-02	6.10E+01		
transcription repressor activity			4.50E-02	5.10E+01						
CELLULAR COMPONENT (CC ALL)	**0.01 mg Roundup/ L**		**0.5 mg Roundup/ L**		**10 mg Roundup/ L**		**0.01 mg glyphosate/ L**		**10 mg glyphosate/ L**	
	P Value	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**
nucleus	8.40E-03	7.50E+00			4.80E-02	5.60E+01				
collagen			1.50E-02	1.60E+01						
endoplasmic reticulum			1.40E-02	1.40E+01			7.30E-02	5.30E+01		
external side of plasma membrane							6.40E-02	4.80E+01		
extracellular matrix			7.40E-04	8.10E-01						
extracellular region			5.60E-03	6.00E+00			4.70E-02	3.80E+01		
proteinaceous extracellular matrix			6.00E-04	6.50E-01						
KEGG PATHWAY	**0.01 mg Roundup/ L**		**0.5 mg Roundup/ L**		**10 mg Roundup/ L**		**0.01 mg glyphosate/ L**		**10 mg glyphosate/ L**	
	P Value	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**	**P Value**	**FDR**
Adipocytokine signaling pathway					4.90E-02	3.50E+01				
Apoptosis			4.80E-02	6.10E+01						
ECM-receptor interaction			7.90E-03	7.80E+00						
Glycerophospholipid metabolism			3.60E-02	3.20E+01						
Insulin signaling pathway					5.90E-03	4.90E+00	4.10E-02	5.30E+01		
MAPK signaling pathway			4.80E-02	5.70E+01			7.20E-03	6.20E+00		
NOD-like receptor signaling pathway			4.80E-02	6.10E+01						
RIG-I-like receptor signaling pathway			3.70E-03	3.80E+00						
Sphingolipid metabolism			8.90E-03	8.80E+00						
TGF-beta signaling pathway	2.80E-03	2.10E+00								
Toll-like receptor signaling pathway			5.00E-03	5.10E+00						

Table S4. Fold changes of all differentially-regulated transcripts in all treatment groups. Values presented are log2 transformed fold changes calculated by EdgeR. Significant differences in expression (FDR <0.1) are indicated by red (up-regulated) and green (down-regulated) shading. Treatments are represented by the following codes: LR, MR and HR represent 0.01, 0.5 and 10 mg/L Roundup, and LG and HG represent 0.01 and 10 mg/L glyphosate.

Name	Symbol	Database	LR Log2 FC	MR Log2 FC	HR Log2 FC	LG Log2 FC	HG Log2 FC
aanat2	NM_001124257.1	refseq	-1.32	-0.74	-0.85	-0.17	-0.32
abcf2a	ENSDARG00000038785	Ensembl	1.23	2.09	1.15	1.33	0.57
abcf2a	ENSDARG00000038785	Ensembl	0.52	0.97	0.34	0.55	0.17
abcf2a	ENSDARG00000038785	Ensembl	0.75	1.27	0.58	0.89	0.35
ABCF3	ENSDARG00000089705	Ensembl	1.30	2.21	1.18	2.00	0.50
acsbg2	ENSDARG00000004094	Ensembl	-1.48	-0.79	-0.56	-1.06	-0.16
acsbg2	ENSDARG00000004094	Ensembl	-1.41	-0.90	-0.80	-0.97	-0.21
acsbg2	ENSDARG00000004094	Ensembl	-1.36	-1.06	-0.56	-0.90	0.18
acss1	ENSDARG00000044142	Ensembl	0.68	1.38	1.59	2.29	0.02
adamts10	ENSDARG00000075188	Ensembl	-0.57	-0.66	-0.33	-0.82	-0.17
AIFM2	ENSDARG00000077549	Ensembl	0.66	0.70	0.33	0.55	0.33
alpk2	ENSDARG00000079637	Ensembl	-0.78	-1.11	-0.91	-0.21	-0.20
amd1	ENSDARG00000043856	Ensembl	0.49	0.81	0.57	0.35	0.21
angptl4	ENSDARG00000035859	Ensembl	0.66	0.69	0.13	0.17	0.08
angptl4	ENSDARG00000035859	Ensembl	0.69	0.54	0.30	0.24	0.19
ankrd16	ENSDARG00000003822	Ensembl	1.13	2.14	1.42	0.65	0.00
ANKRD28	ENSGACG00000006364	Ensembl	0.44	1.06	1.22	0.74	0.30
ANKRD30B	ENSDARG00000013015	Ensembl	-0.52	-0.31	-1.26	-0.21	-1.00
ANTXR1_(1_of_2)	ENSDARG00000025672	Ensembl	0.29	1.07	0.91	0.14	0.71
anxa11a	ENSDARG00000077383	Ensembl	0.43	1.21	0.63	0.64	0.26
anxa11a	ENSDARG00000077383	Ensembl	0.33	1.33	0.60	0.68	0.23
anxa11a	ENSDARG00000077383	Ensembl	0.37	1.14	0.23	0.54	-0.09
apooa	ENSDARG00000046154	Ensembl	0.18	0.98	0.22	0.13	0.30
arf1l	ENSDARG00000016393	Ensembl	0.61	1.01	0.62	1.21	0.49
arhgap21b	ENSDARG00000075673	Ensembl	1.04	1.39	0.59	0.97	0.73
arhgap21b	ENSDARG00000075673	Ensembl	0.14	0.84	0.27	0.22	0.15
ARHGAP32	ENSDARG00000074184	Ensembl	-0.35	-1.06	-0.40	-0.38	-0.52
arhgap5	ENSDARG00000061294	Ensembl	0.13	0.86	0.22	0.49	-0.14
arhgef19	ENSDARG00000078853	Ensembl	-0.92	-0.50	-1.23	-0.73	-0.51
aste1	ENSONIG00000002037	Ensembl	0.00	0.00	1.30	3.02	0.00
atf3	ENSDARG00000007823	Ensembl	1.33	3.02	2.12	2.02	0.83
atf5b	ENSDARG00000077785	Ensembl	0.67	1.17	1.17	1.26	0.32
atg5	NM_001173812.1	refseq	1.90	3.74	2.38	5.26	0.57
atg5	NM_001173812.1	refseq	1.37	3.78	2.35	5.21	0.41
atp1a2a	NM_001124458.1	refseq	2.71	3.07	3.09	2.89	1.93
atp2a2a	ENSDARG00000029439	Ensembl	0.79	1.50	1.21	1.85	0.08
atp2a2b	ENSDARG00000005122	Ensembl	0.96	1.34	0.95	2.00	0.01
atp2a2b	ENSDARG00000005122	Ensembl	0.92	1.11	1.11	1.69	0.35
atp2a2b	ENSDARG00000005122	Ensembl	0.72	1.07	0.80	1.41	0.23
atp2a2b	ENSDARG00000005122	Ensembl	0.56	1.37	0.62	1.47	0.33
bcap29	ENSDARG00000016231	Ensembl	0.62	1.80	1.28	1.35	-0.05
bcap29	ENSDARG00000016231	Ensembl	0.19	1.24	0.78	0.70	-0.13
bhlhe40	ENSDARG00000004060	Ensembl	-0.80	-1.04	-1.39	-0.13	-0.34
bhlhe40	ENSDARG00000004060	Ensembl	-0.65	-0.98	-1.40	-0.18	-0.37
bhlhe40	ENSDARG00000004060	Ensembl	-0.88	-0.92	-1.24	-0.10	-0.41
BICD2	ENSORLG00000011196	Ensembl	-0.40	-0.66	-0.74	-0.01	-1.05
bmp5	ENSDARG00000004965	Ensembl	-1.16	-0.63	-0.54	-0.53	-0.07
bmp5	ENSDARG00000004965	Ensembl	-1.24	-0.93	-0.93	-0.51	0.13
bmp5	ENSDARG00000004965	Ensembl	-1.28	-0.87	-0.66	-0.64	-0.09
bmp5	ENSDARG00000004965	Ensembl	-1.27	-0.61	-1.02	-0.59	-0.01
btbd9	ENSDARG00000068983	Ensembl	-0.77	-0.25	-1.24	0.03	-0.47
btr06	ENSDARG00000054184	Ensembl	0.00	0.00	0.00	1.44	0.00
btr20	ENSDARG00000075603	Ensembl	0.46	-0.61	0.33	-5.21	-0.65
btr22	ENSDARG00000093049	Ensembl	-0.17	-1.03	-0.60	-0.68	-0.47
btr26	ENSDARG00000040860	Ensembl	-0.18	-0.94	-0.14	-5.22	-0.61
BX539340.1	ENSDARG00000089797	Ensembl	-0.76	0.32	-1.71	-4.54	0.00
BX539340.1	ENSDARG00000089797	Ensembl	-0.81	-2.04	0.67	-0.45	-0.44
BX572630.2	ENSDARG00000088251	Ensembl	-0.39	0.25	0.31	1.79	-0.08
BX572630.2	ENSDARG00000088251	Ensembl	-0.60	0.17	0.61	2.08	0.19

BX640584.1	ENSDARG00000086955	Ensembl	-0.24	-0.97	0.26	-2.18	-0.72
BX664721.5	ENSMUSG00000070332	Ensembl	0.09	-0.36	0.10	-5.57	-0.81
BX927253.1	ENSDARG00000089111	Ensembl	1.35	1.57	1.24	1.70	0.24
C18H15orf39	ENSDARG00000069168	Ensembl	-0.47	-0.92	-0.36	-0.09	-0.03
c1qtnf5	ENSDARG00000056134	Ensembl	-0.49	-1.20	-0.38	-0.89	-1.08
C25H1orf51 (2 of 2)	ENSDARG00000088171	Ensembl	1.45	1.36	0.00	1.81	0.00
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.65	2.52	1.84	2.39	0.23
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.50	2.61	1.90	2.43	0.19
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.75	2.81	2.19	2.52	0.06
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.58	2.69	2.03	2.55	0.33
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.67	2.78	2.15	2.56	0.29
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.74	2.59	2.12	2.57	0.33
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.94	2.75	2.10	2.61	0.25
C7 (1 of 2)	ENSDARG00000057121	Ensembl	0.64	2.84	2.15	2.66	0.21
CABZ01040055.1	ENSDARG00000086869	Ensembl	1.69	2.40	1.92	0.55	2.42
CABZ01055715.1	ENSDARG00000087246	Ensembl	0.60	-0.01	0.79	1.60	0.48
CABZ01055715.1	ENSDARG00000087246	Ensembl	0.18	0.84	0.32	0.47	0.14
cacng6b	ENSDARG00000046079	Ensembl	-1.21	-0.33	-0.77	-0.54	-0.77
calcrla	ENSDARG00000011473	Ensembl	0.00	0.00	0.00	1.14	0.87
CARD14	ENSG00000141527	Ensembl	1.24	1.77	1.85	1.06	0.53
CARD14	ENSG00000141527	Ensembl	1.41	1.70	1.80	1.00	1.08
cbln8	ENSDARG00000019294	Ensembl	0.70	1.29	1.44	2.22	-0.38
ccl-c5a	ENSDARG00000058389	Ensembl	0.24	1.27	0.72	2.36	-0.26
ccrn4la	ENSDARG00000077726	Ensembl	0.67	0.39	0.23	0.60	0.80
ccrn4la	ENSDARG00000077726	Ensembl	0.78	0.43	0.20	0.59	0.76
cd40	ENSDARG00000054968	Ensembl	1.05	2.58	1.55	0.85	0.91
cd99l2	ENSDARG00000056722	Ensembl	-1.03	-0.69	-0.47	-1.11	-1.56
cda	ENSDARG00000038199	Ensembl	1.17	1.82	1.53	1.21	1.05
cda	ENSDARG00000038199	Ensembl	1.02	1.82	1.35	0.79	1.01
cda	ENSDARG00000038199	Ensembl	0.77	1.56	1.41	0.92	0.85
cda	ENSDARG00000038199	Ensembl	1.09	1.95	1.37	0.90	1.25
cda	ENSDARG00000038199	Ensembl	0.84	1.71	1.05	0.58	0.87
cda	ENSDARG00000038199	Ensembl	0.95	1.97	1.34	0.89	1.05
CDR2 (1 of 2)	ENSDARG00000035952	Ensembl	0.16	0.37	-2.03	-0.72	0.20
ch25h	ENSDARG00000045190	Ensembl	2.06	3.71	2.65	3.96	0.89
chd4a	ENSDARG00000063535	Ensembl	0.36	1.00	0.38	0.55	-0.35
chka	ENSDARG00000041078	Ensembl	0.95	0.93	0.39	0.08	0.30
chka	ENSDARG00000041078	Ensembl	1.00	0.52	0.28	0.05	-0.15
chka	ENSDARG00000041078	Ensembl	0.93	0.77	0.48	0.63	-0.07
chmp6b	ENSDARG00000021202	Ensembl	0.00	1.07	1.02	0.00	0.00
chp1	ENSDARG00000052859	Ensembl	0.37	0.96	0.50	0.49	0.21
chrm4a	ENSDARG00000069254	Ensembl	-1.27	-1.02	-0.07	-2.00	-0.03
CLEC17A	ENSG00000187912	Ensembl	0.12	1.45	0.95	0.40	0.64
CLEC17A	ENSG00000187912	Ensembl	0.18	1.51	0.98	0.55	0.66
CLEC17A	ENSG00000187912	Ensembl	0.17	1.49	0.98	0.49	0.61
CLEC17A	ENSG00000187912	Ensembl	0.16	1.48	0.96	0.47	0.62
CLEC17A	ENSG00000187912	Ensembl	0.62	1.70	1.23	1.09	0.56
CLEC17A	ENSG00000187912	Ensembl	0.23	1.55	1.01	0.62	0.59
CLEC17A	ENSG00000187912	Ensembl	0.66	1.73	1.25	1.05	0.49
clic2	ENSDARG00000010625	Ensembl	0.51	0.90	1.04	0.33	0.17
clstn1	ENSDARG00000031720	Ensembl	0.61	0.73	0.53	1.27	0.03
col13a1	ENSG00000197467	Ensembl	0.00	0.00	0.00	0.00	1.77
COL16A1	ENSDARG00000009194	Ensembl	0.20	-1.39	0.44	0.09	0.35
col1a1a	ENSDARG00000012405	Ensembl	0.26	-0.91	-0.20	0.11	-0.46
col1a1a	ENSDARG00000012405	Ensembl	0.49	-0.79	-0.09	0.16	-0.26
col1a1a	ENSDARG00000012405	Ensembl	0.17	-0.69	-0.07	0.04	-0.01
col1a1a	ENSDARG00000012405	Ensembl	0.21	-0.68	-0.04	0.12	0.04
col1a1b	ENSDARG00000035809	Ensembl	-0.11	-0.92	-0.06	0.03	0.29
col1a1b	ENSDARG00000035809	Ensembl	0.41	-1.38	-0.12	-0.05	0.13
col1a2	ENSDARG00000020007	Ensembl	0.23	-0.88	-0.04	0.15	-0.19
col1a2	ENSDARG00000020007	Ensembl	0.37	-0.75	0.01	0.26	-0.22
col4a3bp	ENSDARG00000063542	Ensembl	1.17	1.87	1.14	1.05	0.23
col6a2	ENSDARG00000061436	Ensembl	0.16	-0.76	0.03	0.03	-0.28
col6a3	ENSDARG00000077139	Ensembl	0.02	-0.80	-0.21	-0.03	-0.28
col8a1a	ENSDARG00000077403	Ensembl	0.25	0.32	0.90	1.64	-0.49
CR352328.2	ENSDARG00000089432	Ensembl	0.72	0.27	0.46	-7.13	0.03
CR352328.2	ENSDARG00000089432	Ensembl	0.71	-0.10	0.52	-6.92	0.07
CR354432.1	ENSDARG00000091579	Ensembl	0.62	1.44	0.46	0.88	0.37
CR356230.1	ENSDARG00000038872	Ensembl	-0.59	-0.75	-1.35	-0.05	-0.81
CR385063.1	ENSDARG00000044212	Ensembl	0.00	1.11	0.45	0.00	0.49
CREM	ENSMUSG00000063889	Ensembl	0.68	1.70	1.17	0.66	0.33
CT055	NM_001140483.1	refseq	4.84	5.40	4.31	2.07	0.00
ctgfa	ENSDARG00000042934	Ensembl	0.62	1.37	0.97	1.49	1.19
ctgfa	ENSDARG00000042934	Ensembl	0.27	1.20	0.84	1.34	1.02

ctsh	ENSDARG00000041108	Ensembl	-0.42	-1.10	-0.04	-0.19	0.06
CU019646.2	ENSDARG00000091234	Ensembl	0.44	2.81	1.99	1.93	-0.28
CU459095.1	ENSDARG00000086495	Ensembl	-0.10	-0.72	-0.09	-0.19	0.14
cycsb	ENSDARG00000044562	Ensembl	1.08	2.09	0.88	1.95	0.95
cycsb	ENSDARG00000044562	Ensembl	0.75	2.05	0.70	1.68	0.65
cylda	ENSDARG00000060058	Ensembl	0.90	1.86	1.21	1.06	0.63
cylda	ENSDARG00000060058	Ensembl	0.51	1.55	0.90	1.19	0.45
cylda	ENSDARG00000060058	Ensembl	0.32	1.42	0.59	0.68	0.14
cytidine	NM_001146593.1	refseq	1.03	1.69	1.34	0.68	0.79
dcbld1	ENSDARG00000015907	Ensembl	0.36	1.74	0.57	0.93	0.22
ddit4	ENSDARG00000037618	Ensembl	-0.18	1.59	0.58	1.11	3.62
ddit4	ENSDARG00000037618	Ensembl	0.00	0.00	0.00	0.00	3.68
ddit4	ENSDARG00000037618	Ensembl	0.00	0.00	0.00	0.00	3.49
ddit4l	ENSMUSG00000046818	Ensembl	1.21	0.79	0.12	2.07	0.19
DDX17	ENSDARG00000010873	Ensembl	-0.22	-0.84	-0.16	-0.20	-0.13
ddx5	ENSDARG00000038068	Ensembl	0.84	1.54	1.62	1.45	1.41
ddx5	XM_003443093.1	refseq	1.07	2.03	1.72	1.33	1.40
ddx5	ENSDARG00000038068	Ensembl	1.19	1.82	1.74	1.37	1.38
ddx5	ENSDARG00000038068	Ensembl	1.08	2.14	1.74	1.35	1.27
ddx5	ENSDARG00000038068	Ensembl	0.91	2.17	1.55	1.06	1.06
ddx5	ENSDARG00000038068	Ensembl	1.08	2.14	1.77	1.33	1.30
DDX5	BT059556.1	nt	0.00	0.00	1.81	2.05	0.00
desi1a	ENSDARG00000033140	Ensembl	0.29	0.99	0.49	0.61	0.05
dhrs11a	ENSDARG00000046090	Ensembl	0.43	1.06	0.65	0.62	0.23
dio2	ENSDARG00000094857	Ensembl	-1.51	-1.70	-2.61	-1.26	-0.84
dio2	ENSDARG00000094857	Ensembl	-1.64	-1.33	-2.17	-1.39	-1.54
dio2	ENSDARG00000094857	Ensembl	-1.19	-0.95	-1.80	-1.10	-0.58
dio2	ENSDARG00000094857	Ensembl	-1.62	-1.14	-2.27	-1.42	-1.02
dio2	ENSDARG00000094857	Ensembl	-1.49	-1.22	-1.88	-1.07	-0.78
dio2	ENSDARG00000094857	Ensembl	-1.42	-0.93	-2.11	-1.20	-0.72
dio3a	ENSDARG00000089937	Ensembl	1.16	2.24	2.25	1.90	1.55
dio3a	ENSDARG00000089937	Ensembl	-1.53	0.27	0.59	-0.59	0.84
dnajb11	ENSDARG00000015088	Ensembl	1.59	1.52	1.42	3.19	0.15
dnajb11	ENSDARG00000015088	Ensembl	0.76	1.00	0.76	2.29	-0.03
dnajb11	ENSDARG00000015088	Ensembl	0.80	1.07	0.90	2.35	0.09
dnajb11	ENSDARG00000015088	Ensembl	1.50	1.23	1.48	3.19	0.20
DNAJB9 (1 of 2)	ENSDARG00000052072	Ensembl	1.09	1.91	1.30	1.95	0.37
DNAJB9 (1 of 2)	ENSDARG00000052072	Ensembl	1.27	2.00	0.00	1.86	0.45
DNAJB9 (1 of 2)	ENSDARG00000052072	Ensembl	1.52	2.15	1.53	1.82	0.71
dnajc3	ENSG00000102580	Ensembl	1.01	0.74	0.50	2.07	-1.37
dnajc3	ENSG00000102580	Ensembl	0.72	0.63	0.59	1.56	-0.25
drg1	ENSDARG00000039345	Ensembl	0.07	0.69	0.41	0.51	0.38
dusp1	ENSDARG00000007628	Ensembl	0.15	2.24	1.17	1.95	0.77
dusp2	ENSDARG00000007628	Ensembl	0.57	1.78	0.82	1.94	0.43
egr1	ENSDARG00000037421	Ensembl	0.61	2.44	0.71	3.21	0.17
egr1	ENSDARG00000037421	Ensembl	0.92	2.85	0.96	3.71	0.00
eif1b	BT056792.1	nt	0.56	1.80	1.38	0.85	0.77
eif1b	ENSDARG00000012688	Ensembl	0.25	1.29	0.86	0.50	0.34
eif1b	ENSDARG00000012688	Ensembl	0.27	1.14	0.76	0.49	0.31
eif1b	ENSDARG00000012688	Ensembl	0.16	1.24	0.72	0.44	0.24
eif1b	ENSDARG00000012688	Ensembl	0.20	1.17	0.83	0.39	0.21
eif1b	ENSDARG00000012688	Ensembl	0.31	1.17	0.85	0.48	0.20
eif1b	ENSDARG00000012688	Ensembl	0.38	1.29	0.86	0.57	0.25
eif1b	ENSDARG00000012688	Ensembl	0.28	1.23	0.82	0.42	0.25
eif1b	ENSDARG00000012688	Ensembl	0.25	1.26	0.83	0.48	0.26
eif1b	ENSDARG00000012688	Ensembl	0.40	1.08	0.68	0.31	0.27
eif1b	ENSDARG00000012688	Ensembl	0.30	1.18	0.92	0.51	0.42
eif1b	ENSDARG00000012688	Ensembl	0.14	1.13	0.78	0.39	0.24
eif1b	ENSDARG00000012688	Ensembl	0.29	0.86	0.33	0.16	0.06
eif1b	ENSDARG00000012688	Ensembl	0.23	1.10	0.74	0.43	0.09
eif1b	ENSDARG00000012688	Ensembl	0.26	1.05	0.71	0.58	0.15
eif1b	ENSDARG00000012688	Ensembl	0.22	1.13	0.78	0.41	0.29
eif4g1	ENSG00000114867	Ensembl	1.00	0.65	0.77	1.00	0.48
eif4g2a	ENSDARG00000020377	Ensembl	0.91	1.38	1.04	0.88	0.67
eif4g2a	ENSDARG00000020377	Ensembl	0.74	1.23	1.06	0.68	0.48
eif4g2a	ENSDARG00000020377	Ensembl	0.38	1.23	1.00	0.65	0.48
eif4g2a	ENSDARG00000020377	Ensembl	0.61	1.00	1.05	0.70	0.24
eif4g2a	ENSDARG00000020377	Ensembl	0.55	0.99	0.47	0.35	0.50
eif4g2a	ENSDARG00000020377	Ensembl	0.68	1.22	0.77	0.93	0.17
elvol5a	GU238431.1	nt	0.74	3.65	1.36	1.81	0.52
enpp7	ENSDARG00000077225	Ensembl	1.43	1.96	1.24	1.87	1.27
ERGIC1	ENSDARG00000005273	Ensembl	0.56	0.85	0.77	1.27	0.26
evpla	ENSDARG00000019808	Ensembl	0.39	1.27	1.52	0.15	2.15
evpla	ENSDARG00000019808	Ensembl	0.79	1.50	1.17	0.20	2.04
fam108b1	ENSDARG00000035571	Ensembl	0.00	1.29	1.01	0.00	0.00

fam20a	ENSDARG00000079486	Ensembl	-0.33	-0.79	-0.37	-0.98	-0.38
fbxl5	ENSDARG00000043046	Ensembl	0.87	1.71	1.19	1.37	0.49
fbxl5	ENSDARG00000043046	Ensembl	0.94	1.58	1.34	1.34	0.39
fbxl5	ENSDARG00000043046	Ensembl	0.88	1.35	0.69	1.32	0.60
fbxl5	ENSDARG00000043046	Ensembl	0.66	1.45	1.16	1.11	0.29
fbxl5	ENSDARG00000043046	Ensembl	0.84	1.38	0.91	1.38	0.35
Fc	ENSMUSG00000015947	Ensembl	1.24	0.91	1.36	2.01	-0.09
FDFT1	ENSDARG00000060260	Ensembl	1.69	1.32	0.30	1.28	-0.26
fdx1	ENSDARG00000056410	Ensembl	1.75	1.77	0.03	2.35	0.51
ficd	ENSDARG00000035595	Ensembl	0.98	1.25	1.06	2.12	0.21
fnbp1	ENSDARG00000036156	Ensembl	-0.71	-1.41	-1.51	-0.85	-0.57
fnbp1	ENSDARG00000036156	Ensembl	-0.68	-1.52	-1.94	-0.57	-0.70
fnbp1	ENSDARG00000036156	Ensembl	-0.32	-1.34	-2.21	-0.76	-0.38
FNDC3A	ENSDARG00000067569	Ensembl	0.89	0.44	-0.01	0.71	-0.06
fosl2	ENSDARG00000040623	Ensembl	1.87	4.41	2.83	3.34	1.62
fosl2	ENSDARG00000040623	Ensembl	1.18	3.24	2.25	2.74	0.93
fosl2	ENSDARG00000040623	Ensembl	1.44	3.31	2.13	2.79	0.00
FOXO4	ENSDARG00000055792	Ensembl	0.82	0.83	0.61	0.41	0.12
fructose	NM_001173920.1	refseq	1.07	2.39	1.27	1.35	0.59
fth1b	ENSDARG00000007975	Ensembl	0.72	1.21	1.88	0.85	0.96
fxyd5b	NM_001123724.1	refseq	0.69	2.97	1.48	1.90	0.14
FYB (1 of 2)	ENSDARG00000044694	Ensembl	0.00	0.00	1.19	0.00	0.00
G0S2	BT046903.1_	nt	1.87	5.06	3.10	4.58	-0.07
G0S2	BT046903.1_	nt	2.13	5.13	3.21	4.76	0.61
gadd45a	BT046750.2	nt	-0.64	-1.07	-1.07	-0.79	-0.75
gadd45ab	ENSDARG00000069991	Ensembl	-0.01	-0.26	-0.01	-0.96	0.20
gata5	ENSDARG00000017821	Ensembl	-1.15	-2.41	-0.78	-1.32	-0.01
gck	ENSDARG00000068006	Ensembl	3.40	1.53	-2.78	-0.72	0.56
gclm	ENSDARG00000018953	Ensembl	0.97	1.19	0.20	0.73	0.34
gclm	ENSDARG00000018953	Ensembl	0.72	1.17	0.41	0.59	0.20
GIMAP4	ENSG00000133574	Ensembl	-1.50	-2.47	-2.10	-6.23	-1.72
GIMAP4	ENSG00000133574	Ensembl	1.14	1.73	1.00	1.01	0.11
GIMAP9	ENSMUSG00000051124	Ensembl	1.06	1.42	0.00	0.60	0.00
glcci1	ENSDARG00000008503	Ensembl	0.53	0.58	0.74	0.96	0.54
glcci1	ENSDARG00000008503	Ensembl	0.60	0.45	0.55	0.87	0.48
glcci1	ENSDARG00000008503	Ensembl	0.82	0.75	0.80	1.18	0.60
glcci1	ENSDARG00000008503	Ensembl	0.44	0.46	0.68	0.94	0.07
glyg	NM_001139830.1	refseq	0.20	0.99	0.37	2.12	0.01
gmppb	ENSDARG00000017658	Ensembl	0.63	1.16	0.62	2.07	-0.09
gnai1	ENSDARG00000021647	Ensembl	-0.99	-1.06	-0.78	-0.33	-0.89
gopc	ENSDARG00000023117	Ensembl	1.04	2.45	1.90	2.01	0.74
gopc	ENSDARG00000023117	Ensembl	0.79	1.76	1.43	1.55	0.63
gopc	ENSDARG00000023117	Ensembl	1.09	2.32	1.66	1.86	0.38
gopc	ENSDARG00000023117	Ensembl	0.95	1.92	1.43	1.28	0.15
gpd1b	ENSDARG00000043180	Ensembl	-0.43	-0.91	-0.29	-0.54	-0.02
gpd1b	ENSDARG00000043180	Ensembl	0.04	-1.00	-0.16	-0.82	-0.08
Grik2	ENSMUSG00000056073	Ensembl	-0.23	-1.57	0.03	-0.24	0.47
gsr	ENSDARG00000019236	Ensembl	0.41	0.86	0.29	0.19	-0.12
gsr	ENSDARG00000019236	Ensembl	0.53	0.94	0.29	0.32	-0.04
gys2	ENSDARG00000004904	Ensembl	-0.13	-0.82	-0.11	-0.20	-0.28
hdac4	ENSDARG00000041204	Ensembl	-0.47	-0.69	-0.47	-1.34	-0.42
HECT	ENSG00000138646	Ensembl	-0.69	0.31	0.70	1.90	0.21
hect	ENSMUSG00000029804	Ensembl	-0.10	0.32	0.68	1.54	0.00
HELZ2 (2 of 2)	ENSDARG00000016527	Ensembl	-0.66	0.23	0.37	2.35	0.30
HELZ2 (2 of 2)	ENSDARG00000016527	Ensembl	0.22	0.13	1.06	1.58	-0.24
HELZ2 (2 of 2)	ENSDARG00000016527	Ensembl	0.00	0.00	0.00	1.97	0.00
HERC4	ENSG00000138642	Ensembl	-0.73	0.05	0.42	1.67	-0.18
HERC4	ENSG00000138642	Ensembl	-0.88	-0.27	-0.06	1.75	0.07
HERC4	ENSG00000138642	Ensembl	-0.66	-0.19	0.49	1.77	0.12
herp2	NM_001146672.1	refseq	1.30	1.74	1.72	1.22	0.00
HHEX	ENSG00000152804	Ensembl	-0.54	-0.81	-0.57	-0.12	-0.05
hig1	ENSDARG00000022303	Ensembl	0.74	2.10	0.86	1.45	0.77
hig1	ENSDARG00000022303	Ensembl	0.39	1.82	0.75	1.43	0.57
hig1	ENSDARG00000022303	Ensembl	0.26	1.80	0.96	1.27	0.60
hmox1	ENSDARG00000027529	Ensembl	0.76	1.61	0.94	1.02	0.55
hmox1	ENSDARG00000027529	Ensembl	0.70	1.43	0.84	0.74	0.32
hmox1	ENSDARG00000027529	Ensembl	0.89	1.47	0.96	0.74	0.32
homez	ENSDARG00000054304	Ensembl	0.58	1.73	0.82	1.05	0.43
homez	ENSDARG00000054304	Ensembl	0.92	1.76	0.86	0.72	0.57
HoxD	EU025718.1	nt	0.69	1.31	1.66	1.70	0.59
hpcl1	NM_001173876.1	refseq	-0.92	-0.97	-0.79	-0.64	-0.13
hspa13	ENSDARG00000040984	Ensembl	0.00	0.00	0.00	1.85	0.00
hspa5	ENSDARG00000004665	Ensembl	1.14	1.55	1.31	2.40	-0.06
hspa5	ENSDARG00000004665	Ensembl	1.23	1.57	1.39	2.46	0.02
hspa5	ENSDARG00000004665	Ensembl	0.94	1.25	1.14	2.10	-0.16

hspb2	ENSDARG00000052450	Ensembl	0.08	-1.15	-0.14	-0.34	-0.11
huwe1	ENSDARG00000016782	Ensembl	0.43	0.94	0.49	0.60	0.24
hyou1	ENSDARG00000013670	Ensembl	0.60	0.75	0.63	2.31	-0.49
hyou1	ENSDARG00000013670	Ensembl	0.60	0.67	0.81	2.39	-0.41
hyou1	ENSDARG00000013670	Ensembl	0.83	0.77	0.86	2.66	-0.22
hyou1	ENSDARG00000013670	Ensembl	0.84	0.36	0.81	2.40	0.00
hyou1	ENSDARG00000013670	Ensembl	0.51	0.80	0.59	2.44	0.00
hyou1	ENSDARG00000013670	Ensembl	0.64	0.77	0.55	2.50	0.00
hyou1	ENSDARG00000013670	Ensembl	0.00	1.12	0.71	2.58	0.00
hyou1	ENSDARG00000013670	Ensembl	0.00	0.63	1.12	2.69	0.00
hyou1	ENSDARG00000013670	Ensembl	1.14	0.00	1.33	2.92	0.00
id1	ENSDARG00000040764	Ensembl	-0.70	-0.99	-0.29	-0.37	-0.39
id2	ENSDARG00000040764	Ensembl	-0.87	-0.70	-0.34	-0.28	-0.68
id2a	ENSDARG00000055283	Ensembl	-1.46	-0.26	-0.13	0.20	-0.11
idi1	ENSDARG00000019976	Ensembl	0.60	0.66	0.11	0.43	-0.08
ifit5	ENSG00000152778	Ensembl	-0.59	0.51	0.73	2.19	-0.51
igfbp1a	ENSDARG00000014947	Ensembl	1.38	1.07	0.62	3.36	1.67
igfbp1a	ENSDARG00000014947	Ensembl	1.74	1.66	1.03	3.28	1.50
ihhb	ENSDARG00000058815	Ensembl	-0.62	-0.76	-0.41	-0.31	-0.16
IL10RB	ENSDARG00000068711	Ensembl	0.20	0.93	0.42	0.61	0.06
il17r	NM_001165364.1	refseq	0.91	1.83	1.55	0.93	0.34
IL17RA	ENSDARG00000058244	Ensembl	1.01	2.01	1.67	0.98	0.31
il4r	ENSDARG00000031051	Ensembl	0.98	1.56	1.08	0.44	0.19
insig1	ENSDARG00000010658	Ensembl	0.00	1.33	0.00	0.74	0.00
ipmkb	ENSDARG00000029291	Ensembl	0.00	0.91	0.00	0.00	0.00
irak3	ENSDARG00000053131	Ensembl	0.00	1.23	0.00	0.00	0.00
irf7	ENSDARG00000045661	Ensembl	0.00	0.00	1.05	2.31	0.00
irk11	NM_001173964.1	refseq	2.27	2.43	2.60	2.52	1.45
IRS2	ENSDARG00000037099	Ensembl	0.70	0.73	1.29	0.44	0.57
IRS4 (1 of 2)	ENSDARG00000052065	Ensembl	0.00	1.12	1.07	0.79	0.00
ITCH (1 of 2)	ENSDARG00000076149	Ensembl	0.34	0.78	0.31	0.28	-0.23
ITGAX	ENSG00000140678	Ensembl	1.30	0.97	0.96	0.93	-0.14
itln1	ENSG00000158764	Ensembl	1.46	2.66	2.23	1.92	0.71
jak1	ENSDARG00000020625	Ensembl	0.78	1.52	0.86	0.95	0.25
jak1	ENSDARG00000020625	Ensembl	0.57	1.20	0.71	0.52	0.53
junba	ENSDARG00000074378	Ensembl	1.14	2.15	1.64	2.45	1.01
junba	ENSDARG00000074378	Ensembl	1.24	2.27	1.68	2.66	1.06
kifap3a	ENSDARG00000008639	Ensembl	0.14	-1.30	-0.02	-0.12	-0.45
lama4	ENSDARG00000020785	Ensembl	-0.25	-0.97	-0.31	-0.15	-0.11
lamb1b	ENSDARG00000045524	Ensembl	-0.35	-0.61	-0.48	-0.14	-0.29
laptm4b	ENSDARG00000035870	Ensembl	0.36	0.82	0.52	0.40	0.40
lectin	NM_001123579.1	refseq	0.18	1.66	1.04	0.55	0.46
leptin	NM_001145890.1	refseq	1.50	3.81	2.23	4.05	0.85
leptin	NM_001145890.1	refseq	0.69	3.04	1.47	3.26	0.19
leptin	GU584004.1	nt	1.05	3.41	1.93	3.70	0.00
leucine	ENSG00000188993	Ensembl	0.92	0.81	0.81	0.20	0.15
long	NM_001173689.1	refseq	0.86	1.09	1.00	0.91	0.46
lpar5b	ENSDARG00000068638	Ensembl	-0.20	-1.28	-0.51	-0.70	-0.24
lpcat3	ENSDARG00000075178	Ensembl	0.75	1.04	1.08	0.60	0.82
lpcat3	ENSDARG00000075178	Ensembl	0.84	1.02	0.84	0.66	0.56
lphn3.1	ENSDARG00000061121	Ensembl	-0.77	-0.41	-1.20	0.09	-0.42
lrpprc	ENSDARG00000043970	Ensembl	0.24	0.79	0.22	0.61	0.62
LRRC16B	ENSDARG00000086990	Ensembl	-1.30	-1.07	-1.46	-0.18	-1.04
LRRC73	ENSDARG00000063411	Ensembl	-1.05	-0.66	-1.26	-0.23	-0.65
manf	ENSDARG00000063177	Ensembl	0.70	0.86	0.64	2.14	0.05
MAP3K6	ENSDARG00000069933	Ensembl	0.18	0.67	-0.08	0.88	-0.24
mapk14a	ENSDARG00000000857	Ensembl	0.26	1.57	1.18	1.10	0.03
mapk14b	ENSDARG00000028721	Ensembl	0.43	1.41	0.81	0.66	0.07
mapre1b	ENSDARG00000002659	Ensembl	0.00	1.05	0.00	0.00	0.00
marco	ENSDARG00000059294	Ensembl	0.28	-0.06	-0.33	1.41	-0.30
mat2aa	ENSDARG00000040334	Ensembl	0.72	2.39	1.51	1.88	0.22
mat2aa	ENSDARG00000040334	Ensembl	0.63	1.72	1.11	1.34	0.47
mat2aa	ENSDARG00000040334	Ensembl	0.68	1.71	0.77	0.82	0.60
mat2aa	ENSDARG00000040334	Ensembl	0.52	1.42	0.43	0.76	0.30
matn4	ENSDARG00000015947	Ensembl	0.00	0.00	0.00	2.65	0.00
matn4	ENSDARG00000015947	Ensembl	0.00	0.00	0.00	2.92	0.00
mcf2l	ENSDARG00000075859	Ensembl	-1.17	-1.27	-1.45	-1.18	-0.85
mcf2l	ENSDARG00000075859	Ensembl	-0.99	-1.29	-1.25	-0.70	-0.64
mcf2l	ENSDARG00000075859	Ensembl	-0.86	-1.12	-1.05	-0.61	-0.96
mcf2l	ENSDARG00000075859	Ensembl	-1.20	-1.39	-1.05	-0.74	-0.66
mcm6	ENSDARG00000057683	Ensembl	-0.85	-1.00	-0.73	-0.35	-0.21
mertk	ENSDARG00000074695	Ensembl	1.33	0.00	0.00	2.06	0.00
mfsd12a	ENSDARG00000061908	Ensembl	0.76	1.05	1.01	0.84	0.39
MGAM	ENSG00000259858	Ensembl	1.55	1.64	0.56	0.49	1.16
MGAM	ENSMUSG00000068587	Ensembl	0.00	1.84	0.00	0.00	0.00

mgt4a	NM_001173641.1	refseq	-2.92	-1.94	-0.86	-2.50	-0.50
mia3	ENSDARG00000008184	Ensembl	-0.17	0.00	2.66	0.13	0.37
micall2b	ENSDARG00000017834	Ensembl	1.49	1.45	1.27	1.56	0.44
mid1ip1l	ENSDARG00000018145	Ensembl	-1.07	-0.57	-0.94	-0.41	-0.28
mknk1	ENSDARG00000018411	Ensembl	0.96	2.67	1.55	1.73	0.76
mknk1	ENSDARG00000018411	Ensembl	0.76	2.47	1.30	1.41	0.60
mknk2b	ENSDARG00000015164	Ensembl	0.67	0.83	0.87	0.74	0.20
mknk2b	ENSDARG00000015164	Ensembl	0.77	0.73	1.06	0.77	0.43
mknk2b	ENSDARG00000015164	Ensembl	0.66	0.86	0.97	0.72	0.35
mll	ENSDARG00000004537	Ensembl	-0.31	-0.37	-1.11	-0.25	0.02
mmp13a	ENSDARG00000012395	Ensembl	1.20	2.40	1.80	0.51	-0.75
mmp13a	ENSDARG00000012395	Ensembl	1.30	2.21	1.76	0.41	0.01
mmp13a	ENSDARG00000012395	Ensembl	1.02	2.31	0.00	0.00	0.00
MMP19	ENSDARG00000091557	Ensembl	-0.27	2.83	1.01	0.94	-0.54
mmp9	ENSDARG00000042816	Ensembl	1.35	2.76	2.07	0.94	-0.71
mmp9	ENSDARG00000042816	Ensembl	1.65	0.00	2.61	1.59	0.00
mmp9	ENSDARG00000042816	Ensembl	1.60	3.34	2.40	0.00	0.00
MPPED2 (1 of 2)	ENSDARG00000006889	Ensembl	-1.05	-0.55	-0.69	-0.52	-0.80
mtmr8	ENSDARG00000008592	Ensembl	1.11	1.16	1.44	0.72	-0.01
mx2	ENSDARG00000004953	Ensembl	0.00	0.00	0.00	2.44	0.00
mych	ENSDARG00000077473	Ensembl	0.50	1.84	0.60	2.08	0.14
nadkb	ENSDARG00000060362	Ensembl	0.78	1.74	1.64	1.54	0.41
nadkb	ENSDARG00000060362	Ensembl	0.86	1.78	1.56	1.63	0.32
napa	ENSDARG00000020405	Ensembl	0.40	0.74	0.20	0.38	0.04
napepld	ENSDARG00000009252	Ensembl	0.19	0.73	0.40	0.33	-0.13
NAV2	ENSG00000166833	Ensembl	-0.70	-1.33	-0.77	-0.83	0.07
NAV2	ENSMUSG00000052512	Ensembl	-0.73	-1.49	-0.48	-0.58	0.14
ncf1	ENSDARG00000033735	Ensembl	0.00	0.99	1.42	0.00	0.00
ncor1	ENSDARG00000035285	Ensembl	-0.61	-0.38	-1.09	-0.41	-0.57
NDUFA4L2 (2 of 2)	ENSDARG00000087907	Ensembl	-2.06	-1.06	-1.01	-1.19	-0.82
NEDD5	XM_004079979.1	refseq	0.76	1.39	1.19	0.73	0.15
nfil3-5	ENSDARG00000094965	Ensembl	0.63	0.74	0.14	0.45	0.84
nfkb2	ENSDARG00000038687	Ensembl	0.41	1.65	1.36	1.17	0.17
nfkb2	ENSDARG00000038687	Ensembl	0.57	1.01	0.66	1.00	0.41
nfkb2	ENSDARG00000038687	Ensembl	0.18	1.11	0.33	0.66	-0.10
nfkb2	ENSDARG00000038687	Ensembl	0.24	1.21	0.21	0.80	-0.24
NID1 (2 of 2)	ENSDARG00000060675	Ensembl	0.71	2.66	1.26	0.96	0.00
nlrc3	ENSG00000167984	Ensembl	0.55	1.17	1.57	1.51	0.51
nlrc3	ENSG00000167984	Ensembl	0.77	1.17	1.63	1.59	0.06
nlrc3	ENSG00000167984	Ensembl	0.00	1.39	1.67	1.27	0.00
nod2	ENSDARG00000010756	Ensembl	0.38	1.10	0.33	0.60	-0.06
nots	ENSDARG00000052792	Ensembl	0.41	0.23	0.24	0.36	1.03
NOXO1	ENSONIG00000002316	Ensembl	0.00	1.82	0.00	0.00	0.00
noxo1a	ENSDARG00000041294	Ensembl	0.17	1.42	0.24	1.40	-0.28
npsn	ENSDARG00000010423	Ensembl	1.31	1.25	1.92	1.31	0.12
nr1h4	ENSDARG00000057741	Ensembl	0.71	1.40	0.87	0.55	0.78
nr1h4	ENSDARG00000057741	Ensembl	0.78	1.43	0.88	0.66	0.67
nr1h4	ENSDARG00000057741	Ensembl	0.61	1.35	0.79	0.45	0.65
nr1h4	ENSDARG00000057741	Ensembl	0.42	1.15	0.55	0.38	0.55
nr1h4	ENSDARG00000057741	Ensembl	0.65	1.40	0.98	0.43	0.72
nr1h4	ENSDARG00000057741	Ensembl	0.95	1.62	1.10	0.72	0.81
nr1h4	ENSDARG00000057741	Ensembl	0.70	1.48	0.80	0.53	0.59
nr2f1a	ENSDARG00000052695	Ensembl	-0.44	-0.81	-0.56	-0.20	-0.18
nr2f5	ENSDARG00000033172	Ensembl	0.43	0.63	0.26	0.05	0.17
Ocln-001	ENSMUSG00000021638	Ensembl	-0.82	-0.28	-2.12	0.05	-0.83
odc1	ENSDARG00000007377	Ensembl	0.40	1.25	0.32	0.53	0.42
odc1	ENSDARG00000007377	Ensembl	0.03	1.32	-0.01	0.20	0.21
odc1	ENSDARG00000007377	Ensembl	0.07	1.26	0.00	0.22	0.19
odc1	ENSDARG00000007377	Ensembl	0.00	1.31	0.13	0.38	0.02
optn	ENSDARG00000002663	Ensembl	0.74	1.47	0.00	0.74	0.68
ORC2	ENSDARG00000090203	Ensembl	0.75	0.00	0.00	1.05	0.00
pank1a	ENSDARG00000008192	Ensembl	0.29	0.93	0.55	0.45	-0.17
paternally	ENSMUSG00000092035	Ensembl	-0.74	-0.89	-0.35	-0.19	-0.36
pdcd6	ENSDARG00000005220	Ensembl	0.28	1.03	0.63	0.64	0.45
pglyrp6	ENSDARG00000015626	Ensembl	0.42	1.09	-0.23	3.34	0.00
phactr4a	ENSDARG00000015552	Ensembl	-0.29	-0.80	-0.14	-0.13	-0.03
phkb	ENSDARG00000078284	Ensembl	-0.28	-0.21	-0.61	-0.60	-0.96
phkb	ENSDARG00000078284	Ensembl	-0.60	-0.44	-0.63	-1.24	-0.21
phop2	NM_001139857.1	refseq	0.84	1.32	1.47	1.68	0.80
phospho2	ENSDARG00000058675	Ensembl	0.51	1.14	0.82	1.13	0.56
pik3cg	ENSDARG00000017757	Ensembl	0.69	1.24	0.58	1.14	0.32
pik3r3b	ENSDARG00000034409	Ensembl	-1.20	-0.44	-0.69	-0.43	-0.72
pim1	ENSDARG00000059120	Ensembl	0.80	1.37	0.83	1.49	0.55
PIM1	ENSDARG00000059120	Ensembl	0.64	1.27	0.60	1.41	0.50

pion	ENSDARG00000045481	Ensembl	-0.55	-0.28	-0.64	-0.55	-1.28
pisd	ENSDARG00000052462	Ensembl	1.32	2.19	1.57	1.71	-0.72
pisd	ENSDARG00000052462	Ensembl	1.53	2.13	1.58	1.79	-0.66
pisd	ENSDARG00000052462	Ensembl	1.39	2.02	1.61	1.60	-0.09
pisd	ENSDARG00000052462	Ensembl	1.36	2.19	1.64	1.68	-0.33
pisd	NM_001173606.1	refseq	1.36	2.28	1.68	1.55	-0.08
pisd	ENSDARG00000052462	Ensembl	1.39	1.97	1.46	1.50	-0.39
pisd	ENSDARG00000052462	Ensembl	1.34	2.07	1.59	1.61	-0.36
pisd	ENSDARG00000052462	Ensembl	1.48	2.04	1.58	1.73	-0.42
pisd	ENSDARG00000052462	Ensembl	1.22	1.87	1.39	1.50	-0.34
pkn3	ENSDARG00000079585	Ensembl	0.99	1.48	0.79	0.72	0.98
plaua	ENSDARG00000039145	Ensembl	-0.35	-1.07	-0.24	-0.23	-0.37
plaua	ENSDARG00000039145	Ensembl	-0.40	-1.52	-0.34	-0.63	-0.66
plekhg3	ENSDARG00000096613	Ensembl	-0.55	-1.07	-0.26	0.06	-0.60
plekhg4	ENSMUSG00000039713	Ensembl	-0.71	-1.13	-0.71	-0.23	-0.81
plin2	ENSDARG00000042332	Ensembl	1.03	2.35	1.14	1.17	0.82
plin2	ENSDARG00000042332	Ensembl	1.02	2.40	0.94	1.09	0.89
plin2	ENSDARG00000042332	Ensembl	0.48	1.35	0.44	0.50	0.52
plin2	ENSDARG00000042332	Ensembl	0.49	1.24	0.40	0.26	0.57
plin2	ENSDARG00000042332	Ensembl	0.62	1.17	0.53	0.19	0.44
plin2	ENSDARG00000042332	Ensembl	0.27	1.21	0.50	0.04	0.40
plin2	ENSDARG00000042332	Ensembl	0.30	1.33	0.45	0.21	0.34
pltp	ENSDARG00000035768	Ensembl	2.37	0.53	0.31	0.42	0.03
pltp	ENSDARG00000035768	Ensembl	2.68	0.39	0.34	0.63	0.10
pltp	ENSDARG00000035768	Ensembl	2.63	0.55	0.75	0.12	0.74
pnpla3	ENSDARG00000044086	Ensembl	0.99	1.17	0.21	0.00	0.54
pnpla7a	ENSDARG00000062986	Ensembl	0.47	1.06	0.18	0.13	-0.59
PPAP2A (1 of 2)	ENSDARG00000079790	Ensembl	1.61	2.11	1.68	0.00	0.00
PPAP2C (1 of 2)	ENSDARG00000002231	Ensembl	-0.08	-1.23	-0.30	0.07	-0.22
ppargc1a	ENSDARG00000067829	Ensembl	-0.53	-0.49	-1.48	-0.47	-0.33
PPP1R16A	ENSDARG00000076980	Ensembl	-0.70	-1.36	-0.58	-0.57	-0.05
PPP1R16A	ENSDARG00000076980	Ensembl	-0.91	-1.27	-0.80	-0.77	-0.16
praf2	ENSDARG00000032535	Ensembl	-0.62	-0.76	-0.30	-0.35	-0.33
prdm1a	ENSDARG00000002445	Ensembl	1.19	2.44	1.63	3.33	-1.26
prdm1a	ENSDARG00000002445	Ensembl	0.00	2.83	0.00	0.00	0.00
PRODH (3 of 3)	ENSDARG00000086512	Ensembl	0.01	0.72	1.35	0.33	-0.38
prodha	ENSDARG00000044804	Ensembl	0.27	0.94	1.50	0.96	0.28
psmc1b	ENSDARG00000043561	Ensembl	0.00	0.00	1.41	1.35	0.00
psmd11b	ENSDARG00000005134	Ensembl	0.36	0.69	0.47	0.68	0.28
ptbp1a	ENSDARG00000019362	Ensembl	-0.15	-1.45	-0.37	-0.05	-0.31
pycr1	ENSDARG00000053965	Ensembl	0.49	0.78	0.66	0.67	0.13
rab11bb	ENSDARG00000090086	Ensembl	-0.06	-0.29	-0.55	-0.93	-0.40
rab12	ENSDARG00000089428	Ensembl	0.21	0.99	-0.03	-0.09	0.13
rab20	ENSDARG00000005049	Ensembl	0.53	1.25	0.40	0.47	0.09
RAB32	ENSMUSG00000019832	Ensembl	0.47	0.89	1.01	0.53	0.37
rad54l2	ENSDARG00000063031	Ensembl	0.56	0.91	0.86	0.23	0.56
rad54l2	ENSDARG00000063031	Ensembl	0.54	0.67	0.92	0.10	0.29
raf1a	AB204911.1	nt	0.56	1.51	0.89	0.96	0.38
rarg	EU025716.1	nt	2.74	2.24	0.00	2.79	1.33
rarg	EU025716.1	nt	0.78	2.05	1.17	1.09	0.28
rbfox1	ENSDARG00000014746	Ensembl	-0.88	-0.37	-0.26	-0.28	-0.36
RBM6	ENSDARG00000077060	Ensembl	0.00	1.04	0.00	1.18	0.00
rela	ENSDARG00000021907	Ensembl	0.30	1.29	0.81	1.36	-0.21
relb	ENSDARG00000086173	Ensembl	1.02	2.33	2.06	1.76	1.07
relb	ENSDARG00000086173	Ensembl	0.83	2.24	1.74	1.63	0.83
relb	ENSDARG00000086173	Ensembl	1.10	2.17	1.92	1.73	0.79
relb	ENSDARG00000086173	Ensembl	1.15	1.93	1.48	1.38	0.83
relb	ENSDARG00000086173	Ensembl	1.01	2.06	1.49	1.51	0.82
relb	ENSDARG00000086173	Ensembl	0.75	1.95	1.28	1.28	0.45
rev3l	ENSDARG00000058801	Ensembl	-0.78	-0.97	-0.44	-0.43	-1.04
rfk	ENSDARG00000060522	Ensembl	1.77	2.61	1.76	1.56	0.38
rifk	NM_001140512.1	refseq	1.91	2.55	1.88	1.42	0.92
rifk	NM_001140512.1	refseq	1.90	2.64	1.93	1.45	0.69
rifk	NM_001140512.1	refseq	1.86	2.73	1.82	1.51	0.53
RIPK2	ENSG00000104312	Ensembl	0.00	0.00	1.66	0.00	0.00
rnd3a	ENSDARG00000076799	Ensembl	1.02	1.75	1.40	1.16	0.28
rnd3a	ENSDARG00000076799	Ensembl	-0.78	-0.40	-1.36	-0.51	-0.59
rnf126	ENSDARG00000088454	Ensembl	0.53	0.24	0.39	0.75	0.64
rnf170	ENSG00000120925	Ensembl	0.94	0.92	0.55	0.93	0.22
Rnf213	ENSMUSG00000070327	Ensembl	-0.38	0.34	0.32	1.44	-0.17
rsad2	ENSDARG00000004952	Ensembl	-0.17	0.08	0.08	1.88	-0.03
rxraa	ENSDARG00000057737	Ensembl	-0.75	-0.82	-0.64	-0.57	-0.78
sacs	ENSMUSG00000048279	Ensembl	-0.82	-0.35	0.04	3.01	-1.36
sacs	ENSMUSG00000048279	Ensembl	-0.73	-0.64	0.17	2.96	-1.11
sacs	ENSMUSG00000048279	Ensembl	0.00	0.00	0.00	2.94	0.00

sar1ab	ENSDARG00000033320	Ensembl	0.00	1.04	0.00	0.00	0.00
sc5d	NM_001140116.1	refseq	2.10	2.81	2.13	1.80	0.77
scap	ENSDARG00000018096	Ensembl	0.63	0.99	0.52	1.15	-0.08
scinla	ENSDARG00000091639	Ensembl	-0.49	-0.41	-0.15	-0.11	-0.82
sdc4	ENSDARG00000059906	Ensembl	1.12	4.05	2.94	1.45	0.78
sec14l1	ENSDARG00000019301	Ensembl	0.90	1.18	1.16	0.01	0.81
sec23a	ENSDARG00000016636	Ensembl	0.56	0.78	0.54	0.53	0.43
sec24d	ENSDARG00000045946	Ensembl	0.38	0.93	0.44	0.86	0.12
sf3b3	NM_001141594.1	refseq	1.01	4.53	2.69	3.15	-0.13
sgms2	ENSDARG00000052520	Ensembl	0.76	1.80	1.31	1.64	0.99
sh2d4b	ENSDARG00000029443	Ensembl	0.79	1.93	1.70	2.54	0.01
sh2d4b	ENSDARG00000029443	Ensembl	1.58	2.33	1.97	2.08	0.33
shrprbck1r	ENSDARG00000059871	Ensembl	0.39	0.74	0.35	0.28	0.03
si:ch211-154o6.6	ENSDARG00000056379	Ensembl	0.28	1.56	1.00	0.61	0.53
si:ch211-154o6.6	ENSDARG00000056379	Ensembl	0.56	1.76	1.15	0.98	0.55
si:ch211-154o6.6	ENSDARG00000056379	Ensembl	0.88	1.94	1.41	1.45	0.51
si:ch211-154o6.6	ENSDARG00000056379	Ensembl	0.89	1.89	1.43	1.41	0.50
si:ch211-154o6.6	ENSDARG00000056379	Ensembl	0.88	1.92	1.31	1.45	0.45
si:ch211-214c7.4	ENSDARG00000069595	Ensembl	-0.51	-0.49	-1.37	-0.08	-0.40
si:ch211-236p5.3	ENSDARG00000086418	Ensembl	-0.86	-0.82	-0.47	-2.29	-1.73
si:ch211-74m13.1	ENSDARG00000094952	Ensembl	0.85	4.24	2.33	3.02	0.23
si:dkey-10o6.2	ENSDARG00000074628	Ensembl	-0.82	-1.24	-0.64	-0.71	-0.01
skia	ENSDARG00000042151	Ensembl	-0.39	-1.29	-0.56	-0.54	-0.95
SLC13A5	ENSG00000141485	Ensembl	0.41	0.71	1.30	0.95	0.75
SLC13A5 (2 of 2)	ENSDARG00000077691	Ensembl	0.82	0.71	1.08	1.10	0.61
slc16a6b	ENSDARG00000060246	Ensembl	0.40	0.84	1.12	1.62	0.56
slc16a6b	ENSDARG00000060246	Ensembl	0.44	0.81	1.20	1.47	0.46
slc25a28	ENSDARG00000074297	Ensembl	0.00	1.26	0.00	0.48	0.00
slc27a4	ENSDARG00000017047	Ensembl	0.00	1.03	0.66	1.13	0.19
slc31a1	ENSDARG00000013961	Ensembl	0.93	1.59	1.62	1.52	0.29
slc33a1	ENSDARG00000020085	Ensembl	0.40	0.85	0.45	0.78	0.24
SLC39A8 (1 of 2)	ENSDARG00000056757	Ensembl	0.27	0.99	0.36	1.02	0.25
SLC39A8 (2 of 2)	ENSDARG00000087905	Ensembl	0.50	1.10	0.62	1.19	0.09
slc3a2b	ENSDARG00000037012	Ensembl	0.71	1.08	0.89	0.77	0.34
slc43a1a	ENSDARG00000037393	Ensembl	1.37	1.75	1.72	1.98	0.78
slc43a1a	ENSDARG00000037393	Ensembl	2.86	3.33	3.25	3.51	1.61
slc52	ENSMUSG00000027463	Ensembl	4.86	5.50	4.32	2.55	3.49
slc52	ENSMUSG00000027463	Ensembl	4.87	5.24	4.08	2.45	3.34
SLC6A16 (1 of 2)	ENSDARG00000007129	Ensembl	1.15	1.11	0.00	1.78	0.00
smad4	ENSDARG00000023527	Ensembl	-0.83	-1.35	-0.74	-0.54	-0.22
smad6b	ENSDARG00000031763	Ensembl	-0.43	-0.73	-0.44	-0.15	-0.32
smad7	ENSDARG00000016858	Ensembl	-1.26	-1.01	-0.86	-0.44	-0.56
smad7	ENSDARG00000016858	Ensembl	-0.92	-0.96	-0.70	-0.38	-0.53
SMCHD1	ENSG00000101596	Ensembl	0.00	0.00	0.00	1.45	0.00
smpd5	ENSDARG00000059811	Ensembl	1.01	1.43	1.80	0.51	0.46
smpd5	ENSDARG00000059811	Ensembl	0.88	1.54	1.58	0.35	0.15
socs2	ENSDARG00000045557	Ensembl	1.22	0.00	1.62	0.00	0.00
socs3b	ENSDARG00000026611	Ensembl	1.03	0.92	0.86	0.97	0.42
sox11a	ENSDARG00000077811	Ensembl	-0.75	-1.26	-0.10	-0.24	0.01
sqstm1	ENSDARG00000075014	Ensembl	0.93	1.99	1.39	1.41	0.43
sqstm1	ENSDARG00000075014	Ensembl	0.80	1.78	1.12	1.08	0.39
sqstm1	ENSDARG00000075014	Ensembl	0.84	1.87	1.09	1.17	0.44
sqstm1	ENSDARG00000075014	Ensembl	0.78	1.69	1.09	1.06	0.45
sqstm1	ENSDARG00000075014	Ensembl	0.74	1.75	0.97	1.07	0.29
srebf2	ENSDARG00000063438	Ensembl	1.04	1.10	0.67	1.05	0.17
srebf2	ENSDARG00000063438	Ensembl	0.95	0.96	0.50	0.90	0.01
srebf2	ENSDARG00000063438	Ensembl	1.00	1.16	0.71	0.77	0.32
srebf2	ENSDARG00000063438	Ensembl	1.11	1.18	0.86	0.84	-0.04
srebf2	ENSDARG00000063438	Ensembl	0.99	1.13	0.32	0.73	0.06
srebf2	ENSDARG00000063438	Ensembl	1.06	0.90	0.46	0.80	0.02
ssh2b	ENSDARG00000077623	Ensembl	0.91	1.48	1.60	1.38	0.12
ssh2b	ENSDARG00000077623	Ensembl	0.97	1.30	1.59	1.19	0.27
ssh2b	ENSDARG00000077623	Ensembl	1.05	1.26	1.60	1.51	0.11
st3gal1	ENSDARG00000079654	Ensembl	0.51	1.97	1.42	0.44	0.51
stat2	ENSDARG00000031647	Ensembl	0.40	1.19	1.00	1.19	0.37
stat3	ENSDARG00000022712	Ensembl	1.24	0.95	0.96	0.85	0.55
steap4	ENSDARG00000055901	Ensembl	1.59	1.90	1.88	1.88	0.50
steap4	ENSDARG00000055901	Ensembl	1.60	1.95	1.96	1.83	0.56
steap4	ENSDARG00000055901	Ensembl	1.54	1.94	2.04	1.68	0.41
steap4	ENSDARG00000055901	Ensembl	1.44	1.87	1.95	1.68	0.46
steap4	ENSDARG00000055901	Ensembl	1.27	1.82	1.79	1.73	0.32
steap4	ENSDARG00000055901	Ensembl	1.35	1.88	1.77	1.59	0.52
steap4	ENSDARG00000055901	Ensembl	1.34	1.88	1.80	1.60	0.48
steap4	ENSDARG00000055901	Ensembl	1.60	1.93	1.98	1.79	0.42
steap4	ENSDARG00000055901	Ensembl	1.60	1.98	1.99	1.80	0.50

steap4	ENSDARG00000055901	Ensembl	1.26	1.60	1.77	1.57	0.30
steap4	ENSDARG00000055901	Ensembl	1.48	1.82	1.88	1.62	0.38
steap4	ENSDARG00000055901	Ensembl	1.31	1.62	1.74	1.62	0.24
steap4	ENSDARG00000055901	Ensembl	1.48	1.96	1.90	1.62	0.59
steap4	ENSDARG00000055901	Ensembl	1.50	1.74	1.84	1.64	0.29
steap4	ENSDARG00000055901	Ensembl	1.35	1.51	1.66	1.64	0.23
steap4	ENSDARG00000055901	Ensembl	1.57	1.78	1.83	1.68	0.50
steap4	ENSDARG00000055901	Ensembl	1.46	1.80	1.98	1.68	0.52
steap4	ENSDARG00000055901	Ensembl	1.47	1.97	2.02	1.74	0.38
steap4	ENSDARG00000055901	Ensembl	1.48	1.93	2.07	1.74	0.38
steap4	ENSDARG00000055901	Ensembl	1.42	1.69	1.84	1.77	0.16
steap4	ENSDARG00000055901	Ensembl	1.49	1.88	1.98	1.78	0.55
steap4	ENSDARG00000055901	Ensembl	1.58	1.89	1.99	1.84	0.52
steap4	ENSDARG00000055901	Ensembl	1.01	1.44	1.48	1.23	0.42
steap4	ENSDARG00000055901	Ensembl	1.08	1.47	1.51	1.37	0.31
steap4	ENSDARG00000055901	Ensembl	1.15	1.57	1.58	1.40	0.43
steap4	ENSDARG00000055901	Ensembl	1.57	1.73	1.81	1.71	0.64
steap4	ENSDARG00000055901	Ensembl	1.59	1.88	2.08	1.80	0.49
steap4	ENSDARG00000055901	Ensembl	1.59	1.89	1.98	1.84	0.47
stim2	ENSDARG00000001776	Ensembl	1.32	1.72	0.82	1.42	0.62
stk40	ENSDARG00000060318	Ensembl	0.70	1.99	1.27	1.38	0.39
stk40	ENSDARG00000060318	Ensembl	0.46	1.78	1.20	1.03	0.05
stk40	ENSDARG00000060318	Ensembl	0.93	1.87	1.27	1.50	0.44
styx	ENSDARG00000057699	Ensembl	0.12	0.88	0.54	0.62	0.03
SUCO	ENSMUSG00000040297	Ensembl	0.39	1.20	0.54	0.85	0.19
syncrip	ENSDARG00000040184	Ensembl	0.89	1.40	0.79	1.72	0.39
syncripl	ENSDARG00000026723	Ensembl	1.12	1.72	1.11	1.62	0.58
syncripl	ENSDARG00000026723	Ensembl	0.94	1.70	0.40	1.60	0.51
SYNPO2 (1 of 2)	ENSDARG00000079675	Ensembl	-0.23	0.83	0.37	0.20	-0.08
tbx2b	ENSDARG00000006120	Ensembl	-0.57	-0.45	-1.04	-0.32	-0.47
tbx2b	ENSDARG00000006120	Ensembl	-0.64	-0.78	-0.61	-0.14	-0.51
tbx2b	ENSDARG00000006120	Ensembl	-1.20	-0.68	-0.54	-0.29	-0.97
TDG	ENSGMOG00000019080	Ensembl	0.33	1.38	0.74	0.18	0.58
tdh	ENSDARG00000002745	Ensembl	1.01	1.93	1.38	1.69	1.01
tdh	ENSDARG00000002745	Ensembl	0.94	1.88	1.70	1.60	0.69
tdo2a	ENSDARG00000071429	Ensembl	-0.35	-1.39	-0.30	-0.31	0.24
tescb	ENSDARG00000030839	Ensembl	-0.16	0.98	0.49	1.97	-0.03
tfpi2	ENSDARG00000061351	Ensembl	0.27	-0.08	0.71	0.83	-0.02
tial1	ENSDARG00000009525	Ensembl	0.26	0.93	0.30	0.68	0.59
tlr21	ENSDARG00000058045	Ensembl	-0.17	1.58	0.85	0.56	-0.20
tlr5b	ENSDARG00000052322	Ensembl	2.50	4.31	3.31	3.06	1.61
tlr5b	ENSDARG00000052322	Ensembl	3.24	5.07	4.13	3.67	2.02
tlr5b	ENSDARG00000052322	Ensembl	2.52	4.24	3.27	2.84	1.52
tnfr14	NM_001141866.1	refseq	1.40	3.18	2.87	3.03	0.25
tnfr21	ENSDARG00000001807	Ensembl	0.20	1.72	1.27	0.53	0.25
tnip1	ENSDARG00000015653	Ensembl	0.96	1.49	1.30	1.60	0.34
tnip1	ENSDARG00000015653	Ensembl	0.63	0.95	1.00	0.53	0.35
tnip1	ENSDARG00000015653	Ensembl	0.94	1.34	1.29	1.45	-0.05
tnip1	ENSDARG00000015653	Ensembl	0.67	1.34	1.13	1.08	0.71
TNIP2	ENSDARG00000074501	Ensembl	0.65	3.27	1.64	1.83	-0.19
tp53	ENSDARG00000035559	Ensembl	0.00	0.00	0.00	1.91	0.00
traf2b	ENSDARG00000017812	Ensembl	0.29	1.30	0.90	0.81	0.04
traf3	ENSDARG00000022000	Ensembl	0.58	1.33	0.90	0.66	0.06
trak1	ENSG00000182606	Ensembl	-0.06	-1.31	-0.53	-0.46	-0.23
trim32	ENSDARG00000076553	Ensembl	-0.18	-0.73	-0.04	-0.10	-0.39
trim39	ENSMUSG00000045409	Ensembl	-0.87	-0.63	-1.19	-0.28	-0.68
Tstd1	ENSMUSG00000091166	Ensembl	0.62	0.47	-0.13	1.12	0.40
txndc11	ENSDARG00000076938	Ensembl	0.64	0.00	0.00	1.60	0.00
txndc9	ENSDARG00000069853	Ensembl	-1.37	-1.36	-0.83	-1.38	-0.87
u2af2b	ENSDARG00000011740	Ensembl	-0.74	-1.31	-0.56	-0.58	-0.11
ubald1a	ENSDARG00000002362	Ensembl	0.37	0.77	0.37	0.24	0.28
ubap2	ENSDARG00000088318	Ensembl	0.65	0.48	0.46	1.03	0.41
ugt5c1	ENSDARG00000061444	Ensembl	-0.20	-1.13	-0.30	-0.80	-0.30
unc93a	ENSDARG00000041554	Ensembl	0.61	1.16	0.45	0.74	0.06
uncharacterised	BT045802.1	nt	-0.43	-0.27	0.17	0.02	1.27
uncharacterised	BT059612.1	nt	-0.01	0.19	-0.07	0.29	0.99
uncharacterised	DQ156150.1	nt	0.02	0.00	0.02	0.20	0.94
uncharacterised	DQ246664.1	nt	0.13	0.38	0.12	0.34	1.44
uncharacterised	ENSORLG00000000574	Ensembl	0.07	0.32	0.17	0.86	1.90
uncharacterised	NM_001141471.1	refseq	0.18	0.25	0.15	1.22	1.43
uncharacterised			-0.30	0.94	1.44	1.04	2.35
uncharacterised			0.51	0.49	0.56	0.24	1.28
uncharacterised			0.50	0.65	0.34	-0.09	0.84
uncharacterised	NP_001118004.1	refseq	-0.19	-0.24	0.96	0.96	2.07
uncharacterised	EU853449.1	nt	0.00	0.00	0.00	0.76	1.61

uncharacterised			0.00	0.00	0.56	0.00	1.26
uncharacterised			0.00	0.81	0.00	0.00	1.23
uncharacterised			0.00	0.00	0.00	0.00	1.75
uncharacterised	AC203446.12	nt	1.07	3.06	2.81	3.24	0.31
uncharacterised	AC203446.12	nt	1.06	2.33	1.27	1.48	0.28
uncharacterised	AC203446.12	nt	1.16	2.81	2.63	2.79	0.51
uncharacterised	AF055440.1	nt	1.56	2.22	1.97	1.75	1.05
uncharacterised	BT047041.1	nt	-0.02	1.71	1.27	1.55	0.36
uncharacterised	BT059080.1	nt	1.10	2.67	1.65	1.82	0.78
uncharacterised	BT072619.1	nt	0.80	1.39	1.35	1.37	0.20
uncharacterised	EU221180.1_	nt	1.46	3.15	2.03	1.85	1.04
uncharacterised	GU129139.1	nt	1.33	1.78	1.67	1.38	1.29
uncharacterised	GU817337.1	nt	1.16	2.23	2.73	2.86	0.66
uncharacterised			1.17	2.44	1.74	1.45	1.25
uncharacterised	EU221177.1	nt	2.56	2.60	2.77	2.82	1.71
uncharacterised	EU481821.1	nt	3.12	4.72	3.80	3.03	2.23
uncharacterised	BX511086.5	nt	0.92	0.83	0.85	1.03	0.65
uncharacterised			1.76	2.37	2.33	2.62	1.27
uncharacterised	FJ969489.1	nt	0.86	1.41	0.71	1.11	0.29
uncharacterised	GU294488.1	nt	0.35	1.55	0.57	1.81	0.13
uncharacterised	AB162343.1	nt	0.48	0.11	0.30	1.18	0.19
uncharacterised	AC203446.12	nt	0.09	0.92	0.28	2.02	-0.19
uncharacterised	AY493348.1	nt	0.54	0.56	0.74	2.10	-0.73
uncharacterised	BT045214.1	nt	0.74	0.83	0.38	1.89	1.14
uncharacterised	CAB51372.1	nt	-0.18	0.17	0.33	1.60	0.12
uncharacterised	CAB51372.1	nt	0.20	0.81	0.60	1.95	-0.05
uncharacterised	DQ156149.1	nt	0.63	1.20	0.99	1.44	0.70
uncharacterised	EU025708.1	nt	2.14	1.52	0.28	2.70	0.51
uncharacterised	EU816603.1	nt	0.76	0.12	0.99	1.26	0.62
uncharacterised	GU129140.1	nt	1.58	0.20	1.63	2.11	1.15
uncharacterised	HM159473.1	nt	1.58	0.73	1.61	2.22	0.92
uncharacterised			0.82	0.94	1.07	2.60	1.62
uncharacterised			0.64	1.26	0.67	1.46	0.51
uncharacterised			0.52	0.86	0.47	1.08	0.25
uncharacterised	XM_003455175.1	refseq	0.33	1.60	0.21	2.44	-0.15
uncharacterised	XP_003458266.1	refseq	1.02	0.38	0.46	1.19	0.32
uncharacterised	XP_003458962.1	refseq	1.06	0.34	0.73	1.31	-0.19
uncharacterised	CAB51372.1	nt	0.34	1.30	0.00	2.31	0.42
uncharacterised	NP_001119851.1	refseq	0.68	1.09	0.00	1.14	0.47
uncharacterised	NM_001123619.1	refseq	0.00	0.00	0.00	2.06	0.21
uncharacterised	EU221178.1	nt	-1.21	-1.65	-0.79	-1.45	-0.34
uncharacterised	EU481821.1	nt	-0.70	-0.81	-0.68	-1.14	-0.19
uncharacterised	HM159471.1	nt	-1.25	-1.87	-0.75	-1.68	-0.93
uncharacterised			-0.13	-0.90	-0.58	-1.17	-0.57
uncharacterised	FP016154.2	nt	-1.49	-1.78	-0.71	-1.82	-0.56
uncharacterised	AB162342.1	nt	0.68	0.46	0.95	-5.06	0.01
uncharacterised	AF232215.1	nt	-0.24	-0.22	-0.25	-1.59	0.21
uncharacterised	EU481821.1	nt	-0.48	-0.02	-0.42	-0.95	-0.28
uncharacterised	NM_001165397.1	refseq	-0.15	-0.08	0.07	-1.10	-0.14
uncharacterised	NM_001172281.1	refseq	-1.03	-0.25	0.00	-4.84	-0.62
uncharacterised			-0.35	-0.26	-0.26	-1.71	-1.11
uncharacterised			-0.67	-0.47	-0.73	-1.17	-0.52
uncharacterised			-0.62	-0.68	-0.53	-1.39	-0.46
uncharacterised			-0.34	-0.62	-0.58	-1.31	-0.45
uncharacterised			0.50	0.24	0.47	-6.63	-0.63
uncharacterised			1.34	0.66	1.09	-5.37	0.40
uncharacterised			-0.37	0.24	-0.54	-2.23	-0.04
uncharacterised			-0.64	-0.60	-0.41	-1.09	-0.17
uncharacterised	XP_003448688.1	refseq	-0.17	-0.08	-0.30	-0.74	-0.25
uncharacterised	AC203446.12	nt	1.48	3.50	2.48	2.17	0.86
uncharacterised	BT044988.1	nt	0.52	2.32	1.25	1.11	0.65
uncharacterised	BT045198.1	nt	1.80	2.60	1.86	1.46	0.70
uncharacterised	BT045418.1	nt	1.77	2.50	1.74	1.40	0.68
uncharacterised			0.44	0.84	0.87	0.35	0.89
uncharacterised			0.31	0.90	0.85	-0.01	0.66
uncharacterised			0.53	2.00	1.30	1.32	0.70
uncharacterised			0.78	2.47	1.42	1.02	0.55
uncharacterised			1.41	2.41	1.91	1.10	0.72
uncharacterised			0.23	0.91	0.66	0.73	0.07
uncharacterised	XM_003450362.1	refseq	0.45	1.68	1.19	0.79	0.46
uncharacterised	XM_004553799.1	refseq	1.09	1.41	1.32	1.01	1.05
uncharacterised	XM_004575248.1	refseq	1.11	1.75	1.31	1.36	0.81
uncharacterised	AAX28478.2	nt	1.05	0.88	1.62	1.05	0.76
uncharacterised	BT048706.1	nt	1.35	2.73	3.46	3.33	0.28
uncharacterised	CX354065.1	nt	0.01	0.03	1.38	0.30	1.32

uncharacterised	DN047920.1	nt	0.51	0.65	1.25	1.32	0.87
uncharacterised	DQ246664.1	nt	1.69	1.96	2.04	1.99	0.68
uncharacterised	DQ246664.1	nt	1.76	2.24	2.06	2.00	0.64
uncharacterised	DW537532.1	nt	1.23	1.23	1.28	0.66	0.87
uncharacterised	DY702037.1	nt	0.84	0.75	1.10	0.93	0.42
uncharacterised	DY729066.1	nt	0.82	1.05	1.24	0.82	0.79
uncharacterised	DY733338.1	nt	0.37	1.01	1.37	1.28	0.93
uncharacterised	DY738636.1	nt	0.10	0.09	1.09	0.34	0.93
uncharacterised	EF210363.1	nt	1.68	1.83	1.95	1.74	0.81
uncharacterised	EF210363.1	nt	1.41	1.43	1.58	1.44	0.28
uncharacterised	EG831757.1	nt	1.29	1.40	2.17	2.16	1.16
uncharacterised	ENSORLG00000004811	Ensembl	1.79	1.77	2.00	2.15	0.75
uncharacterised	ENSORLG00000004811	Ensembl	1.45	1.84	1.77	1.88	0.70
uncharacterised	ENSORLG00000004811	Ensembl	1.55	1.44	1.73	1.84	0.58
uncharacterised	ENSORLG00000015212	Ensembl	0.57	0.67	1.09	0.82	0.42
uncharacterised	EU025706.1	nt	0.64	1.25	1.33	0.91	0.65
uncharacterised	EU025708.1	nt	1.67	1.82	2.01	1.94	0.56
uncharacterised	EU025716.1	nt	1.48	1.64	1.87	1.68	0.73
uncharacterised	EU025719.1	nt	1.79	1.98	2.14	2.07	0.70
uncharacterised	EU025719.1	nt	0.75	1.00	1.08	0.75	0.00
uncharacterised	EU025719.1	nt	1.74	1.95	2.09	1.99	0.60
uncharacterised	EU221177.1	nt	1.82	1.82	1.94	2.03	0.66
uncharacterised	EU221180.1	nt	1.77	1.99	2.15	1.97	0.66
uncharacterised	EV374524.1	nt	0.81	0.52	0.98	0.67	0.39
uncharacterised	FF845837.1	nt	1.37	1.10	2.04	2.26	0.98
uncharacterised	FJ356137.1	nt	0.35	0.71	1.04	1.20	0.66
uncharacterised	GQ505860.1	nt	1.69	1.66	1.78	1.88	0.54
uncharacterised	GQ925552.1	nt	0.42	1.72	1.71	2.01	0.87
uncharacterised	GU817336.1	nt	1.42	1.36	1.70	1.61	0.20
uncharacterised	HM159472.1	nt	1.73	1.78	1.91	2.01	0.67
uncharacterised	HM159473.1	nt	1.77	1.80	2.05	1.98	0.52
uncharacterised	HQ287746.1	nt	0.83	2.16	2.13	2.02	1.21
uncharacterised	NM_001124458.1	refseq	1.64	1.75	1.92	1.87	0.68
uncharacterised	NM_001146488.1	refseq	0.45	1.16	2.21	0.67	1.15
uncharacterised	NM_001173968.1	refseq	1.73	1.82	1.92	1.98	0.48
uncharacterised			0.36	1.75	1.71	1.76	0.70
uncharacterised	XP_002933173.1	refseq	1.10	1.91	1.55	0.89	0.47
uncharacterised	NM_001173828.1	refseq	1.15	1.12	1.58	1.12	0.46
uncharacterised			1.15	0.43	1.04	0.70	1.21
uncharacterised	XM_004553799.1	refseq	1.06	0.79	1.22	0.73	0.72
uncharacterised	BT058802.1	nt	-0.13	-1.37	-1.65	-0.99	-0.32
uncharacterised	BT125491.1	nt	-0.07	-0.19	-1.30	-0.23	0.18
uncharacterised	CA038505.1	nt	-0.07	-0.39	-1.27	-0.38	-0.59
uncharacterised	CA052404.1	nt	-1.58	-1.19	-2.04	-1.35	-0.98
uncharacterised	CB510837.1	nt	-0.74	-0.54	-1.10	-0.62	-0.56
uncharacterised	CB517258.1	nt	-1.02	-0.95	-1.15	-0.65	-0.54
uncharacterised	CR381643.18	nt	-0.42	-0.59	-1.14	-0.37	-0.54
uncharacterised	DN047751.1	nt	-1.69	-0.33	-2.83	-0.51	-0.43
uncharacterised	DN047751.1	nt	-1.57	-0.23	-3.32	-0.23	-0.27
uncharacterised	DY736041.1	nt	-0.69	-0.57	-1.49	-0.89	-0.62
uncharacterised	EG818439.1	nt	-0.27	-0.75	-1.32	-0.28	-0.11
uncharacterised	EG856747.1	nt	-0.32	-0.40	-1.28	-0.17	0.28
uncharacterised	EG862378.1	nt	-0.35	-0.20	-1.16	-0.10	0.28
uncharacterised	FJ969488.1	nt	0.06	-0.25	-1.73	0.02	0.24
uncharacterised	NM_001124249.1	refseq	3.17	1.65	-2.56	-0.31	0.31
uncharacterised	NM_001140310.1	refseq	-0.68	-0.67	-1.09	-0.33	-0.30
uncharacterised	NM_001141267.2	refseq	-1.03	-0.73	-1.27	-0.49	-0.41
uncharacterised	NM_001141481.1	refseq	-0.18	0.06	-1.13	-0.41	-0.06
uncharacterised	NM_001173941.1	refseq	-0.21	-0.27	-1.12	0.53	0.21
uncharacterised			-0.61	-0.91	-1.44	-1.01	-0.57
uncharacterised			-0.92	-1.09	-1.33	-1.11	0.09
uncharacterised			-1.38	-0.45	-3.18	-0.45	-0.41
uncharacterised			-1.23	-0.46	-1.75	0.07	-0.14
uncharacterised			-0.69	-0.05	-1.09	0.12	-0.23
uncharacterised	XP_001923568.1	refseq	-1.26	-0.14	-2.85	-0.48	-0.43
uncharacterised	AB204911.1	nt	0.78	2.30	1.12	1.12	0.42
uncharacterised	AC203446.12	nt	0.71	1.97	1.15	0.65	0.50
uncharacterised	AC203446.12	nt	0.40	1.27	0.30	0.62	0.09
uncharacterised	AC203446.12	nt	0.48	1.21	0.44	0.68	0.03
uncharacterised	ACI66788.1	nt	0.49	1.38	0.88	0.47	0.48
uncharacterised	ACI68549.1	nt	0.85	2.43	1.59	0.77	0.92
uncharacterised	ACN10093.1	nt	1.06	1.92	1.36	0.78	0.83
uncharacterised	ACN10793.1	nt	0.64	1.44	0.51	0.74	0.61
uncharacterised	BT045054.1	nt	0.58	1.22	0.87	0.83	0.04
uncharacterised	BT045136.1	nt	0.15	0.64	0.27	0.37	0.27

uncharacterised	BT048122.1	nt	0.62	1.35	1.06	0.68	0.16
uncharacterised	BT059080.1	nt	0.73	1.82	1.05	1.04	0.72
uncharacterised	BT059080.1	nt	0.49	1.48	0.40	0.56	0.68
uncharacterised	BT059080.1	nt	0.34	1.59	0.61	0.74	0.65
uncharacterised	BT071883.1	nt	1.05	2.41	1.42	1.66	0.27
uncharacterised	BT072281.1	nt	0.10	0.93	0.38	0.63	0.14
uncharacterised	BT072361.1	nt	0.74	1.95	1.07	0.98	0.89
uncharacterised	BT072377.1	nt	0.66	1.07	0.76	0.92	0.49
uncharacterised	BT072377.1	nt	0.61	1.42	0.69	1.01	0.51
uncharacterised	BT072598.1	nt	0.97	2.15	0.89	0.99	0.99
uncharacterised	CB484574.1	nt	0.34	1.02	0.90	1.00	0.68
uncharacterised	DQ778606.1	nt	0.66	1.52	0.79	0.80	0.46
uncharacterised	DQ778606.1	nt	0.91	2.05	1.02	1.05	0.49
uncharacterised	DQ778606.1	nt	0.42	1.46	0.72	0.80	0.06
uncharacterised	DQ778606.1	nt	0.35	1.32	0.62	0.82	0.21
uncharacterised	DW535658.1	nt	0.56	1.27	1.17	0.40	0.55
uncharacterised	DW538554.1	nt	0.53	1.41	0.42	0.77	0.47
uncharacterised	DW557959.1	nt	0.26	1.57	0.78	0.50	0.35
uncharacterised	DW564872.1	nt	0.23	1.04	-0.20	0.75	0.48
uncharacterised	DW564872.1	nt	0.20	1.14	0.24	0.99	-0.20
uncharacterised	DW565550.1	nt	0.71	1.11	-0.01	0.47	0.34
uncharacterised	DY719183.1	nt	0.99	1.90	1.04	0.16	0.39
uncharacterised	DY724465.1	nt	1.61	3.63	1.60	2.08	0.84
uncharacterised	DY729630.1	nt	0.48	1.77	1.05	1.09	0.30
uncharacterised	EF467296.1	nt	1.53	2.35	1.87	1.50	1.12
uncharacterised	EG778722.1	nt	0.04	0.86	0.61	0.54	0.16
uncharacterised	EG787053.1	nt	1.06	1.89	1.22	1.39	0.44
uncharacterised	EG858211.1	nt	0.83	1.62	0.76	1.05	0.40
uncharacterised	EG876529.1	nt	0.63	1.01	0.68	0.39	0.41
uncharacterised	EG891444.1	nt	0.18	1.59	0.54	0.98	0.20
uncharacterised	EG939846.1	nt	0.42	1.33	0.81	0.58	0.25
uncharacterised	ENSONIG00000020760	Ensembl	0.31	0.88	0.34	0.46	0.26
uncharacterised	ENSONIG00000020760	Ensembl	0.43	0.95	0.33	0.44	0.21
uncharacterised	ENSONIG00000020760	Ensembl	0.50	1.48	0.61	0.70	0.23
uncharacterised	ENSONIG00000020760	Ensembl	0.21	0.81	0.18	0.33	0.16
uncharacterised	ENSONIG00000020760	Ensembl	0.41	1.17	0.50	0.60	0.15
uncharacterised	ENSORLG00000004337	Ensembl	0.38	1.01	0.40	0.50	0.25
uncharacterised	ENSORLG00000004811	Ensembl	0.99	1.09	0.83	0.83	0.57
uncharacterised	EU025709.1	nt	-0.08	1.09	0.16	-0.06	0.13
uncharacterised	EU025714.1	nt	0.38	1.35	0.71	0.77	0.55
uncharacterised	EU025715.1	nt	0.97	1.74	1.21	0.53	0.55
uncharacterised	EU025715.1	nt	0.76	1.70	1.03	0.80	0.20
uncharacterised	EU025717.1	nt	0.73	1.72	1.21	0.56	0.37
uncharacterised	EU221176.1	nt	-0.17	0.70	0.25	-0.11	0.34
uncharacterised	EU221178.1	nt	0.56	1.28	0.69	0.75	0.33
uncharacterised	EU481821.1	nt	0.55	0.90	0.39	0.34	0.02
uncharacterised	EU621898.1	nt	0.68	1.10	0.54	0.62	0.20
uncharacterised	FM207658.1	nt	0.28	2.01	1.15	0.71	0.86
uncharacterised	GQ505859.1	nt	-0.13	3.45	-0.10	0.24	0.08
uncharacterised	GU129139.1	nt	0.68	2.55	1.57	1.34	0.10
uncharacterised	GU129140.1	nt	0.17	1.22	0.50	0.63	-0.01
uncharacterised	HM159473.1	nt	1.06	1.95	1.39	0.91	0.62
uncharacterised	HM208332.1	nt	0.19	1.15	0.55	0.43	0.34
uncharacterised	NM_001139612.1	refseq	0.40	1.11	0.62	0.53	0.29
uncharacterised	NM_001139997.1	refseq	0.56	1.39	0.65	0.52	0.45
uncharacterised	NM_001160619.1	refseq	0.63	1.27	0.10	0.49	0.49
uncharacterised	NM_001173566.1	refseq	0.64	1.48	0.75	0.73	0.16
uncharacterised			0.70	1.10	0.44	0.42	0.51
uncharacterised			-0.56	1.74	0.65	0.48	0.17
uncharacterised	NR_030020.1	refseq	0.51	1.30	0.91	0.96	0.66
uncharacterised	U58910.1	nt	0.50	2.53	1.15	1.49	-0.55
uncharacterised	FJ969489.1	nt	1.20	1.63	0.93	1.26	0.68
uncharacterised			1.19	2.02	1.06	1.33	0.55
uncharacterised	AB162342.1	nt	-0.47	-0.86	-0.78	0.08	0.00
uncharacterised	ABV31710.1	nt	-0.36	-0.86	-0.37	-0.47	-0.21
uncharacterised	AC203456.8	nt	-0.44	-1.06	-1.03	-0.08	0.20
uncharacterised	BAB55662.1	nt	0.05	-0.88	-0.15	0.12	-0.43
uncharacterised	BAB55662.1	nt	0.20	-0.93	-0.16	0.09	-0.38
uncharacterised	BAB55662.1	nt	0.30	-0.77	-0.20	0.10	-0.26
uncharacterised	BAB55662.1	nt	0.20	-1.32	-0.45	-0.22	-0.35
uncharacterised	BAB55662.1	nt	0.24	-0.77	-0.10	0.28	-0.18
uncharacterised	BT044936.1	nt	-0.43	-1.05	-0.42	-0.34	-0.16
uncharacterised	BT057777.1	nt	0.22	-1.03	-0.32	-0.66	0.05
uncharacterised	BT059209.1	nt	-0.87	-1.09	-0.38	-0.40	-0.30
uncharacterised	BT059282.1	nt	-1.59	-1.53	-0.67	-1.25	-0.65

uncharacterised	BT059667.1	nt	-0.03	-0.86	0.17	-0.09	0.03
uncharacterised	BT072251.1	nt	-0.28	-1.04	-0.04	0.05	0.39
uncharacterised	BT072255.1	nt	-1.77	-1.87	-0.97	0.31	0.14
uncharacterised	BT072255.1	nt	-1.50	-1.93	-1.69	0.32	0.00
uncharacterised	CA063502.1	nt	-0.52	-1.13	-0.72	-0.75	-0.33
uncharacterised	CR318614.7	nt	-0.54	-1.04	-0.15	-0.16	0.21
uncharacterised	DQ025547.1	nt	-0.10	-0.84	0.03	-0.41	-0.17
uncharacterised	DQ156151.1	nt	-0.55	-2.13	-1.45	-0.55	-1.65
uncharacterised	DQ246664.1	nt	-0.34	-0.96	0.10	0.11	-0.10
uncharacterised	DW570469.1	nt	-0.64	-1.28	-0.38	-0.39	-0.30
uncharacterised	DY700139.1	nt	-0.81	-2.19	-0.17	-0.67	-0.70
uncharacterised	DY714549.1	nt	-0.36	-0.92	-0.47	-0.37	-0.42
uncharacterised	DY722844.1	nt	-0.99	-1.08	-0.82	-0.67	-0.29
uncharacterised	DY730167.1	nt	-1.44	-2.48	-0.57	-1.17	-0.45
uncharacterised	EF427377.1	nt	-0.32	-1.25	0.11	-0.24	0.15
uncharacterised	EF467300.1	nt	-0.83	-1.50	-0.32	-0.14	-0.11
uncharacterised	EG760823.1	nt	-0.55	-1.04	-0.39	-0.23	-0.63
uncharacterised	EG795246.1	nt	-0.70	-1.19	-0.24	-0.75	-0.53
uncharacterised	EG818374.1	nt	-0.46	-0.89	-0.50	-0.33	0.00
uncharacterised	EG860955.1	nt	-0.57	-0.93	-0.20	-0.31	-0.14
uncharacterised	EG930769.1	nt	-0.40	-1.47	-0.49	-0.44	-0.42
uncharacterised	ENSGACG00000002729	Ensembl	-0.69	-1.91	0.75	-0.45	-0.33
uncharacterised	ENSORLG00000017674	Ensembl	-0.50	-1.06	-0.01	-0.23	-0.15
uncharacterised	ENSORLG00000017674	Ensembl	-0.22	-0.87	0.12	-0.05	0.09
uncharacterised	EU025716.1	nt	-0.23	-0.87	-0.46	-0.22	-0.61
uncharacterised	EU025717.1	nt	-0.62	-1.18	-0.76	-0.26	-0.19
uncharacterised	EU025717.1	nt	-0.26	-1.06	0.13	-0.22	0.05
uncharacterised	EU025717.1	nt	-0.35	-0.97	-0.07	0.03	-0.24
uncharacterised	EU221177.1	nt	-0.84	-2.15	-1.20	-0.75	-0.21
uncharacterised	EU221179.1	nt	-0.61	-1.06	0.13	-0.04	-0.68
uncharacterised	EV394677.1	nt	-0.27	-0.94	-0.31	-0.25	0.13
uncharacterised	EV394848.1	nt	-0.18	-0.97	-0.53	0.03	-0.30
uncharacterised	FJ969488.1	nt	-1.05	-1.07	-0.61	-0.75	-0.71
uncharacterised	FJ969490.1	nt	-0.26	-0.99	0.00	-0.23	0.02
uncharacterised	FJ969490.1	nt	-0.16	-1.08	0.13	-0.16	-0.01
uncharacterised	GQ505860.1	nt	-0.99	-1.55	0.23	-0.52	-0.80
uncharacterised	GQ505860.1	nt	-0.86	-1.68	0.51	-0.59	-0.54
uncharacterised	GQ505860.1	nt	-0.66	-1.74	0.72	-0.58	-0.18
uncharacterised	GQ505860.1	nt	-0.59	-1.88	0.47	-0.39	-0.54
uncharacterised	GQ505860.1	nt	-0.71	-1.53	0.69	-0.25	-0.22
uncharacterised	GQ925642.1	nt	-0.14	-1.02	0.21	-0.21	0.10
uncharacterised	GQ925642.1	nt	-0.29	-1.01	-0.09	-0.06	0.05
uncharacterised	HM159473.1	nt	-0.33	-1.15	-0.01	-0.15	-0.61
uncharacterised	HQ287745.1	nt	-0.31	-1.38	0.12	-0.21	0.18
uncharacterised	NM_001129986.1	refseq	-2.39	-4.82	1.58	-1.20	2.77
uncharacterised			-0.96	-1.11	-0.41	-0.35	-0.69
uncharacterised			-0.73	-1.51	-0.55	-0.27	-0.63
uncharacterised			-2.01	-2.00	-0.72	-1.69	-0.94
uncharacterised			-1.55	-1.99	-1.24	-1.89	-0.79
uncharacterised			-0.43	-1.19	-0.69	-0.49	-0.42
uncharacterised			-0.38	-0.92	-0.52	-0.35	-0.10
uncharacterised			0.01	-0.76	-0.30	-0.22	-0.09
uncharacterised	NP_957363.1	refseq	-0.22	-0.90	-0.55	-0.21	-0.05
uncharacterised	NR_029981.1	refseq	-0.44	-0.81	-0.60	-0.23	-0.43
uncharacterised	XP_003458662.1	refseq	0.20	-1.03	-0.28	-0.28	-0.05
uncharacterised	BT059787.1	nt	-1.22	-0.32	0.11	-0.68	-0.30
uncharacterised	BX571969.5	nt	-1.11	-0.13	-0.51	-0.52	-0.08
uncharacterised	CT033841.18	nt	-0.98	-0.48	-0.52	-0.59	0.02
uncharacterised	DQ156150.1	nt	-1.06	-0.85	-0.97	-0.12	-0.08
uncharacterised	DQ849941.1	nt	-0.80	-0.39	-0.42	-0.14	-0.19
uncharacterised	DW582826.1	nt	-1.98	-0.95	-0.80	-0.69	-0.71
uncharacterised	DY701683.1	nt	-1.42	-1.00	-0.46	-0.51	0.20
uncharacterised	DY710134.1	nt	-0.92	-0.69	-0.43	-0.50	0.03
uncharacterised	EG801741.1	nt	-1.62	-0.49	-0.08	-0.96	0.11
uncharacterised	EU025718.1	nt	-1.22	-0.73	-0.81	-0.62	-0.30
uncharacterised	EU481821.1	nt	-1.05	-0.57	-0.11	-0.30	0.26
uncharacterised	EV392641.1	nt	-1.88	-0.47	-0.23	-0.59	-0.80
uncharacterised	HM159471.1	nt	-0.94	-0.92	-0.99	-0.57	-0.59
uncharacterised	HQ287746.1	nt	-0.93	-0.47	-0.48	-0.45	-0.32
uncharacterised	JN755268.1	nt	-1.25	-0.87	-0.89	-0.29	-0.09
uncharacterised			-1.64	-0.82	-0.38	-0.10	-0.83
uncharacterised	ACN11142.1	nt	1.14	0.90	1.01	0.81	0.92
uncharacterised	BX649540.7	nt	3.22	-0.63	1.54	-1.18	0.02
uncharacterised	CA046791.1	nt	1.27	1.07	1.16	0.66	0.72
uncharacterised	CAZ39956.1	nt	0.92	0.21	0.26	0.74	0.69

uncharacterised	DW571201.1	nt	1.66	0.75	0.61	0.62	0.18
uncharacterised	BT059485.1	nt	0.00	2.06	0.00	0.62	0.27
uncharacterised	GU817335.1	nt	0.38	0.73	1.41	0.00	0.92
uncharacterised	BT045014.1	nt	0.94	0.00	1.11	0.00	1.10
uncharacterised	GU129140.1	nt	0.21	0.00	1.34	0.00	0.56
uncharacterised	EG766368.1	nt	0.27	1.77	1.23	0.00	0.52
uncharacterised	CB508615.1	nt	0.68	1.05	0.00	0.00	0.05
uncharacterised			0.00	1.22	0.00	0.00	0.52
uncharacterised	AF004739.1	nt	2.13	4.38	3.42	6.00	0.00
uncharacterised			1.59	3.01	2.08	2.72	0.00
uncharacterised	AC203446.12	nt	0.89	0.00	0.70	1.16	0.00
uncharacterised	EU481821.1	nt	0.00	0.00	0.77	1.12	0.00
uncharacterised	BT047517.1	nt	0.00	0.93	0.00	1.02	0.00
uncharacterised			0.00	1.11	0.00	2.22	0.00
uncharacterised			0.00	0.00	0.00	2.70	0.00
uncharacterised	XP_002740391.1	refseq	0.00	0.00	0.00	1.69	0.00
uncharacterised	CB494419.1	nt	1.26	2.43	2.84	2.20	0.00
uncharacterised	EF210363.1	nt	0.47	1.23	1.51	1.54	0.00
uncharacterised	ACN58700.1	nt	0.72	0.00	1.35	1.08	0.00
uncharacterised	AC203446.12	nt	0.27	1.64	0.86	0.72	0.00
uncharacterised			1.22	1.21	0.00	0.94	0.00
uncharacterised	EU025706.1	nt	0.00	1.79	0.00	0.74	0.00
uncharacterised	HQ287747.1	nt	0.00	1.20	0.00	1.72	0.00
uncharacterised			0.77	2.51	1.69	0.00	0.00
uncharacterised	ENSONIG00000015881	Ensembl	1.73	0.00	2.26	0.00	0.00
uncharacterised	BT059181.1	nt	0.00	0.00	1.69	0.00	0.00
uncharacterised	BT072122.1	nt	0.00	0.00	1.80	0.00	0.00
uncharacterised	EG850879.1	nt	0.00	0.00	2.46	0.00	0.00
uncharacterised	ENSGACG00000017205	Ensembl	0.00	0.00	1.77	0.00	0.00
uncharacterised	GQ505860.1	nt	0.00	0.00	2.79	0.00	0.00
uncharacterised	GU817336.1	nt	0.00	0.00	2.69	0.00	0.00
uncharacterised	NM_001140220.1	refseq	0.00	0.00	1.60	0.00	0.00
uncharacterised	EU025719.1	nt	1.37	0.00	1.11	0.00	0.00
uncharacterised	HM159473.1	nt	1.43	0.00	0.77	0.00	0.00
uncharacterised	EG877986.1	nt	1.37	1.99	0.00	0.00	0.00
uncharacterised	BT071996.1	nt	0.00	1.13	0.00	0.00	0.00
uncharacterised	DW561968.1	nt	0.00	1.18	0.00	0.00	0.00
uncharacterised	DW573624.1	nt	0.00	2.21	0.00	0.00	0.00
uncharacterised	NM_001140385.1	refseq	0.00	1.05	0.00	0.00	0.00
uncharacterised			0.00	1.36	0.00	0.00	0.00
URGCP (1 of 3)	ENSDARG00000078731	Ensembl	-0.08	-0.02	0.10	1.67	-0.74
USP2 (2 of 3)	ENSDARG00000087495	Ensembl	0.78	0.55	-0.05	2.20	0.17
vcam1	ENSDARG00000062479	Ensembl	-0.36	-0.50	-0.58	-1.01	-0.18
vps37a	ENSDARG00000017119	Ensembl	0.59	0.79	0.61	0.42	0.50
vtg1	ENSDARG00000092233	Ensembl	2.73	1.60	1.08	1.71	1.51
wasb	ENSDARG00000026350	Ensembl	-0.56	-0.57	-0.52	-0.20	-1.39
wbscr27	ENSDARG00000069507	Ensembl	-0.56	-1.01	-0.34	-0.37	-0.11
xbp1	ENSDARG00000035622	Ensembl	0.84	1.04	0.75	0.59	0.21
XM_004550138.1	XM_004550138.1	refseq	1.45	3.20	2.89	3.20	0.52
XM_004550138.1	XM_004550138.1	refseq	1.65	3.26	3.06	3.50	0.41
XM_004551111.1	XM_004551111.1	refseq	0.34	1.96	1.07	0.68	0.60
XM_004551111.1	XM_004551111.1	refseq	0.40	2.00	1.13	0.78	0.50
zbtb21	ENSDARG00000043285	Ensembl	0.69	1.27	1.04	0.66	0.24
zdhhc23b	ENSDARG00000003899	Ensembl	0.87	0.83	0.37	0.69	0.72
zgc:152863	ENSDARG00000069338	Ensembl	1.31	2.10	1.66	1.05	1.42
zgc:162608	ENSDARG00000069375	Ensembl	2.10	1.03	1.33	0.16	1.65
zgc:162608	ENSDARG00000069375	Ensembl	2.11	1.54	0.78	1.44	1.60
zgc:162608	ENSDARG00000069375	Ensembl	2.07	1.03	1.29	0.20	1.61
zgc:162608	ENSDARG00000069375	Ensembl	2.00	1.02	1.32	0.19	1.55
zgc:162608	ENSDARG00000069375	Ensembl	1.99	0.91	1.27	0.08	1.60
zgc:162608	ENSDARG00000069375	Ensembl	1.85	0.79	1.06	-0.07	1.43
zgc:162608	ENSDARG00000069375	Ensembl	2.03	1.02	1.24	0.07	1.52
zmym4	ENSDARG00000035823	Ensembl	-0.06	-1.02	-0.24	-0.16	-0.25

CHAPTER 6

Effects of glyphosate and its formulation, Roundup, on reproduction in zebrafish (*Danio rerio*)

Manuscript provisionally accepted by Environmental Science and Technology

subject to minor revisions

Effects of glyphosate and its formulation, Roundup, on reproduction in zebrafish (*Danio rerio*).

T. M. Uren Webster, L. V. Laing, H. Florance and E. M. Santos

Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

Abstract

Roundup, and its active ingredient glyphosate, are among the most widely used herbicides worldwide, and may contaminate surface waters. Research suggests both Roundup and glyphosate induce oxidative stress in fish, and may also cause reproductive toxicity in mammalian systems. We aimed to investigate the reproductive effects of Roundup and glyphosate in fish, and the potential associated mechanisms of toxicity. To do this, we conducted a 21-day exposure of breeding zebrafish (*Danio rerio*) to 0.01, 0.5 and 10 mg/L (glyphosate acid equivalent) Roundup and 10 mg/L glyphosate. 10 mg/L glyphosate reduced egg production, but not fertilisation rate in breeding colonies. Both 10 mg/L Roundup and glyphosate increased early-stage embryo mortalities and premature hatching. However, exposure during embryogenesis alone did not increase embryo mortality, suggesting that this effect was caused primarily by exposure during gametogenesis. Transcript profiling of the gonads revealed 10 mg/L Roundup and glyphosate induced changes in the expression of *cyp19a1* and *esr1* in the ovary, and *hsd3b2*, *cat* and *sod1* in the testis. Our results demonstrate that these chemicals cause reproductive toxicity in zebrafish, although only at high concentrations unlikely to occur in the environment, and likely mechanisms of toxicity include disruption of the steroidogenic biosynthesis pathway and oxidative stress.

Introduction

Glyphosate is extensively used worldwide, topping lists of agricultural herbicide usage in Europe [1] and the US [2]. It is a broad-spectrum, post emergence herbicide, which acts by binding phosphoenolpyruvate, the substrate of EPSP synthase, and subsequently inhibiting aromatic amino acid synthesis via the shikimate pathway in plants [3, 4]. Glyphosate is generally applied as part of a formulated product, the most widely used of which are the Roundup herbicides. Roundup formulations contain glyphosate in the form of an isoproylamine salt, which aids solubility but does not affect its properties as the active ingredient, together with various adjuvants which enhance its herbicidal properties. One of the most important and commonly used adjuvants is polyethoxylated tallow amine (POEA), a surfactant that enhances penetration of glyphosate through the plant cuticle [5, 6]. Glyphosate and Roundup are also extensively used as domestic and urban-area weed-killers [2]. Commercial glyphosate formulations vary in composition with country and purpose and the properties of these formulations, including their toxicity, can be compared using the concentration of glyphosate present, expressed as glyphosate acid equivalent (a.e.).

Glyphosate is known to strongly adsorp to soil, where it is subject to microbial degradation. This is one of glyphosate's advantageous herbicidal properties, limiting agricultural input to surface waters in ideal conditions. However, pulses of contamination can be expected when rainfall occurs directly after application and when flood events increase river sediment load [6]. Urban runoff and wastewater treatment effluent also account for considerable glyphosate input into rivers [7]. Despite its widespread use, concentrations of glyphosate, or its associated formulation components, are not routinely monitored in surface waters. However, glyphosate concentrations worldwide have been regularly reported to occur up to ~10-15 µg/L in rivers [e.g.8, 9]. Considerably higher peaks in concentration, in the range of 500-800 µg/L, have also been measured, but are mainly associated with direct aquatic application, and in isolated wetland environments [6, 10].

Although the target mechanism of action of glyphosate and glyphosate-based formulations is specific to plants, they have been shown to induce diverse biological

effects in a range of non-target organisms. In fish, much of previous research assessing effects of Roundup and glyphosate has focused on their induction of oxidative stress through ROS generation and/or interference with cellular antioxidant production. Short-term exposures (up to 6 days) to 1-20 mg/L of several Roundup formulations in a number of fish species altered levels of cellular antioxidants and induced oxidative damage of DNA, lipids and proteins [e.g. 11, 12-15]. Environmentally relevant concentrations of Roundup, glyphosate and POEA have also induced DNA damage in blood and liver cells of eel and catfish after up to 9 days exposure [16-19]. Other studies have found that Roundup, and in some cases glyphosate, induce other effects in fish including neurotoxicity and immunotoxicity [e.g. 20, 21, 22]. Similar evidence of Roundup and glyphosate toxicity has been found for other vertebrate species, and demonstrated effects include occurrence of developmental abnormalities, especially in amphibians [23, 24]. Roundup formulations have largely been found to be more toxic than pure glyphosate. The inherent toxicity of POEA [17, 23], and potentially other formulation components, is likely to contribute to this, although a modulating effect on glyphosate toxicity is also possible. Few studies, however, have directly compared equivalent concentrations of Roundup and glyphosate.

The potential of Roundup to disrupt the endocrine system *in vitro* has been demonstrated in mammalian cell lines. In mouse Leydig cells, sub-lethal concentrations of Roundup (from 25 mg/L) altered the transcription and activity of steroidogenic acute regulatory protein (StAR), resulting in disruption of progesterone production [25]. A number of studies demonstrated consistent inhibition of aromatase activity by Roundup in various human cell lines. The concentrations required to cause these effects varied depending on the cell type and formulation, but included from 4 mg/L in liver cells and 72 mg/L in placental and embryonic cells [26-28]. Less consistently, and to a smaller extent than Roundup, aromatase inhibition by glyphosate alone has also been reported (approximately 10 fold higher concentrations) [28]. Additionally, both glyphosate and Roundup were reported to reduce testosterone production in rat testicular cells at concentrations from 0.36 mg/L [29], but it is difficult to relate these results to the potential effects of these chemicals *in vivo*. Few studies have investigated the effects of glyphosate and its

commercial formulations on the endocrine system *in vivo.* Drakes treated with 5 and 100 mg Roundup/kg (body weight) exhibited reduced levels of testosterone, corresponding with alterations in testis structure. In rats, maternal and juvenile treatment from 5 and 50 mg/kg (body weight) of Roundup impaired male reproductive development, with effects including alteration in testis structure, sperm production and sex steroid production [30-32].

Reproductive effects of Roundup and glyphosate in fish have seldom been investigated and are far from clear, despite their potential for environmental exposure. While no evidence of altered gonadal development was evident in juvenile stickleback exposed to 0.1-100 µg/L glyphosate [33], treatment with 3.6 mg/L Roundup had some negative impact on offspring production in Silver catfish [34]. The mechanisms contributing to this reproductive effect have not been investigated.

This study aimed to examine the effects of Roundup formulation on reproduction in fish and to determine to what extend these effects were associated with the toxicity of glyphosate alone. To do this, we conducted a 21 day reproductive test in breeding colonies of zebrafish, to determine if reproduction, embryo development and embryo survival, were affected by exposure to 0.01, 0.5 and 10 mg/L (glyphosate acid equivalent) Roundup and 10 mg/L glyphosate. We hypothesised that the mechanisms of toxicity resulting in effects on reproduction might include oxidative stress and disruption of steroid biosynthesis, and to investigate this we conducted transcript profiling of a suite of genes involved in these processes in the gonads.

Materials and Methods

Fish maintenance

Colonies of 4 male and 4 female adult (20 week old) WIK strain zebrafish were established in individual 15 L glass tanks and allowed to breed naturally during a 7 day acclimation period. Fish were maintained according to Paull et al. [35] and a full description of husbandry procedures is provided in the supporting information.

Chemical exposures

Chemical exposure was conducted in the 15 L tanks where the breeding colonies had been established via a flow through system for a period of 21 days in accordance with OECD guidelines for fish reproductive tests, preceded by a 10 day pre-exposure period [36]. The treatment groups consisted of three concentrations of Roundup; 0.01, 0.5 and 10 mg/L glyphosate acid equivalent (using Roundup® GC liquid glyphosate concentrate containing 120 g/L glyphosate acid; Monsanto, Cambridge, UK); 10 mg/L glyphosate (analytical grade; Molekula, Wimborne, UK); and a control group. The two lower Roundup concentrations were chosen to represent concentrations that can be expected to occur in the environment regularly (0.01 mg/L) and during occasional peak contamination events (0.5 mg/L). The highest concentration tested (10 mg/L) is unlikely to occur in surface waters, and was included to facilitate the analysis of the mechanisms of toxicity. We included a treatment group exposed to 10 mg/L glyphosate alone to allow for a direct comparison of its toxicity with the equivalent glyphosate acid equivalent (a.e.) concentration of Roundup. Each treatment group was comprised of three replicate breeding colonies (4 males and 4 females) in 15L tanks. Water samples were collected from each tank on days 7, 14 and 21 of the exposure period and stored at -20 °C prior to chemical analysis. Details of the analytical chemistry procedures are provided in supplemental material.

Reproductive test and embryo exposures

Group spawning occurred daily at dawn and eggs were collected 1 hour post fertilisation (hpf), rinsed thoroughly to remove detritus and incubated in water containing the same chemical exposure concentrations as their tank of origin, at 28

°C. Exposure water for the embryo experiments was made according to the ISO 7346−3:1996 guidelines [37], fully oxygenated and supplemented with 2.5 µl/L of the antifungal agent Methylene Blue (Interpet; Dorking, UK) to avoid mortalities caused by fungal infections. The eggs from each colony were examined using light microscopy between 2 ½ and 3 ½ hours after dawn, when all fertilised eggs had reached at least the 16-cell stage during early cleavage [38], and the total number of fertilised and unfertilised eggs were quantified on each day throughout the pre-exposure and exposure periods. During the 21-day chemical exposure, fertilised eggs displaying cellular necrosis were counted and recorded as early-stage mortalities (<3.5 hpf). Fifty fertilised eggs from each tank were selected randomly and incubated in 50 ml exposure water until 72 hpf. During this period, embryo mortality was recorded at 24, 54 and 72 hpf and embryo hatching was recorded at 54 and 72 hpf.

In order to determine if the observed effects of Roundup and glyphosate on embryos were due to the effects of exposure during gametogenesis or during embryogenesis, embryos collected from a control population were exposed to the same range of concentrations of glyphosate and Roundup as used above for the adults. Chemical treatment was initiated between 10 and 20 minutes post fertilisation. In addition to the exposure concentrations used for the adult exposures, embryos were also treated with higher concentrations (50, 100, 250, 500 and 1000 mg/L a.e. Roundup and glyphosate) to determine the concentration thresholds for embryo mortalities and developmental toxicity. Experiments were conducted in triplicate; each replicate contained 50 embryos and observations of mortalities and hatching were performed as described above.

Sampling

All fish were humanely sacrificed on day 21 of the exposure period by a lethal dose of benzocaine (0.5 g L^{-1}; Sigma-Aldrich) followed by destruction of the brain, in accordance with UK Home Office regulations. Wet weight and fork length were recorded and the condition factor (k= (weight (g) x 100)/ (fork length $(cm)^3$)) was calculated for individual fish. Livers were dissected and weighed, and the hepatosomatic index (HSI) (liver weight (mg)/ total weight (mg)) x 100)) was

determined for individual fish. Gonads were dissected, weighed and one gonad from each fish was snap frozen in liquid nitrogen and stored at -80°C prior to transcript profiling. The remaining gonad was fixed in Bouin's solution (Sigma-Aldrich) for histological analysis. The gonadosomatic index (GSI; gonad weight (mg)/ total weight (mg)) x 100)) was determined for both males and females.

Transcript profiling and histological analysis

Transcript profiling of genes encoding steroidogenic enzymes, sex steroid receptors and antioxidant enzymes, was conducted using RT-QPCR in the gonads of exposed fish according to [39]. Histological analysis of the gonads was conducted according to [40]. A full description of these methodologies is presented in the supporting information.

Statistical analysis

Statistical analyses were conducted with SigmaStat (version 12.0). Before analysis, proportional data (embryo survival and hatching) were subjected to variance-stabilising square-root or arcsine transformations as appropriate. All reproductive output and sampling data met assumptions of normality and equal variance. Outliers in transcript expression data were identified and removed according to Chauvenet's criterion [41] prior to statistical analysis. Transcript expression data that did not meet normally-distributed criteria was log transformed before statistical analysis. All data was analysed using single factor one way analysis of variance (ANOVA), followed by the Holm-Sidak post hoc test using a pairwise comparison method. Data were considered to be significant when $P < 0.05$.

Results

Water chemistry

The mean measured concentrations of glyphosate in the tank water were between 88-140 % of the nominal values for all treatments (quantification of glyphosate in tanks receiving 0.01 mg/L Roundup was below the detection limit of our method), and are presented in Table S2.

Morphometric parameters

The mean mass and length of male and female fish were 375.0 ± 6.3 mg/ 32.6 ± 0.2 mm and 402.6 ± 9.3 mg/ 31.7 ± 0.2 mm respectively. There were no significant differences in size or condition factor (mean 1.08 and 1.25 for males and females, respectively) between treatment groups. Additionally, we observed no alteration of general health or behaviour in any colony. The GSI of females was significantly lower in the fish treated with 10 mg/L glyphosate compared to the control group (Figure 1c). There was no significant difference in the GSI of males between treatment groups, or in the HSI of males or females.

Reproductive test and embryo exposures

During the 10 day pre-exposure period, there was no difference in cumulative egg production between the treatment groups (P=0.468). During the exposure period, colonies in the control group consistently spawned the greatest number of eggs per female, while those treated with 10 mg/L glyphosate spawned the least. From day 10 of the exposure period, cumulative egg production was significantly reduced in colonies exposed to 10 mg/L glyphosate compared to the controls, and this difference intensified throughout the remainder of the exposure period. At the end of the 21 day exposure, cumulative egg production was significantly lower in colonies exposed to 10 mg/L glyphosate compared to the control, and also compared to the 10 and 0.01 mg/L Roundup groups (Figure 1a,b). Additionally, egg output significantly correlated (R^2= 0.79; P= 0.043) with female GSI across all treatment groups. Fertilisation rate remained consistently high throughout the exposure period with no significant differences between treatment groups and an overall mean value of 83.4%.

There was a significant increase in embryo mortalities occurring before 3.5 hpf in embryos from both the 10 mg/L Roundup and glyphosate treatment groups (Figure 2a). Additionally, there was a significant correlation between early embryo mortality and the concentration of Roundup (R^2= 0.52; P=0.008). There were no significant differences between treatments in embryo mortality between the start of epiboly (3.5 hpf) and the end of somitogenesis at 24 hpf (Figure 2b). However, there was a significant increase in the percentage of embryos that had hatched at 54 hpf in

groups treated with 10 mg/L Roundup and 10 mg/L glyphosate compared to the control group (Figure 2b).

For embryos originating from a control population, exposure to glyphosate and Roundup at the concentrations used in the adult reproductive test (0, 0.01, 0.5 and 10 mg/L Roundup and 10 mg/L glyphosate) did not result in increased mortality rate at either 3.5 hpf or 24 hpf (Figure 2a,b), but there was a significant increase in 3.5-24 hpf mortality in embryos exposed to concentrations ≥ 100 mg/L glyphosate and ≥ 500 mg/L Roundup (Figure S4a). We also observed evidence of developmental delay and abnormalities from concentrations ≥ 50 mg/L glyphosate and ≥250 mg/L Roundup at 24 hpf. There was a trend towards increased hatching at 54 hpf in groups exposed to 10 and 50 mg/L Roundup and glyphosate, and there was a significant correlation between hatching rate at 54hpf and exposure concentration of Roundup up to 50 mg/L (R^2= 0.27; P=0.04) (Figure S4b). For embryos exposed to ≥ 100 mg/L Roundup and glyphosate, we found evidence of progressive delay in development and hatching with increasing concentration.

Gonad transcript profiling

In the ovary, the transcript encoding aromatase (*cyp19a1*) was significantly up-regulated in the 10 mg/L Roundup treatment group compared to the controls. Estrogen receptor 1 (*esr1*) in the 10 mg/L Roundup group was significantly up-regulated compared to the 10 mg/L glyphosate group. There were similar, but not statistically significant, decreasing trends in expression of other steroidogenic enzymes including cytochrome P450, subfamilies 17 and 11 (*cyp17a1, cyp11a1*) and 3β-hydroxysteroid dehydrogenase (*hsd3b2*) in groups exposed to both Roundup and glyphosate. In contrast, for the antioxidants glutathione peroxidase (*gpx1a*), catalase (*cat*) and glutathione-S-transferase pi (*gstp1)* non-significant, increasing trends in transcript expression were observed (Figure 3a, Figure S1a).

In the testis, *hsd3b2* was significantly up-regulated following exposure to 10 mg Roundup/L compared to all other treatment groups. The expression pattern of steroidogenic acute regulatory protein (*star*)*, cyp17a1, cyp11a1* and the androgen receptor (*ar*) additionally appeared to follow an expression pattern similar to *hsd3b2*

across treatment groups. *cat* was significantly up-regulated in groups exposed to both 10 mg/L Roundup and 10 mg/L glyphosate compared to those treated with 0.5 mg/L Roundup. In addition, *sod1* was significantly up-regulated in the 10 mg/L compared to 0.5 mg/L Roundup groups (Figure 3b, Figure S1b).

Gonad Histology

Histological examination of females from all treatment groups showed that the ovaries of all individuals contained oocytes at all stages of development (oogonia, primary oocytes, cortical alveoli stage oocytes, secondary oocytes and mature vitellogenic oocytes) and the majority contained recent post-ovulatory follicles. We found evidence of ovarian abnormalities in 9.1, 18.2, 9.1, 50.0 and 63.6 % of females in the control, 0.01 mg/L Roundup, 0.5 mg/L Roundup, 10 mg/L Roundup and 10 mg/L glyphosate treatment groups, respectively (Figure S3). The majority of abnormalities were relatively mild and included accumulation of eosinophilic fluid and presence of abnormal tissue. In addition, the proportion of fish containing atretic oocytes in their ovaries also appeared to be increased (Figure S2).

Histological examination of males showed that testes of all individuals from all treatment groups contained germ cells at all stages of spermatogenesis (including spermatogonia, spermatocytes, spermatids and mature spermatozoa) (Figure S2). There were no abnormalities and no differences between stages of development between treatment groups.

Discussion

Reproductive effects on adult zebrafish

This study provides evidence that glyphosate caused a reduction in the number of eggs spawned by female zebrafish exposed to high concentrations (10 mg/L) of glyphosate. However, this concentration is well above concentrations measured to date in the environment and unlikely to occur in aquatic systems, except when glyphosate is directly applied to control algal populations. In addition, our study also showed an apparent reduction, albeit not significant, in egg production in all three Roundup treated groups. Therefore, the potential for adverse effects of Roundup on

reproductive output and impact on wild populations cannot be ruled out. A number of potential mechanisms may contribute to the observed effect of glyphosate on egg production, including disruption of normal progression through oogenesis, inhibition of ovulation and increased rate of oocyte atresia. In order to explore this, we conducted histological analysis of the gonads of exposed females and observed a trend towards an increase in the incidence of ovarian abnormalities as a result of exposure to both Roundup and glyphosate. Ovarian follicle atresia is an apoptotic process leading to re-absorption of maturing oocytes rather than ovulation. It is a highly-regulated, natural process thought to have a role in maintaining ovarian homeostasis; however various environmental stressors, as well as disruption of the hormonal control of oogenesis and ovulation, have been shown to increase atresia [42]. We found atretic vitellogenic oocytes in all treatment groups, but this incidence tended to increase in both the 10 mg/L Roundup and 10 mg/L glyphosate treatment groups. Similarly, in these groups we also found an increased trend in the incidence of abnormal ovarian tissue, including excess connective tissue and putative haemopoietic tissue. In some females treated with 0.01 and 10 mg/L Roundup and 10 mg/L glyphosate we observed the presence of areas containing eosinophilic fluid. Previously, accumulated proteinaceous fluid in the ovary has been found to contain vitellogenin, and this has been associated with a disruption in the endocrine control of oogenesis in zebrafish through exposure to elevated levels of 17β-oestradiol [43].

It is important to note that despite the trends towards increased incidence of atretic follicles and ovarian abnormalities following exposure to glyphosate and Roundup, the majority of fish were only moderately affected and their ovaries contained oocytes at all stages of maturation, including mature vitellogenic oocytes and post-ovulatory follicles. Moreover, we found no differences in the ovarian expression of bcl2-associated X protein (*baxa*) and tumour protein 53 (*tp53*), which are typical marker genes of apoptosis. This indicates that oocyte atresia was unlikely to be the major mechanism responsible for the decline in egg production rate induced by glyphosate treatment. Corresponding with this, a similar degree of atresia in fish exposed to Roundup was not accompanied by a significant decline in egg-production in this treatment group. Therefore, we hypothesise that the observed decrease in egg production following exposure to glyphosate was more likely to be due to a

reduction in the number of follicles undergoing oogenesis. The strong correlation between egg production and female GSI, including a significant reduction in GSI in females exposed to 10 mg/L glyphosate, which indicates reduced gonadal volume, provides support for this hypothesis.

Sex steroids are essential for the regulation of oogenesis, and alterations in sex steroid biosynthesis may have contributed to the reduction of egg production in colonies exposed to glyphosate. To test this hypothesis, we investigated the effects of glyphosate and Roundup on the expression of a number of transcripts encoding enzymes involved in steroid biosynthesis, several of which have previously been shown to be targets of their toxicity [25-28]. We found a significant increase in the expression of ovarian aromatase, an enzyme which catalyses the conversion of testosterone to oestradiol in granulosa cells, in the gonads of females exposed to 10 mg/L Roundup, and also an increasing trend in those exposed to 10 mg/L glyphosate. Several previous studies have demonstrated that Roundup disrupts both aromatase activity and *cyp19a1* expression levels in a number of human cell lines, and there is some evidence that glyphosate can also inhibit aromatase activity, especially with the addition of small percentages of Roundup, which may facilitate its cellular entry [26-28]. Romano et al. [32] proposed inhibition of aromatase as a causative mechanism for disruption of steroidogenesis and adverse reproductive impacts in the male offspring of rats exposed to Roundup during pregnancy. The stimulatory effect of Roundup on *cyp19a1* expression observed in the present study contrasts with the predominantly inhibitory effects found in the *in vitro* studies. This may reflect the complex nature of feedback mechanisms governing steroid biosynthesis pathways *in vivo* or, possibly, a compensatory transcriptional response to an inhibition of aromatase enzyme. Additionally, it is difficult to equate the concentrations used in the present study with those used in the *in vitro* studies. It is possible that differential stimulatory and inhibitory responses occur with concentration, and also with time. Although not significant, there were also similar decreasing trends in expression of steroidogenic enzymes, *hsd3b2, cyp17a1* and *cyp11a1,* in females treated with both 10 mg/L of Roundup and 10 mg/L glyphosate, indicating a possible wider effect on steroidogenic pathways. The differential regulation of ovarian *esr1* by Roundup and glyphosate is interesting and may reflect

the effect of other chemicals present in Roundup formulation on this receptor. Increased *esr1* expression following Roundup exposure may have resulted from compensatory mechanisms in the ovary to maintain or restore oestrogen signalling pathways. This may explain, at least in part, the differences in the effects of these chemicals on egg production, with glyphosate having a more pronounced effect than Roundup. Using human liver HepG2 cells, Gasnier [26] showed that Roundup and glyphosate antagonistically bind oestrogen receptors (*ERα* and *ERβ*), although Kojima [44] found no evidence of agonistic or antagonistic interaction with oestrogen receptors in Chinese hamster ovary cells. A recent study showed glyphosate actively bound oestrogen receptors and induced proliferative growth of oestrogen-dependent breast cancer cells, and also increased protein levels of ERα and ERβ [45]. Taken together, our ovarian transcript profiling data suggests that Roundup and glyphosate may have disrupted steroid hormone biosynthesis and also potentially modulated the biological effects of oestrogens via alterations in the expression of *esr1*, the predominant oestrogen receptor in the ovary.

Despite having no significant effect on egg production, it is interesting to note that exposure to 10 mg/L Roundup also elicited alterations in gene expression often in the opposite direction of those induced by exposure to the equivalent concentration of glyphosate alone. This might suggest the presence of compensatory mechanisms ameliorating the adverse effects of glyphosate when in the presence of the other constituents of Roundup. A possible mechanism could be increased synthesis of aromatase to maintain sex steroid ratios and oestrogen signalling in the ovary in order to promote oogenesis, and maintain egg production.

There was no effect of exposure to Roundup or glyphosate on fertilisation rate. Corresponding with this, histological examination revealed no evidence of any disruption of spermatogenesis, or abnormalities in the testis following exposure to glyphosate or Roundup. Therefore, we found no indication that these chemicals affect the ability of the sperm produced to fertilise eggs. This contrasts with several previous *in vivo* studies that have found some evidence that Roundup disrupts spermatogenesis in rats, resulting in testis pathology, sperm abnormalities and altered sperm production [30-32]. It is important to note, however, that our

experimental conditions are optimised to maximise reproduction and may not detect subtle changes in sperm quality that may be sufficient to cause effects under the conditions found in the natural environment.

Previous Roundup-induced testicular toxicity has been associated with alterations in steroidogenesis and sex steroid levels in rats and drakes [30-32, 46]. In the current study, analysis of transcripts encoding steroidogenic enzymes in the testes showed that *hsd3b2* was significantly up-regulated in males exposed to 10 mg/L Roundup compared to those exposed to 0.01 and 0.5 mg/L Roundup, and 10 mg/L glyphosate (Figure 3b). Moreover, although not statistically significant, the expression patterns of the other steroidogenic enzymes profiled (*star, cyp17a1* and *cyp11a1*), as well as *ar*, followed a similar expression pattern to *hsd3b2* across treatment groups. This pattern, of apparent down-regulation in the 0.5 mg/L Roundup treatment and up-regulation in the 10 mg/L Roundup group was robust across tank replicates. Walsh et al. [25] found evidence that Roundup, but not glyphosate, disrupted StAR and P450scc (Cyp11a1) in mouse testis cells, primarily through alteration of protein expression and activity, suggesting that such post-transcriptional regulatory changes should also not be ruled out. Additionally, we found 10 mg/L Roundup significantly increased expression *cat* and *sod1* compared to the lower Roundup treatments, and 10 mg/L glyphosate also significantly increased *cat* expression in the testis. Together, these changes in the transcription of antioxidant enzymes provide evidence that both Roundup and glyphosate induce oxidative stress in the testis.

Therefore, despite no apparent impacts on fertilisation success, we have found some evidence that high concentrations of Roundup and glyphosate cause disruption of steroidogenesis and oxidative stress in the testis, suggesting that their potential to cause adverse impacts on male reproductive health should not be ruled out. It is interesting to note that exposure to 10 mg/L Roundup elicited differential responses, in terms of the magnitude and direction of transcript expression changes, compared to 10 mg/L glyphosate, possibly suggesting greater compensatory mechanisms of response following exposure to Roundup, similarly to that observed in females.

Effects on embryo survival and development

We found evidence that treatment with both 10 mg/L Roundup and glyphosate induce an increased rate of embryo mortality during very early development. We observed necrosis of the fertilised embryos during cleavage and early blastula stages, prior to progression to epibioly at ~3.5 hpf (as described by Kimmel et al. [38]). In order to assess if the early stage mortality was caused as a direct result of the chemical exposure on embryos or by the parental exposure, we exposed embryos originating from a control population of untreated adults and found that concentrations of up to 10 mg/L of Roundup and 10 mg/L glyphosate had no effect on embryo survival at <3.5 or 3.5-24 hpf. This corresponds with previous work showing exposure of zebrafish embryos to up to 10 mg/L glyphosate for 5 days had no effect on survival or development [47]. We only found a significant increase in embryo mortality at concentrations of 100 mg/L glyphosate and 1000 mg/L Roundup, which are 10 and 1000 times higher than the concentrations used in the reproductive study. Moreover, this mortality predominantly occurred between 3.5-24 hpf, rather than in the earlier stages of development. These high concentrations of glyphosate, and to a lesser extent Roundup formulation, result in a pronounced decrease in pH in the exposure water (to 3.8 (100 mg/L glyphosate) and 4.9 (1000 mg/L Roundup)), which may be responsible for the embryo toxicity seen. Overall, these results suggest that the increase in early stage mortalities observed in embryos originating from fish exposed to 10mg/L Roundup and glyphosate is attributable to potential damage of the gametes occurring during gametogenesis and/or fertilisation, rather than as a result of direct embryo exposure. Additionally, it is possible that maternal transfer of glyphosate, Roundup or formulation products, via the yolk, might contribute to this embryo toxicity.

As discussed above, gonadal transcript profiling revealed significant up-regulation of transcripts encoding antioxidant enzymes in response to exposure to 10 mg/L Roundup and 10 mg/L glyphosate in the testes and increasing trends in transcripts encoding antioxidant enzymes in the ovary. Oxidative stress induced in the testis by chemical exposure has been shown to cause DNA damage in developing sperm [48]. Pérez-Cerezales et al. [49] showed that DNA damage in rainbow trout sperm did not impair fertilisation success, but resulted in a high rate of embryo mortality in

early stages of embryogenesis, particularly during gastrulation. This is consistent with our findings that fertilisation success was unaffected, but that an increased rate of embryo mortalities occurred during early stages of development and before transition to epiboly. Therefore, we hypothesise that oxidative stress generation in the testis during spermatogenesis is likely to be an important causative mechanism responsible for the increase in early-stage embryo mortality. Additionally, the increase in ovarian histological abnormalities and the increased trends in ovarian antioxidant transcript expression suggest similar damage during oogenesis is also possible, although oocytes are thought to have greater response and repair mechanisms to counter-act oxidative stress than sperm [49, 50]. DNA damage after spermiation cannot be ruled out, but probably has a minimal effect compared to damage during spermatogenesis, given the brief period of less than 65 seconds that sperm remains motile before fertilisation (Van Look et al., personal communication).

We found an increased percentage of hatching at 54 hpf in groups exposed to 10 mg/L Roundup and 10 mg/L glyphosate. Additionally, embryos originating from the unexposed control population showed a significant increasing trend in hatching rate at 54 hpf with concentrations up to 50 mg/L Roundup, as well as an apparent increase in hatching in those treated with 10 mg/L glyphosate. This suggests an independent impact of Roundup and glyphosate on embryos, not entirely attributable to toxicity during gametogenesis. Hatching is variable, and dependent on a number of environmental factors. Various chemical and other environmental stressors, such as temperature, are known to affect developmental rate and, subsequently, time to hatch. However, in this study, observations at 24h, 48h, 54 and 72 hpf showed no obvious change in development rate between treatment groups, indicating that exposure to 10 mg/L Roundup and 10 mg/L glyphosate induces premature hatching in zebrafish. At 72 hpf, more than 90 % of embryos from all treatment groups had hatched (both those originating from exposed and non-exposed adults), and there were no obvious behavioural or morphological differences between treatments. In natural populations, premature hatching could potentially result in detrimental impacts for population sustainability, for example by increasing the susceptibility to predation.

We found no obvious signs of developmental toxicity at exposure concentrations up to 10 mg/L Roundup or glyphosate, which corresponds with the findings of Stehr [47]. We did find evidence of developmental delay in embryos exposed to concentrations ≥ 50 mg/L glyphosate and ≥250 mg/L Roundup and hypothesise that the increased toxicity of glyphosate may be attributed to its greater acidity than the buffered Roundup formulation. With the exception of amphibians, which appear particularly sensitive [e.g. 23, 24], these results show that only extremely high concentrations of Roundup and glyphosate induce developmental toxicity in zebrafish and are generally in accordance with evidence from other species, including rats [51] and sea urchins [52].

Overall, we have found evidence that both 10 mg/L Roundup and 10 mg/L glyphosate have similar adverse impacts on embryo survival and hatching. This, together with the effects of glyphosate on egg production, demonstrates both glyphosate and Roundup have a detrimental impact on a number of measures of reproductive health in zebrafish, although only at very high concentrations that are unlikely to occur in the environment based on the currently available measurements. We have found some evidence that these reproductive effects occur via multiple mechanisms of toxicity which appear to differ, to some extent, between Roundup and its active ingredient glyphosate. These mechanisms may include disruption of the steroidogenic pathway and sex steroid signalling, and generation of oxidative stress.

Acknowledgements

We wish to thank Dr James Cresswell for advice on the statistical analysis and Dr Gregory Paull for help with the fish husbandry and experimental design. This work was funded by a Natural Environment Research Council CASE PhD studentship (Grant number NE/I528326/1) and the Salmon & Trout Association.

Supporting Information

The supporting information contains: Supplemental Experimental Section; Target genes, primer sequences and assay details for RT-QPCR analysis, Table S1; Measured concentrations of glyphosate in tank water, Table S2, Transcript profiling of target genes in the gonads, Figure S1; Gonad histology of control and exposed fish, Figure S2; Occurrence of ovarian histological abnormalities, Figure S3; Effects of glyphosate and Roundup on embryos originating from a control population, Figure S4. This material is available free of charge via the Internet at http://pubs.acs.org.

References

1. European Commission, The use of plant protection products in the European Union. Eurostat 2007.

2. US EPA, Pesticides Industry Sales and Usage: 2006 and 2007 Market Estimates. 2011.

3. Schonbrunn, E.; Eschenburg, S.; Shuttleworth, W. A.; Schloss, J. V.; Amrhein, N.; Evans, J. N. S.; Kabsch, W., Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. Proceedings of the National Academy of Sciences 2001, 98, (4), 1376-1380.

4. Steinrucken, H. C.; Amrhein, N., The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. Biochemical and Biophysical Research Communications 1980, 94, (4), 1207-1212.

5. Brausch, J. M.; Smith, P. N., Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, Thamnocephalus platyurus. Archives of Environmental Contamination and Toxicology 2007, 52, (2), 217-221.

6. Giesy, J. P.; Dobson, S.; Solomon, K. R., Ecotoxicological risk assessment for Roundup herbicide. Reviews of environmental contamination and toxicology 2000, 167, 35-120.

7. Botta, F.; Lavison, G. l.; Couturier, G.; Alliot, F.; Moreau-Guigon, E.; Fauchon, N.; Guery, B. n. d.; Chevreuil, M.; Blanchoud, H. l. n., Transfer of glyphosate and its degradate AMPA to surface waters through urban sewerage systems. Chemosphere 2009, 77, (1), 133-139.

8. Byer, J. D.; Struger, J.; Klawunn, P.; Todd, A.; Sverko, E., Low cost monitoring of glyphosate in surface waters using the ELISA method: An evaluation. Environmental Science & Technology 2008, 42, (16), 6052-6057.

9. Struger, J.; Thompson, D.; Staznik, B.; Martin, P.; McDaniel, T.; Marvin, C., Occurrence of glyphosate in surface waters of southern Ontario. Bulletin of Environmental Contamination and Toxicology 2008, 80, (4), 378-384.

10. Battaglin, W. A.; Rice, K. C.; Focazio, M. J.; Salmons, S.; Barry, R. X., The occurrence of glyphosate, atrazine, and other pesticides in vernal pools and adjacent streams in Washington, DC, Maryland, Iowa, and Wyoming, 2005-2006. Environ. Monit. Assess. 2009, 155, (1), 281-307.

11. Cavalcante, D.; Martinez, C.; Sofia, S., Genotoxic effects of Roundup on the fish Prochilodus lineatus. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 2008, 655, (1), 41-46.

12. Modesto, K. A.; Martinez, C. u. B., Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish Prochilodus lineatus. Chemosphere 2010, 78, (3), 294-299.

13. Cavas, T.; Konen, S., Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 2007, 22, (4), 263-268.

14. Lushchak, O. V.; Kubrak, O. I.; Storey, J. M.; Storey, K. B.; Lushchak, V. I., Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. Chemosphere 2009, 76, (7), 932-937.

15. Ferreira, D.; Costa da Motta, A.; Kreutz, L. C.; Toni, C.; Loro, V.; Barcellos, L., Assessment of oxidative stress in Rhamdia quelen exposed to agrichemicals. Chemosphere 2010, 79, (9), 914-921.

16. Guilherme, S.; Gaivao, I.; Santos, M.; Pacheco, M., European eel (Anguilla anguilla) genotoxic and pro-oxidant responses following short-term exposure to Roundup, a glyphosate-based herbicide. Mutagenesis 2010, 25, (5), 523-530.

17. Guilherme, S.; Santos, M.; Barroso, C.; Gaivao, I.; Pacheco, M., Differential genotoxicity of Roundup formulation and its constituents in blood cells of fish (Anguilla anguilla): considerations on chemical interactions and DNA damaging mechanisms. Ecotoxicology 2012, 1-10.

18. Guilherme, S.; Gaivao, I.; Santos, M.; Pacheco, M., DNA damage in fish (Anguilla anguilla) exposed to a glyphosate-based herbicide “Elucidation of organ-specificity and the role of oxidative stress. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 2012.

19. de Castilhos Ghisi, N.; Cestari, M. M., Genotoxic effects of the herbicide Roundup in the fish Corydoras paleatus (Jenyns 1842) after short-term, environmentally low concentration exposure. Environ. Monit. Assess. 2013, 185, (4), 1-7.

20. Glusczak, L.; Miron, D. d. S.; Moraes, B. S.; Simaues, R. l. R.; Schetinger, M. R. C.; Morsch, V. M.; Loro, V. n. L., Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish Rhamdia quelen. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 2007, 146, (4), 519-524.

21. Kreutz, L. C.; Gil Barcellos, L. J.; de Faria Valle, S.; de Oliveira Silva, T. l.; Anziliero, D.; Davi dos Santos, E.; Pivato, M.; Zanatta, R., Altered hematological and immunological parameters in silver catfish Rhamdia quelen following short term

exposure to sublethal concentration of glyphosate. Fish & Shellfish Immunology 2011, 30, (1), 51-57.

22. Kelly, D. W.; Poulin, R.; Tompkins, D. M.; Townsend, C. R., Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival. Journal of Applied Ecology 2010, 47, (2), 498-504.

23. Howe, C. M.; Berrill, M.; Pauli, B. D.; Helbing, C. C.; Werry, K.; Veldhoen, N., Toxicity of glyphosate based pesticides to four North American frog species. Environmental Toxicology and Chemistry 2004, 23, (8), 1928-1938.

24. Relyea, R. A., New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. Ecol. Appl. 2012, 22, (2), 634-647.

25. Walsh, L. P.; McCormick, C.; Martin, C.; Stocco, D. M., Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environmental Health Perspectives 2000, 108, (8), 769.

26. Gasnier, C.; Dumont, C.; Benachour, N.; Clair, E.; Chagnon, M. C.; Seralini, G. E., Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 2009, 262, 184-191.

27. Richard, S.; Moslemi, S.; Sipahutar, H.; Benachour, N.; Seralini, G.-E., Differential effects of glyphosate and roundup on human placental cells and aromatase. Environmental Health Perspectives 2005, 113, (6), 716.

28. Benachour, N.; Sipahutar, H.; Moslemi, S.; Gasnier, C.; Travert, C.; Seralini, G., Time-and dose-dependent effects of roundup on human embryonic and placental cells. Archives of Environmental Contamination and Toxicology 2007, 53, (1), 126-133.

29. Clair, A.; Mesnage, R.; Travert, C.; Seralini, G. E., A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicology in Vitro 2012, 26, (2), 269-279.

30. Romano, R. M.; Romano, M. A.; Bernardi, M. M.; Furtado, P. V.; Oliveira, C. A., Prepubertal exposure to commercial formulation of the herbicide glyphosate

alters testosterone levels and testicular morphology. Archives of Toxicology 2010, 84, 309-317.

31. Dallegrave, E.; Mantese, F. D.; Oliveira, R. T.; Andrade, A. J.; Dalsenter, P. R.; Langeloh, A., Pre-and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Archives of Toxicology 2007, 81, (9), 665-673.

32. Romano, M. A.; Romano, R. M.; Santos, L. D.; Wisniewski, P.; Campos, D. A.; de Souza, P. B.; Viau, P.; Bernardi, M. M.; Nunes, M. T.; de Oliveira, C. A., Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Archives of Toxicology 2012, 86, (4), 663-673.

33. Le Mer, C.; Roy, R. L.; Pellerin, J.; Couillard, C. M.; Maltais, D., Effects of chronic exposures to the herbicides atrazine and glyphosate to larvae of the threespine stickleback (Gasterosteus aculeatus). Ecotoxicology and Environmental Safety 2013.

34. Soso, A. B.; Barcellos, L. J. G.; Ranzani-Paiva, M. J.; Kreutz, L. C.; Quevedo, R. M.; Anziliero, D.; Lima, M.; Silva, L. B. d.; Ritter, F.; Bedin, A. C., Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundia (Rhamdia quelen). Environmental Toxicology and Pharmacology 2007, 23, (3), 308-313.

35. Paull, G. C.; Van Look, K. J. W.; Santos, E. M.; Filby, A. L.; Gray, D. M.; Nash, J. P.; Tyler, C. R., Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. Aquat. Toxicol. 2008, 87, 115-126.

36. OECD, OECD Guideline for the Testing of Chemicals: Fish Short Term Reproductive Assay. 2009.

37. ISO Water Quality, Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [Brachydanio rerio Hamilton-Buchanan (Teleostei, cyprinidae)], Part 3. Flow-Through Method; International Organization for Standardization. 1996.

38. Kimmel, C. B.; Ballard, W. W.; Kimmel, S. R.; Ullmann, B.; Schilling, T. F., Stages of embryonic development of the zebrafish. Developmental Dynamics 1995, 203, (3), 253-310.

39. Uren-Webster, T. M.; Lewis, C.; Filby, A. L.; Paull, G. C.; Santos, E. M., Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquatic Toxicology 2010, 99, 360-369.

40. Santos, E. M.; Workman, V. L.; Paull, G. C.; Filby, A. L.; Van Look, K. J. W.; Killie, P.; Tyler, C. R., Molecular basis of sex and reproductive status in breeding zebrafish. Physiol. Genomics 2007, 30, 111-122.

41. Chauvenet, W., A manual of spherical and practical astronomy. Lippincott: Philadelphia, 1863.

42. Lubzens, E.; Young, G.; Bobe, J.; Cerda , J., Oogenesis in teleosts: how fish eggs are formed. General and Comparative Endocrinology 2010, 165, (3), 367-389.

43. van der Ven, L. T. M.; Wester, P. W.; Vos, J. G., Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (Danio rerio). Environmental Toxicology and Chemistry 2003, 22, (4), 908-913.

44. Kojima, H.; Katsura, E.; Takeuchi, S.; Niiyama, K.; Kobayashi, K., Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environmental Health Perspectives 2004, 112, (5), 524.

45. Thongprakaisang, S.; Thiantanawat, A.; Rangkadilok, N.; Suriyo, T.; Satayavivad, J., Glyphosate induces human breast cancer cells growth via estrogen receptors. Food Chem. Toxicol. 2013, 59, 129-136.

46. Oliveira, A. G.; Telles, L. F.; Hess, R. A.; Mahecha, G. n. A.; Oliveira, C. A., Effects of the herbicide Roundup on the epididymal region of drakes Anas platyrhynchos. Reproductive Toxicology 2007, 23, (2), 182-191.

47. Stehr, C. M.; Linbo, T. L.; Baldwin, D. H.; Scholz, N. L.; Incardona, J. P., Evaluating the effects of forestry herbicides on fish development using rapid

phenotypic screens. North American Journal of Fisheries Management 2009, 29, (4), 975-984.

48. Lewis, C.; Galloway, T., Reproductive consequences of paternal genotoxin exposure in marine invertebrates. Environmental Science & Technology 2009, 43, (3), 928-933.

49. Perez-Cerezales, S.; Martinez-Paramo, S.; Beirao, J.; Herraez, M., Fertilization capacity with rainbow trout DNA-damaged sperm and embryo developmental success. Reproduction 2010, 139, (6), 989-997.

50. Menezo, Y.; Dale, B.; Cohen, M., DNA damage and repair in human oocytes and embryos: a review. Zygote 2010, 18, (4), 357-365.

51. Dallegrave, E.; Mantese, F. D.; Coelho, R. S.; Pereira, J. n. D.; Dalsenter, P. R.; Langeloh, A., The teratogenic potential of the herbicide glyphosate-Roundup in Wistar rats. Toxicology Letters 2003, 142, (1), 45-52.

52. Marc, J.; Mulner-Lorillon, O.; Boulben, S.; Hureau, D. e.; Durand, G. l.; Bella, R., Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chemical Research in Toxicology 2002, 15, (3), 326-331.

Figure Legends

Figure 1. (A) Cumulative egg production during the 10 day pre-exposure and 21 day chemical exposure periods (n=3 replicate colonies per treatment); (B) Mean number of eggs laid per female per day throughout the 21 day exposure period (n=3 replicate colonies per treatment); and (C) Mean gonad-somatic index of females in each treatment group (n=12 individual females per treatment). Data plotted are mean values ± SEM. Asterisks indicate significant differences between treatment groups (*P<0.05 **P<0.01 ***P<0.001).

Figure 2. Effects of Roundup and glyphosate on embryo survival and development. Black bars represent embryos originating from exposed parental populations (n= 3 replicate colonies, for each colony data was collected every day for 21 days of exposure and averaged) and grey bars represent embryos originating from a control parental population (n=3 replicate exposures, each replicate containing 50 embryos). **(A)** Percentage of embryo mortalities that occurred before 3.5 hpf; **(B)** percentage of embryo mortalities that occurred between 3.5-24 hpf; and **(C)** percentage of embryos that had hatched at 54 hpf in each treatment group. Data plotted are mean values ± SEM. Asterisks represent significant differences from the control treatment (***P<0.001).

Figure 3. Transcript profiling of target genes in the ovary **(A)** and testis **(B)** following exposure to Roundup (R) and glyphosate (G). Data are presented as fold change relative to expression in the control group, whereby red shading indicates up-regulation and green shading represents down-regulation. Relative expression was calculated as ratio of target gene /rpl8 mRNA concentration. For each treatment, n= 6–8 fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis. Lettering indicates significant differences between treatment group, with groups identified with different letters being significantly different from each other (P< 0.05).

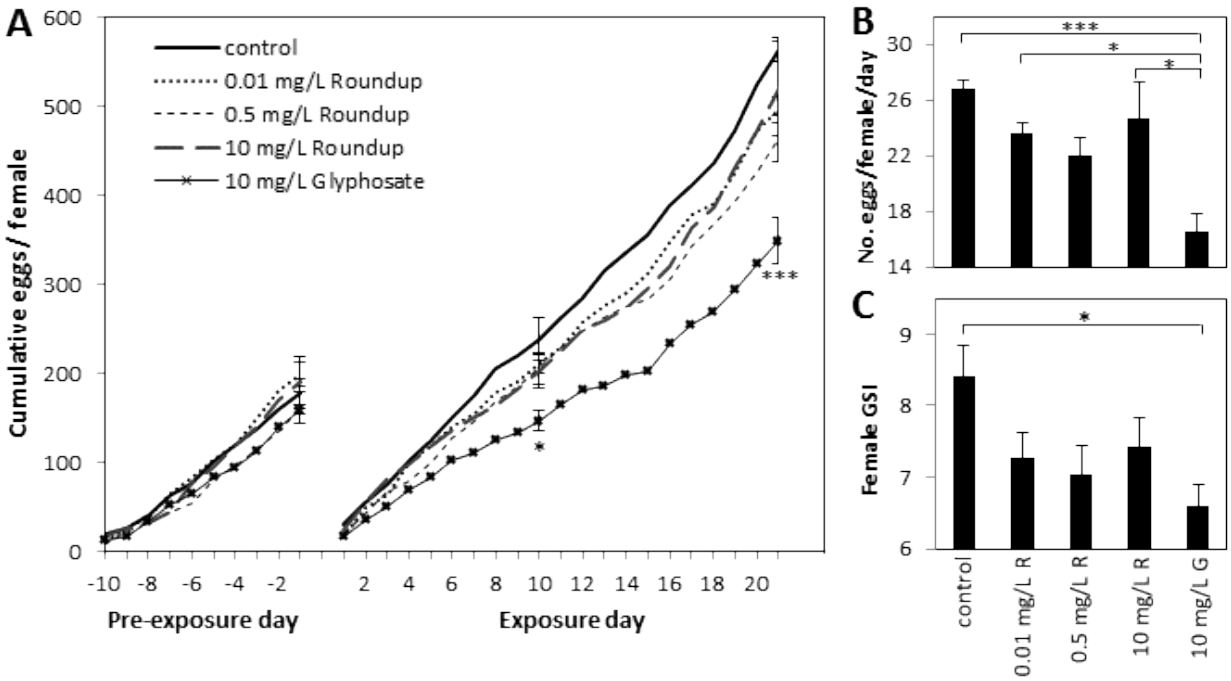

A
Cumulative eggs / female
control
0.01 mg/L Roundup
0.5 mg/L Roundup
10 mg/L Roundup
10 mg/L Glyphosate
Pre-exposure day
Exposure day
B
No. eggs/female/day
C
Female GSI
control
0.01 mg/L R
0.5 mg/L R
10 mg/L R
10 mg/L G

Figure 1

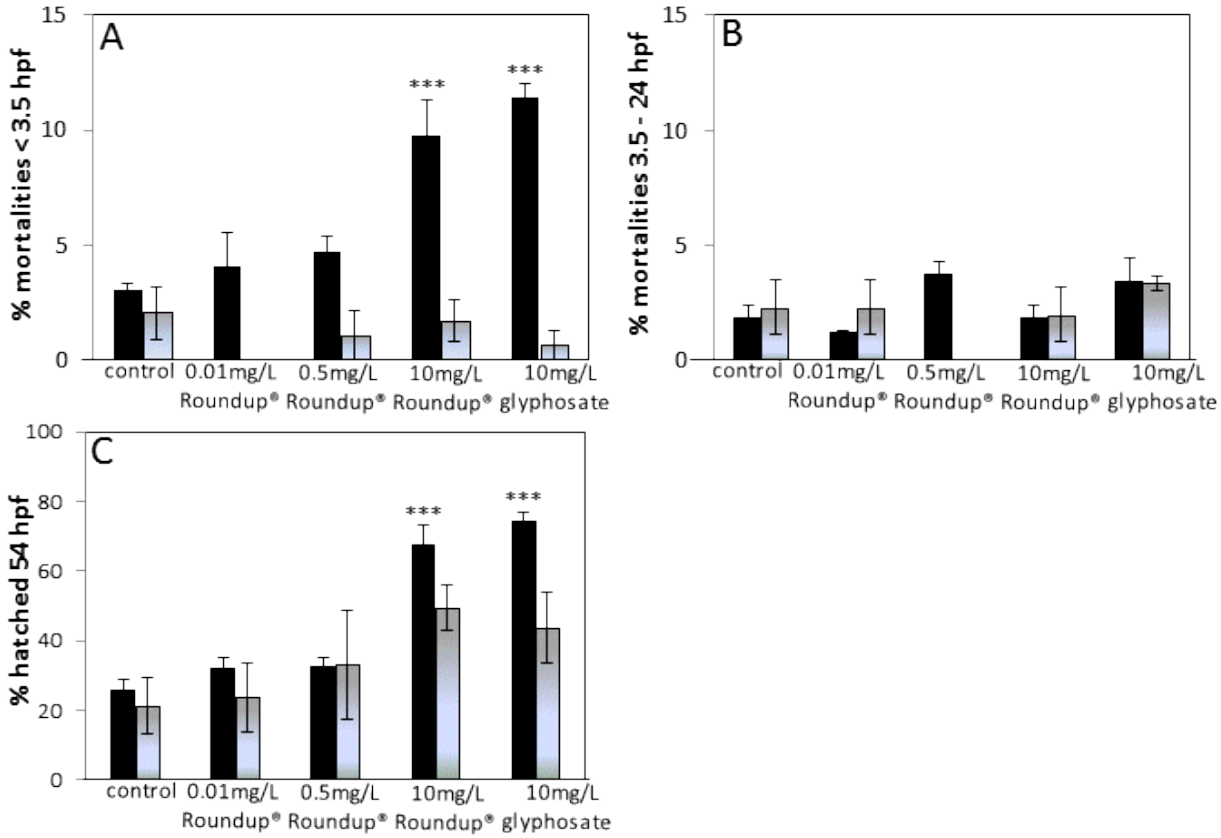

A
% mortalities < 3.5 hpf
15
10
5
0
***
***
control
0.01mg/L
0.5mg/L
10mg/L
10mg/L
Roundup®
Roundup®
Roundup®
glyphosate
B
% mortalities 3.5 - 24 hpf
15
10
5
0
control
0.01mg/L
0.5mg/L
10mg/L
10mg/L
Roundup®
Roundup®
Roundup®
glyphosate
C
% hatched 54 hpf
100
80
60
40
20
0
***
***
control
0.01mg/L
0.5mg/L
10mg/L
10mg/L
Roundup®
Roundup®
Roundup®
glyphosate

Figure 2

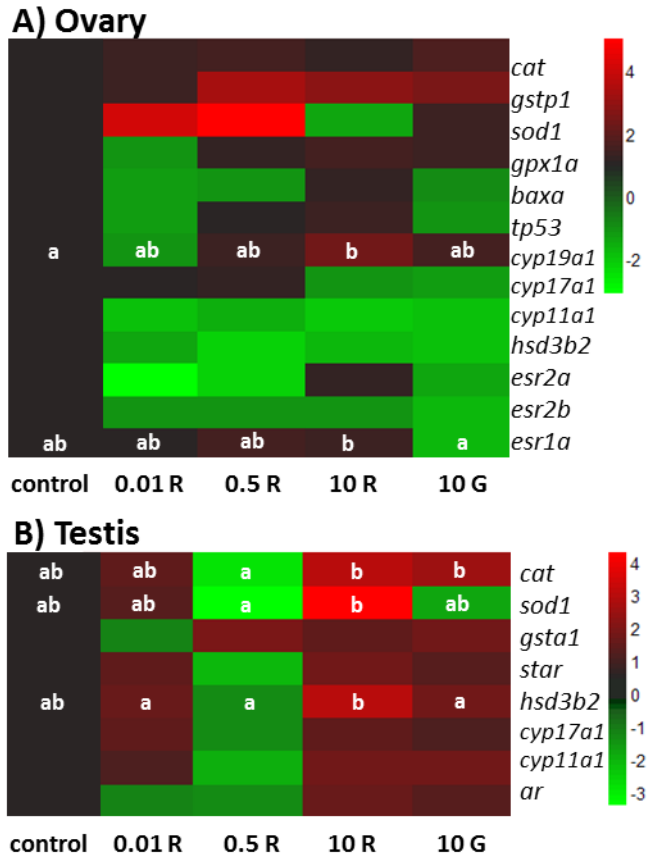

A) Ovary
cat
gstp1
sod1
gpx1a
baxa
tp53
cyp19a1
cyp17a1
cyp11a1
hsd3b2
esr2a
esr2b
esr1a
a ab ab b ab
ab ab ab b a
4
2
0
-2
control 0.01 R 0.5 R 10 R 10 G
B) Testis
ab ab a b b
ab ab a b ab
ab a a b a
cat
sod1
gsta1
star
hsd3b2
cyp17a1
cyp11a1
ar
4
3
2
1
0
-1
-2
-3
control 0.01 R 0.5 R 10 R 10 G

Figure 3

Supporting Information

Effects of the herbicides Roundup and glyphosate on reproduction in zebrafish (*Danio rerio*)

Tamsyn. M. Uren Webster[1]*, Lauren V. Laing[1], Hannah Florance[1] and Eduarda M. Santos[1]*

1. Biosciences, College of Life & Environmental Sciences, Geoffrey Pope Building, University of Exeter, Exeter, EX4 4QD

* Corresponding authors

This Supporting Information contains:

Supplemental Experimental Section

Fish maintenance

Colonies of 4 male and 4 female adult WIK strain zebrafish (20 weeks old; originating form a stock kept at the University of Exeter) were established in individual 15 L glass tanks and allowed to breed naturally during a 7 day acclimation period. Each tank was aerated and supplied with a water flow rate of 48 L /day. The aquarium water supply was reverse-osmosis treated tap water reconstituted with analar-grade salts to produce standardized synthetic freshwater according to OECD guidelines, as described in Paull et al. [1], and maintained at 28 ± 0.5 °C and pH 7-7.5. Fish were kept under a 12h light:dark cycle (with 30 minute dawn/dusk transitional periods) and fed twice daily with live *Artemia nauplii* and flake food (Tetra; Melle, Germany) to satiation.

Water chemistry

Glyphosate quantification was carried out using a 6420B Triple Quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 20 µl of sample were loaded onto a Zorbax Eclipse Plus C_{18} 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. Glyphosate was eluted from the column using the following gradient: 0-3 min, 0-75% B, 0.15 ml/min; 3-4 min – 100% B, 0.25 ml/min; 4.1 min – 0% B, 0.15 ml/min. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 l min^{-1}, nebuliser pressure 35 psig, capillary voltage +4 kV. All analysis was carried out in positive ion mode with the precursor ion *m/z* of 170.1 and a transition *m/z* of 87.8. Fragmentor and collision energy voltages were optimised to 60 and 5 respectively. Glyphosate was used to generate a standard curve. All samples were diluted accordingly to ensure peaks areas were within the linear range of the curve (0.05 – 1.0 mg/L).

Transcript profiling

Transcripts of genes encoding steroidogenic enzymes, sex steroid receptors and antioxidant enzymes were quantified in the gonads of exposed fish using real time quantitative PCR (RT-QPCR). Primers for each target gene were designed with Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA) using zebrafish NCBI refseq sequences, and purchased from MWG-Biotech (Ebersburg, Germany). Primer specificity throughout the range of detection was confirmed by the observation of single amplification products of the expected size and Tm, and optimised by performing a standard curve for each primer pair as described previously [40]. Over the detection range, the linear correlation (R^2) between the mean Ct and the logarithm of the cDNA dilution was > 0.99 in each case, and efficiencies were between 1.95 and 2.18. The primer sequences, PCR product sizes, annealing temperatures and PCR efficiencies for each primer pair are shown in Table S1.

RNA was extracted from the gonads of eight male and eight female fish from each treatment group using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA concentration and purity were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). cDNA was synthesised according to manufacturer's instructions from 2 µg of total RNA treated with RQ1 DNase (Promega, Southampton, UK) using random hexamers (MWG-Biotech) and M-MLV reverse transcriptase (Promega). cDNA was diluted (ovary 1:4, testis 1:2) and RT-QPCR was performed in duplicate in an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green chemistry as described [40]. A template-minus negative control was run in duplicate on each plate to verify the absence of cDNA contamination. Efficiency-corrected relative expression levels were determined by normalizing to a control gene, ribosomal protein l8 (*rpl8*), which was previously shown to have consistent expression in ovaries and testis [2].

Histological analysis

Gonads were fixed in Bouin’s solution for four hours, then washed and stored in 70 % ethanol prior to histological analysis. Samples were dehydrated in 70-100% industrial methylated spirit (IMS) and ethanol using a Shandon (Citadel 2000) tissue

processor, and then embedded in paraffin wax. The embedded samples were cut in 5 µm sections, floated in 30% IMS in a water bath at 45 °C, and then laid on glass slides and dried on a 40 °C heat tray overnight. Sections were stained with Harris' non-acidified Haemoatoxylin and Eosin Y (both Thermoshandon, Pittsburg, U.S.) and mounted with Histomount (National Diagnostics, Hull, UK). Analysis was conducted with a light microscope (Zeiss Axioskop 40, Carl Zeiss, Oberkochen, Germany) connected to an Olympus DP70 camera (Olympus Optical) and using analySIS image processing 3.2 software (Soft Imaging System, Munster, Germany). Two slides, each containing multiple sections, were prepared from portions of ovary 100 µm apart, to provide a representative analysis of the ovarian tissue, while single slides were prepared for each testis.

References

1. Paull, G. C.; Van Look, K. J. W.; Santos, E. M.; Filby, A. L.; Gray, D. M.; Nash, J. P.; Tyler, C. R., Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. *Aquatic Toxicology* **2008,** *87*, 115-126.
2. Filby, A.; Tyler, C., Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. *Bmc Molecular Biology* **2007,** *8*, (1), 10.

Table S1: Target genes, primer sequences and assay details for RT-QPCR analysis.

Gene Name	Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR efficiency
Ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
Catalase	*cat*	AGTTCCCTCTGATTCCTGTG	ATGGCGATGTGTGTCTGG	173	61.0	2.00
Superoxide dismutase	*sod1*	TTCACTCTCTCACAACTTCTC	GTCACCTTCACTGGCTTC	142	58.0	2.18
Glutathione peroxidase	*gpx1a*	CTGCGTGTTGCCCTTTGAG	GGTGTAATCCCTGACTGTTGTG	189	58.5	1.98
Glutathione S transferase pi	*gstp1*	AACGACAGTGAGGCTTCC	GCATTTGAGGTGGTTGGG	141	56.0	1.85
Glutathione S transferase alpha	*gsta1*	GGTGGCTCTTGGCTGTTG	TGCGATGTAGTTCAGGATGG	170	61.0	2.03
Steroidogenic acute regulatory protein	*star*	TTCTTGAGGACCAGGATG	GACTTGCTTGACATTGGG	197	58.0	2.03
Cytochrome P450, subfamily XIA, polypeptide 1	*cyp11a1*	TGAGTGCTGTGTTGTATG	AAATGTTGGACCCTATGG	159	57.0	2.12
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	*hsd3b2*	GCAGCATTGAGGTAGCGTGTC	AGGATAAGAGGAGTAAGGCGTGTC	83	60.0	2.12
Cytochrome P450, subfamily XVIIA, polypeptide 1	*cyp17a1*	CGACAGTAAGATTGGGAAAGAAAG	GATGAGGAGCGGAGAAACAG	118	60.5	1.98
Estrogen receptor 1	*esr1a*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
bcl2-associated X protein a	*baxa*	CTACTTTGCCTGTCGCCTTG	GTCCCATCCACCCTGTTCC	136	60.0	2.14
Tumour protein p53	*tp53*	GCTTGGTGCTGAATGGAC	GAGTGATGATTGTGAGGATGG	98	56.0	2.09

Table S2: Water chemistry analysis of glyphosate in the exposure tank water. Data presented are the measured concentrations for the three replicate treatment tanks on days 7, 14 and 21 are presented as mean values ± SEM. Analysis was conducted using a 6420B Triple Quadrupole (QQQ) mass spectrometer coupled to a 1200 series Rapid Resolution HPLC system.

Nominal concentration	**control**	**0.01 mg/L Roundup**	**0.5 mg/L Roundup**	**10 mg/L Roundup**	**10 mg/L glyphosate**
Day 7	< 0.05	< 0.05	0.43 ± 0.1	8.8 ± 4.2	14.2 ± 1.9
Day 14	< 0.05	< 0.05	0.40 ± 0.1	13.3 ± 0.6	10.0 ± 0.6
Day 21	< 0.05	< 0.05	0.50 ± 0.1	16.3 ± 2.0	17.7 ± 1.5
Mean	**< 0.05**	**< 0.05**	**0.44**	**12.8**	**13.9**

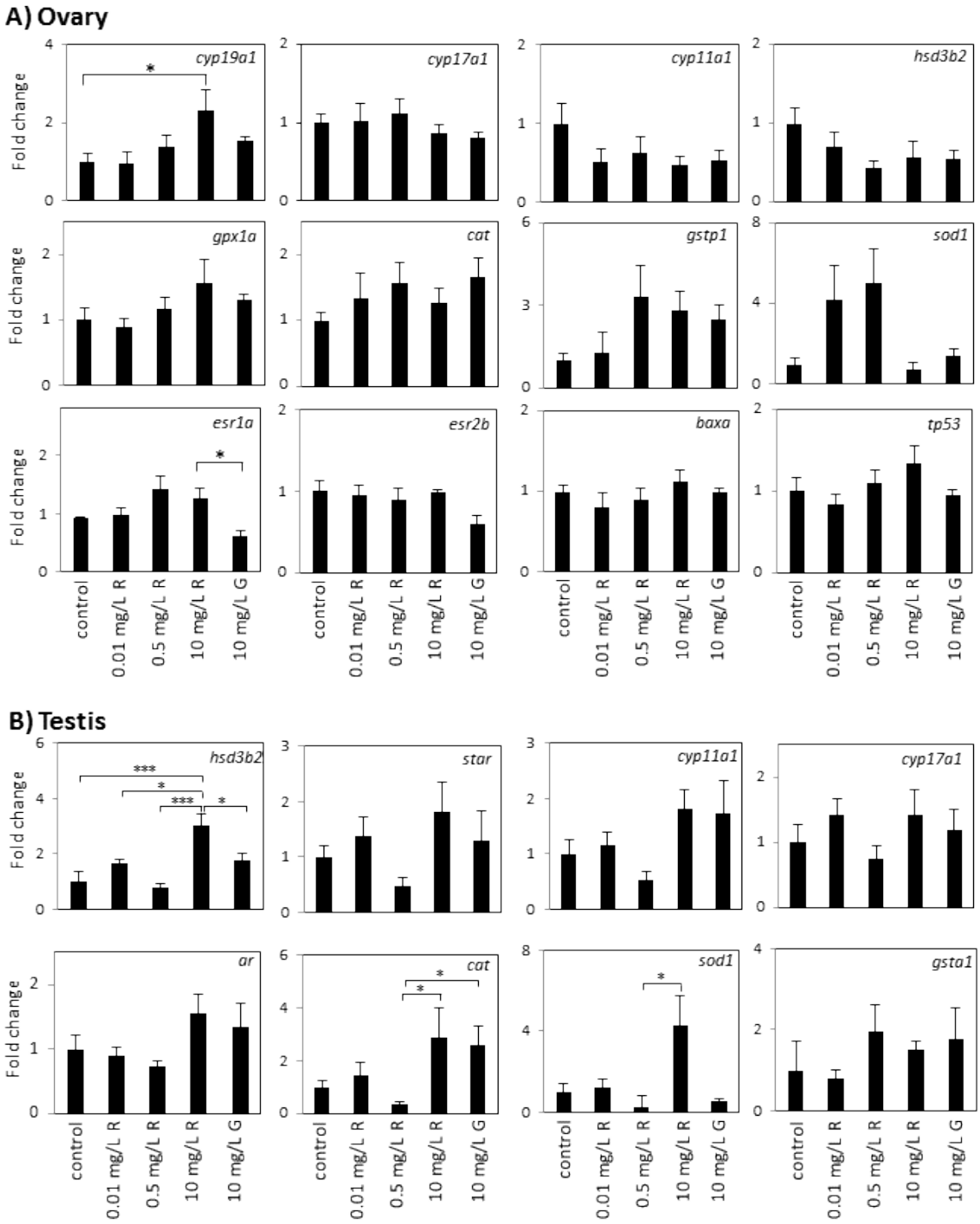


Figure S1. Transcript profiling of target genes in the ovary (A) and testis (B). Data are presented as fold change relative to expression in the control group. Relative expression was calculated as ratio of target gene /rpl8 mRNA concentration. For each treatment, data was collected for 6–8 fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis. Asterisks represent significant differences between treatment groups (*P<0.05 **P<0.01 ***P<0.001).

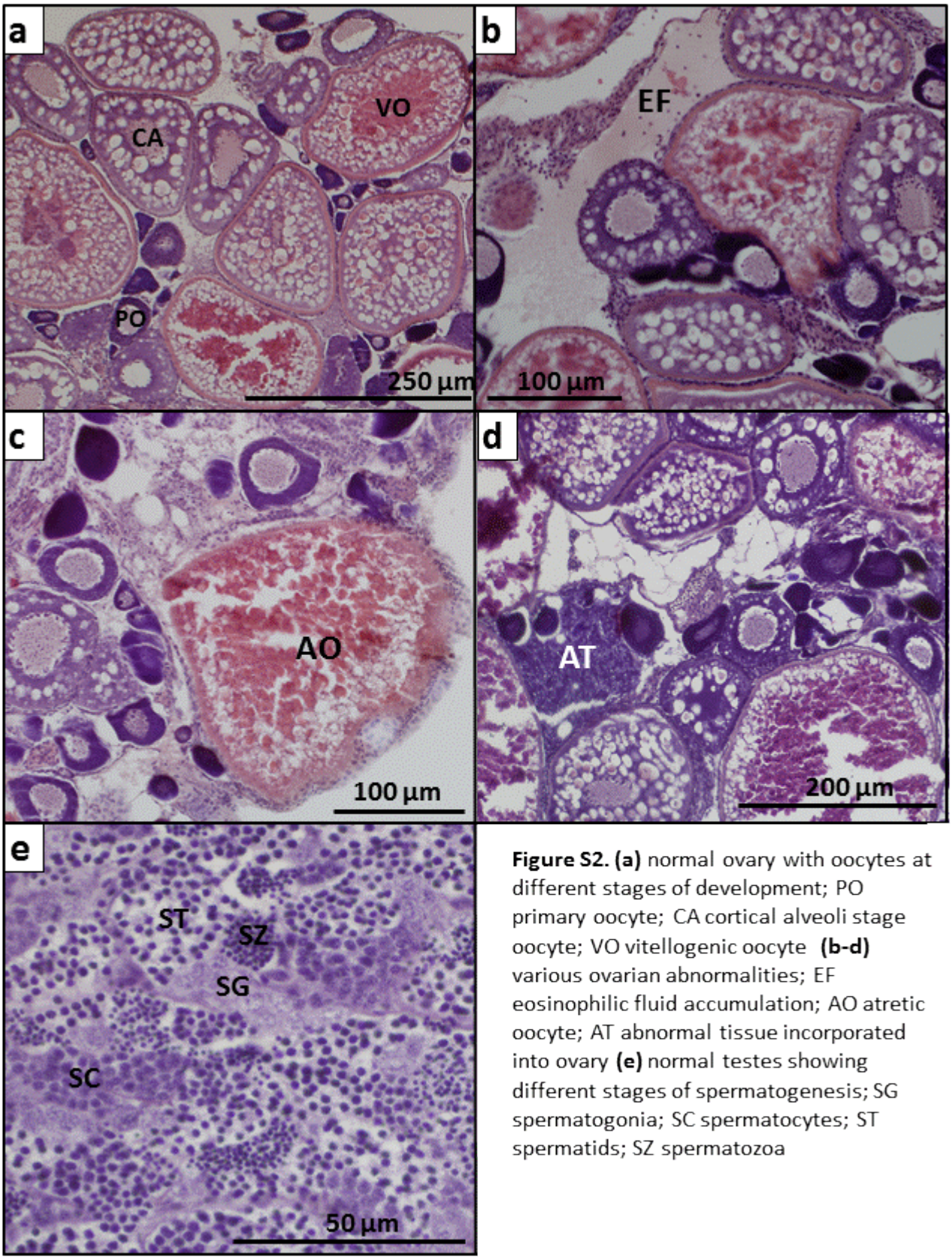


Figure S2. (a) normal ovary with oocytes at different stages of development; PO primary oocyte; CA cortical alveoli stage oocyte; VO vitellogenic oocyte **(b-d)** various ovarian abnormalities; EF eosinophilic fluid accumulation; AO atretic oocyte; AT abnormal tissue incorporated into ovary **(e)** normal testes showing different stages of spermatogenesis; SG spermatogonia; SC spermatocytes; ST spermatids; SZ spermatozoa

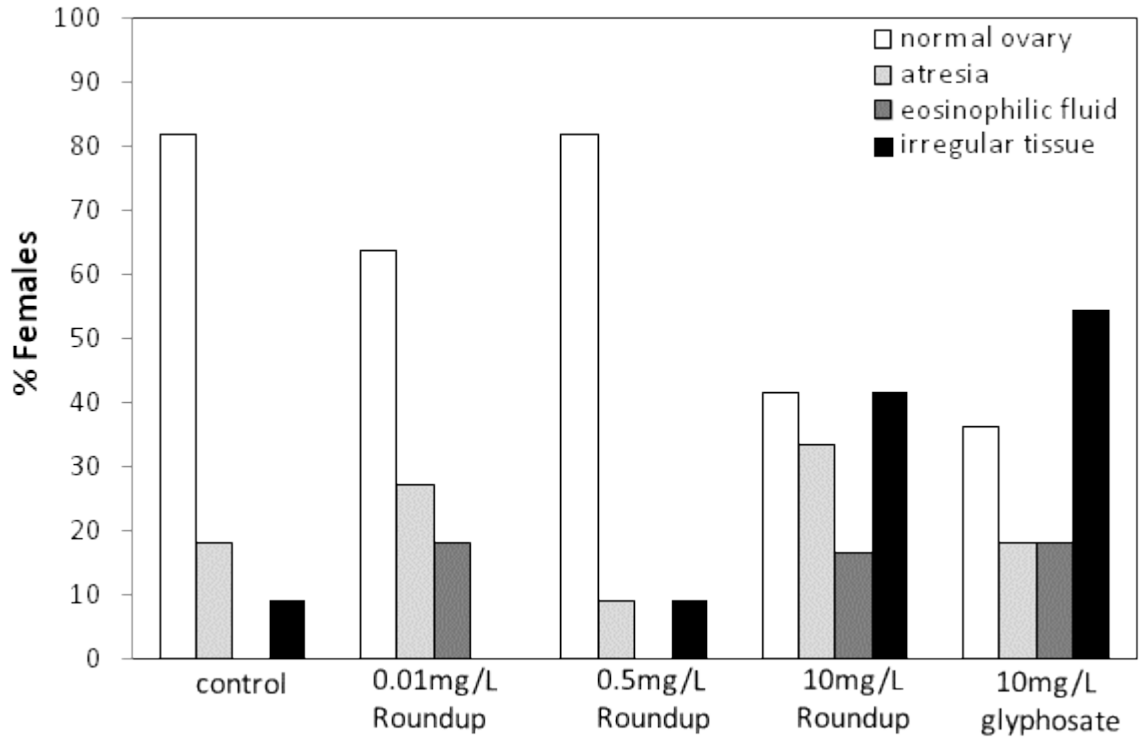


Figure S3. Histological analysis of the ovaries of females exposed to glyphosate and Roundup. Proportion of females in each treatment group showing absence or presence ovarian abnormalities (n=11-12 fish per treatment).

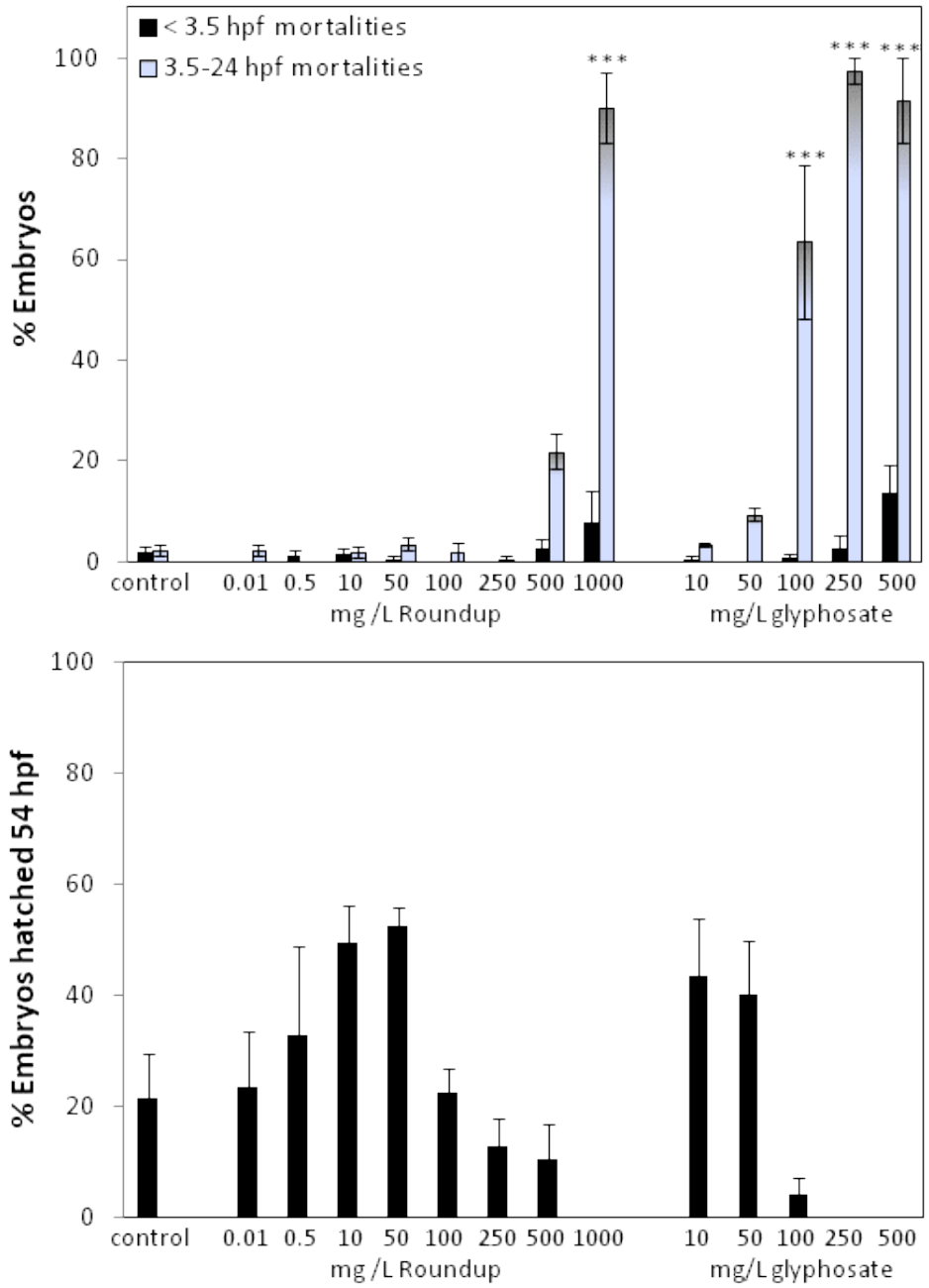


Figure S4. Effects of Roundup and glyphosate on the survival and development of embryos originating from the unexposed control parental population; **(A)** percentage of embryo mortalities that occurred before 3.5 hpf and between 3.5-24 hpf; and **(B)** percentage of embryos that had hatched at 54 hpf. Treatment concentrations include those used during the adult exposure, and higher concentrations (> 10 mg/L) to investigate the thresholds for mortality and development abnormalities to occur. Data presented are mean values ± SEM (n = 3 replicates per treatment concentration, each replicate consisted of 50 embryos). Asterisks indicate significant difference from the control group (*P<0.05 **P<0.01 ***P<0.001).

CHAPTER 7

General Discussion

CHAPTER 7: GENERAL DISCUSSION

Brown trout are an important native species known to be sensitive to environmental stressors but there has been very limited previous research into the potential impacts of chemical pollutants on this species, particularly of underlying molecular mechanisms of toxicity. RNA-seq facilitates such research on non-model species because it can be applied in the absence of existing genomic sequence information, but it has so far been underused in ecotoxicology. The major objectives of this PhD were, therefore, to investigate the molecular mechanisms of toxicity for a selection of environmentally relevant chemicals likely to impact on natural brown trout populations using RNA-seq, and also to evaluate the potential application of this technology in ecotoxicological research.

In this discussion, I provide a critical synopsis of how these objectives were addressed, and the results achieved, including a discussion of the technical challenges and limitations of this work. I also discuss the relevance of this data in the context of wider ecotoxicological research, its potential application in environmental management and possible directions for future research.

7.1 Effects of environmentally relevant chemical stressors on brown trout

At the start of my PhD, I utilised Illumina technology to sequence and assemble a transcriptome for the brown trout, then used this information to investigate molecular mechanisms of metal tolerance in a wild population of brown trout chronically exposed to extremely high concentrations of a mixture of metals (*Chapter 2*). Global transcriptomic profiling and analysis of metal accumulation revealed that these fish have a high degree of metal tolerance and, in contrast to other metal-exposed fish populations, show relatively limited evidence of overt metal toxicity. Whether this tolerance is due to an inherent ability to acclimate to metal exposure, or due to inherited genetic adaptation is unclear. This work also demonstrates the strong connection between this population and its environment, and highlights the importance of considering this relationship for the sustainable management of this population and also, potentially, of other tolerant populations of brown trout that may exist in similarly polluted rivers.

Following this work, I selected a number of test chemicals based on their likely relevance and potential threat to natural brown trout populations and these included the natural oestrogen E2 and two pesticides, glyphosate and linuron (*Chapters 3-5*). The selection of treatment concentrations used was a vital part of the experimental design, and I employed the same strategy in all three chapters. In each case I selected three nominal concentrations. These consisted of a low treatment within the range of concentrations measured in the environment, an intermediate treatment that might represent concentrations associated with short-term peaks in surface water contamination, and a high treatment, chosen to facilitate mechanistic analysis at a concentration where unambiguous effects were likely to occur. I selected an appropriate high treatment concentration based on existing evidence in the literature and also by conducting preliminary exposures of brown trout to linuron and glyphosate/Roundup. These high concentrations (250 ng E2/L, 250 µg linuron/L and 10 mg glyphosate/L) were selected because they were associated with induction of phenotypic effects, including negative impacts on growth rate and condition, but were considerably lower than concentrations known to induce acute stress or mortality in brown trout.

The use of such high concentrations could be criticised for its lack of environmental relevance, and there are also concerns that they may be associated with different mechanisms of toxicity. However, using high treatment concentrations can be very useful to determine well-defined mechanisms of chemical toxicity, in a similar way to the benefits of using a positive control. Comparing transcriptomic profiles of response across treatment concentrations can link similar mechanisms of toxicity occurring at high concentrations with those occurring at low concentrations. Effects at low concentrations may be less pronounced and, therefore, difficult to identify in the absence of the information provided by the data obtained for the higher concentrations. Identifying similar molecular changes at environmentally relevant concentrations might indicate the potential for more pronounced effects to occur following chronic exposure in the environment. These changes may also be useful in identifying molecular ‘early warning signs’ of exposure. Importantly, comparison of transcriptomic response can also be used to identify whether different mechanisms of toxicity are occurring at high concentrations. For example, in *Chapter 5,* I found evidence of similar transcriptomic response in fish exposed to 0.01-10 mg/L Roundup

and 0.01 mg/L glyphosate, but a very different transcript profile in those exposed to 10 mg/L glyphosate, possibly indicating a different mechanism of toxicity at this high concentration.

I found evidence of considerable transcriptomic responses in male brown trout exposed for four days to 34 ng E2/L (measured concentration), but no evidence of effects in fish treated with 2 or 18 ng/L of E2 (*Chapter 3*). Among the most up-regulated transcripts, well-characterised oestrogen-responsive genes (vitellogenins, zona pellucida proteins and estrogen receptor 1) were identified, while up-regulated hepatic processes included those associated with vitellogenesis (lipid metabolism, cell proliferation and growth, transcription, translation and ribosome biogenesis). Overall, these results demonstrate that E2 exposure induces a conserved transcriptional response in brown trout compared to that previously reported in other species. Importantly, although 34 ng E2/L is unlikely to regularly occur in surface waters, this concentration is within a range of values measured in sewage effluent. These results, therefore, highlight the potential threat that E2 contamination can pose to brown trout populations, especially when considering the oestrogenic equivalent concentrations of the complex mixtures of chemicals that can contaminate rivers inhabited by this species.

Exposure of male brown trout to 250 µg/L linuron induced a striking down-regulation of the majority of enzymes involved in the cholesterol biosynthesis pathway (*Chapter 4*). Additionally, there was evidence of an up-regulation of a number of transcripts involved in cellular stress response, including CYP1A (by up to 560 fold), molecular chaperones involved in binding and stabilising damaged proteins, and the antioxidant system. Although there were less pronounced changes in fish exposed to 2.5 µg linuron/L, comparison of transcriptional profiles revealed evidence of similar mechanisms of toxicity occurring at this environmentally relevant concentration, including down-regulation of the cholesterol biosynthesis pathway. Finding this evidence of disruption of cholesterol biosynthesis was an unexpected and novel result. This demonstrates the value of including high treatment concentrations for mechanistic analysis, and also highlights disruption of cholesterol biosynthesis as a potential mechanism of toxicity of other anti-androgens as an area of future research.

I found evidence that exposure to 0.01, 0.5 and 10 mg/L of Roundup and 0.01 mg/L glyphosate induced cellular stress responses in brown trout (*Chapter 5*). Importantly, these results suggest that similar mechanisms of toxicity are induced by environmentally relevant concentrations of both Roundup and glyphosate. This cellular stress response was characterised by a dominant up-regulation of transcripts involved in the antioxidant system, the repair of damaged cell components, regulation of cell proliferation and turnover, apoptosis and compensatory metabolic changes. These changes are consistent with generation of oxidative stress being a central, underlying mechanism of glyphosate and Roundup toxicity. Oxidative stress is often considered a generic effect that is common to many chemicals at high concentrations, and our results therefore demonstrate the importance of considering the environmental relevance of this mechanism of toxicity. The up-regulation of a compensatory cellular stress response to oxidative stress, as characterised in these results, is likely to be associated with a significant energetic cost. This, in turn, might be expected to have a negative impact on other biological processes such as growth, reproduction and immune function.

Overall, the results described in *Chapters 3-5* demonstrate that environmentally relevant concentrations of all of these tested chemicals may potentially impact on natural brown trout populations. This provides valuable information on the toxicological response of brown trout that may, ultimately, contribute to the sustainable management of this environmentally relevant species.

There are a number of limitations with using brown trout as a model species in ecotoxicological research. This species is extremely sensitive to environmental stressors, including hypoxia and disturbance, and adult fish, in particular, are challenging to keep in a laboratory environment. Ideally, I would have investigated the potential endocrine disruption and reproductive toxicity of glyphosate and Roundup in brown trout, but this is not practical due to the life-history strategy of this species, which spawns only once a year following a period of approximately 9 months to complete gametogenesis. Instead, I used zebrafish as a surrogate model species to conduct this investigation in *Chapter 6*. In this study, I investigated the potential reproductive toxicity of Roundup® at a range of concentrations (0.01-10mg/L) and glyphosate (10mg/L) to breeding colonies of zebrafish, and investigated for specific mechanisms of toxicity using RT-QPCR. Evidence of endocrine disruption and

reproductive toxicity was found in fish treated with very high concentrations (10 mg/L) of Roundup and glyphosate, but not in fish exposed to environmentally relevant concentrations. However, given the growing concern about the potential reproductive toxicity of glyphosate and Roundup, in humans as well as wildlife, it is important to report that reproductive effects in fish do occur, but only at these high, environmentally unrealistic, concentrations, at least in the conditions employed in our study.

7.2 Evaluating the use of RNA-seq in ecotoxicology

In the context of this research, the primary advantage of RNA-seq is its applicability in species without existing genomic sequence information. Fundamentally, this has enabled the successful completion of the first major objective of this PhD; to conduct transcriptional profiling of a selection of environmentally relevant chemicals in the brown trout, an ecologically and environmentally important species for which sequencing data was scarce. Furthermore, the sequencing conducted in my PhD project has also generated extensive, valuable gene sequence information which may benefit many areas of wider biological research in this species.

Since the start of this PhD, there has been a continual and rapid development in RNA-seq, in terms of both the sequencing technology and the bioinformatics tools/strategies available for data analysis. This is clear from the considerable increase in the quality and number of RNA-seq experiments reported in the literature. These improvements have also led to a growing body of evidence that RNA-seq has a number of technical advantages over other transcriptomic approaches, which may include sensitivity, accuracy, reproducibility and dynamic range in the quantification of transcript expression. As a result, RNA-seq is now routinely used in many fields, particularly medical research, which in turn has driven the development of this technology.

Throughout this thesis, there is also a clear progression in terms of the sequencing technology used, the experimental design and the results achieved. In *Chapter 2* sequencing was conducted on an Illumina GA2 platform, and generated 147.5 million reads from two lanes. Based on the expected coverage, samples were pooled and multiplexed 12x, and the resulting mean number of reads per sample was 6.6 million.

In contrast, sequencing in *Chapters 3, 4* and *5* was conducted using an Illumina HiSeq 2500, which increased the quality of these later RNA-seq experiments. The substantial increase in typical read output allowed samples to be multiplexed 24x per lane, which in turn enabled the use of biological replicates in each treatment group rather than pooling samples. For *Chapters 4* and *5*, a total of 225.3 million reads were generated from one lane, averaging 9.4 million per sample, and three biological replicates were employed per treatment group. For *Chapter 6,* I generated 969.4 million sequence reads, averaging 20.1 million per sample, and six biological replicates were used for each treatment. In particular, experimental design of the latter experiments was significantly improved by the use of replicates, which improved the statistical power of transcript expression analysis. In parallel to the improvement in this sequencing technology, a greater range of improved bioinformatics tools were available for the analysis of the latter datasets.

In species with no reference genome, the quality of the *de novo* transcriptome assembly is essential for the quality of the downstream transcript expression analysis, and optimising assembly strategy constituted a major part of this work. At the start of this PhD, there were scarce reports of RNA-seq in species without a reference genome in the literature and fewer software packages designed specifically for *de novo* transcriptome assembly of short sequence reads. Despite recent progression in this field, there is still no consensus on the best approach for *de novo* assembly, and it is likely that this will vary based on the characteristics of each dataset and the aims of the experiment.

For *Chapters 3, 4* and *5,* I optimised the transcriptome assembly strategy for the data generated from brown tout liver using the Illumina HiSeq platform. The large number of reads present in these datasets may introduce systematic sequencing errors in abundant transcripts into the *de novo* assembly and exceeded computer memory capacity. Therefore, I compared different strategies for reducing the number of reads input into transcriptome assembly, and found that digital normalisation resulted in higher quality assemblies than using stricter quality filtering or random subsampling methods. Digital normalisation involves a targeted reduction of high coverage reads, which does not compromise rare transcript discovery and also reduces accumulation of sequencing errors in abundant transcripts. In terms of the software used for the assembly itself, *Chapter 5* describes a detailed comparison between assemblies

conducted using Velvet-Oases and Trinity. I conducted a series of initial optimising steps to determine the most appropriate k-mer range to use for Velvet-Oases assemblies, and concluded that this was achieved using a k-mer range of 33-69 and a K-merge of 27. For Trinity, there are fewer adjustable parameters, and I determined that using the default settings generated the best results.

I used measures of transcriptome assembly quality to determine the most appropriate assembly strategy. As I describe in the introduction to this thesis, measures of assembly quality have often been poorly defined in the literature, and vary based on the aims of the experiment. In *Chapter 5,* I concluded that transcriptome redundancy was the most important measure of assembly quality because it significantly impacted on the quality of downstream expression analysis. This is why Trinity was used to assemble the final transcriptome in this chapter, and also in *Chapters 3* and *4.* However, the assembly comparison conducted in *Chapter 5* also revealed that the Velvet-Oases assembly included representation of a greater number of unique gene annotations, and this might be considered a more important measure of assembly in other circumstances. Transcript completeness is also another measure that could be of primary importance depending on the aims of the experiment.

Therefore, a major conclusion from this work is that when conducting RNA-seq in a non-model species, consideration of transcriptome assembly strategy is essential in order to construct the best possible quality assembly and maximise the accuracy and sensitivity of downstream transcript quantification and expression analysis. However, this does not seem to be widely considered, or at least documented, in many existing RNA-seq experiments in the literature. Although, it is clear that the optimum assembly strategy determined in *Chapter 5* is likely to soon be superseded, the results obtained highlight the importance of considering the optimum assembly strategy for a given dataset and set of experimental aims.

From conducting this work, it is also clear that use of RNA-seq is associated with a number of challenges and limitations. As discussed in *Chapter 5* and in the introduction, redundancy reduces statistical power in expression analysis, and some degree of redundancy is always associated with *de novo* transcriptome assemblies due to incorporation of sequence errors or regions of low coverage. I observed that the majority of redundant transcripts consist of incomplete fragments. Genuine

alternative transcript isoforms are very difficult to correctly identify and analyse on a mass scale.

A high level of biological variation between individuals is a challenge associated with using transcriptomics in ecotoxicology, and can limit statistical power and/or increase susceptibility to discovery of false positives. I observed that RNA-seq seems to be susceptible to problems caused by high levels of biological variation, although it is difficult to compare this with other transcriptomic approaches. It is possible that a contributing factor in this is that differential expression algorithms (such as EdgeR) were originally developed for RNA-seq using replicate clonal model organisms and *in vitro* toxicological studies, which are associated with considerably lower levels of biological variation (MacCarthy et al. 2012). For example, in *Chapter 4,* one individual in the intermediate Linuron treatment group showed a different expression profile from the others. It is likely that the low number of identifiable differentially expressed transcripts reflected the high level of biological variation in this group. In *Chapter 5,* one individual in the intermediate glyphosate treatment group showed an even more distinct expression profile form the others, but in this case this resulted in an unrealistically high number of differentially expressed transcripts, presumably including a large number of false positives. These results highlight the importance of maximising the number of replicates in RNA-seq experimental design in order to minimise the impact of this inevitable biological variation and allow for accurate detection of the transcriptional changes associated with the biological question of interest.

The results showed no clear dose-dependent response to chemical exposure (in terms of number of differentially expressed transcripts) in any of the RNA-seq experiments conducted in *Chapters 3, 4* or *5.* In addition to the possible influence of biological variation, another contributing factor to this may be limitations associated with the statistical analysis of expression, as described by Black et al. (2013). There is no standardised, 'best' approach for statistical analysis, and the results can vary based on the algorithm employed and the characteristics of the dataset.

A very recent comparison of RNA-seq and microarrays suggests that RNA-seq may be less sensitive for detecting differential expression at low treatment concentrations (Black et al. 2013). I also found some evidence in agreement with this. For example,

no statistically significant changes were observed in the transcript profiles for fish exposed to the lower concentrations of E2 in *Chapter 3*, although there were strong trends in up-regulation in some of the well-characterised estrogen-responsive genes. Transcript redundancy, which reduces the statistical power when employing multiple test correction, is a likely explanation for this lack of sensitivity.

Another particular challenge associated with analysis of data from RNA-seq experiments is the functional analysis of differentially expressed transcripts. Unfortunately the extent and quality of functional annotation for fish species is much lower than for humans. Therefore, I found that the quality of results from automated functional analysis packages such as DAVID and IPA were relatively limited, and extensive manual gene searching was required to comprehensively interrogate the data.

While there are still challenges and limitations associated with RNA-seq, these will undoubtedly continue to be improved with the development of RNA-seq technology, both in terms of the sequencing chemistry and bioinformatics analysis. Overall, the work conducted during this PhD demonstrates the major advantages of RNA-seq as a technique that can be used to conduct transcriptomic analysis on environmentally-relevant species without a sequenced genome. In *Chapter 3,* I specifically established that RNA-seq can be used to determine a conserved response to oestrogen exposure, which provides biological validation for its use as a tool in ecotoxicology. In terms of the technical advantages of RNA-seq, for *Chapters 3, 4* and *5* I calculated the dynamic range in expression quantification to be far in excess of that typically associated with microarrays, and using ERCC spike-control analysis, also confirmed the accuracy of transcript expression level quantification. The bioinformatics expertise and computation requirements of RNA-seq, together with cost, are undoubtedly reasons contributing to the relatively slow take up of this technique in ecotoxicology, particularly where there are well-established microarrays available for a species. However, this work contributes to the growing number of reports that RNA-seq can be used as a very valuable tool in ecotoxicology, and it can be expected to replace microarrays as the dominant technology used for transcriptomics in the future.

7.3 Relevance and future research

The capacity of the Hayle brown trout to survive lethal concentrations of metals in their environment is remarkable. This population is apparently healthy therefore the metal stress that these individuals incur must be within their physiological limits of tolerance. The transcriptome analysis conducted here sheds some light on the mechanisms employed to avoid the adverse effects usually caused by elevated metals. However, it is unclear whether this tolerance is due to physiological acclimation to metals induced by exposure during the individual's life-span or due to inherited genetic and/or epigenetic adaptation. Additionally, the Hayle trout may have developed behavioural strategies for avoidance of metal exposure. For example, we found some evidence that these fish had a preferential diet of terrestrial slugs rather than aquatic, presumably metal-contaminated, prey.

Some evidence of an inherent ability of individuals to acclimate to metal exposure has been demonstrated for several fish species in laboratory-based experiments (e.g. Xie and Klerk 2004, Gale et al. 2003). However, I believe it is more likely that inherited adaptation is responsible for the remarkable tolerance observed in the Hayle trout which have been exposed to elevated metal concentrations for thousands of years. In contrast, North American yellow perch populations that have been chronically exposed to metals for less than two hundred years show evidence of greater toxic effect (e.g. Pierron et al. 2011, Couture et al. 2003), perhaps because these fish have had considerably less time to adapt to metal stress than the Hayle brown trout. This hypothesis deserves further investigation and potential avenues to explore this include population genetics approaches. For example, SNP genotyping using high-throughput Rad-tag sequencing could be combined with transcriptomic data to investigate the hypothesis that genes positively selected for metal tolerance are also up-regulated in tolerant populations. Additionally, controlled laboratory experiments could be conducted to assess the genetic basis of metal tolerance, for example by testing whether successive generations of individuals originating from a metal-tolerant population, but raised in clean water, retain an ability to tolerate metals. Recent research also suggests that inherited epigenetic change may significantly contribute to tolerance of populations exposed to environmental stressors (e.g. Sahu et al. 2013). To investigate this, a high-throughput sequencing approach could be used to conduct genome-wide profiling of methylation and histone modifications in metal-tolerant fish.

Integration of this information with transcriptomic data would then allow investigation of the relationship between genetic adaptation, epigenetic regulation and transcription. Furthermore, it would also be interesting to investigate whether other populations of brown trout in similarly polluted rivers are also tolerant, and if so, whether the mechanisms of tolerance have similar physiological/genetic/epigenetic basis. This information is fundamental for the conservation and sustainable management of these, potentially unique, populations. The case of the Hayle trout investigated here provides a good example of the importance of considering the links between a population and its specific environment in terms of management approach, and hopefully will support the development of management strategies that take into consideration these relationships in order to more effectively protect fish populations .

Understanding the mechanisms of chemical toxicity is fundamental to effectively predict the potential adverse effects of environmental chemicals and, ultimately, elucidate how they may affect fish populations in isolation or as part of environmentally realistic mixtures. However, objectively interpreting changes in gene expression in terms of the potential effects at the population level is a considerable challenge. Changes in gene expression are not necessarily translated into direct changes at a protein and cellular level, and are also often influenced by complex feedback and compensatory effects at multiple levels of biological organisation. It is also difficult to directly associate the changes occurring following short-term laboratory studies with those occurring in chronically-exposed wild fish. As we found in Chapter 2, it is also inevitable that gene expression in natural fish populations will be affected by natural environmental variation, and this increases the challenge in identifying gene expression patterns associated with pollutant exposure and response.

To determine the most accurate mechanisms of toxicity of environmental chemicals and determine potential threats to wild fish populations, transcriptomic data can be combined with other biochemical, physiological and morphological effects. In terms of environmental relevance, isolated transcriptomic data has limitations, but in combination with these other endpoints it can be used to build a comprehensive signature of chemical exposure across multiple levels of biological organisation, and assess potential adverse health effects of environmental stressors on the health of individuals and populations.

Combination of data on the impact of chemicals on different levels of biological organisation forms the basis for construction of adverse outcome pathways (AOPs), which consist of a series of linked biological effects that are anchored by both specific molecular initiating events (i.e. chemical-molecular interactions) and adverse impacts at an individual and/or population level. AOPs can be constructed for groups of chemicals with similar mechanisms of toxicity, and this provides a framework that can be directly useful in ecological risk assessment and management (Ankley et al 2010). Transcriptomic data is of central importance in the construction of AOPs. This includes reverse engineering approaches which use transcriptomic data to construct networks of connected genes that are differentially regulated in response to chemical exposure (Perkins et al. 2011). The incorporation of microarray data into AOP construction has been well established. In the future there is likely to be increased incorporation of RNA-seq data into AOP frameworks, although this is also likely be associated with some challenges including integration of RNA-seq count data with existing data and also the adaptation of existing, well established pipelines for network and pathway construction.

This data on the global transcriptomic changes following exposure to three diverse chemicals may be used directly in populating existing AOPs with data specific for the brown trout, a very sensitive environmentally relevant species, or to construct novel AOPs. In particular, if the hypothesis that linuron causes down-regulation of the cholesterol biosynthesis through anti-androgenic signalling is confirmed, this may constitute the basis of a novel AOP in fish.

Natural fish populations are typically exposed to a complex mixture of chemicals, and other environmental stressors, which can act via similar or interacting mechanisms. Establishing comprehensive transcriptomic signatures, which are firmly anchored in AOPs, for groups of stressors that share similar mechanisms of toxicity is a key priority for environmental toxicology. This approach can ultimately be used to assess health of wild populations and determine the requirement for ecological management.

7.4 Conclusions

During the course of this PhD I have established the global transcriptomic response of brown trout to environmental chemicals that are likely to be amongst the most relevant to natural populations, providing the first comprehensive mechanistic analysis of toxicity in this species. These results, which include effects induced at environmentally relevant concentrations, may contribute to the risk assessment of the tested chemicals as environmental pollutants and their potential contribution as chemical stressors within the complex mixtures present in surface waters. This data has the potential to inform regulatory decisions governing their use and discharge into the environment, and promote monitoring programs measuring their occurrence in surface waters. For glyphosate, in particular, I hope that this data will help reinforce the importance of monitoring its presence in surface waters, which currently is not routinely conducted, despite the widespread usage of this pesticide.

I have conducted some of the first studies employing RNA-seq in ecotoxicology, and the results provide strong biological and technical evidence that this technology is a very valuable tool for conducting transcriptomics in this field. The successful application of RNA-seq to investigate mechanisms of chemical tolerance and toxicity in the brown trout has also demonstrated the potential utility of this technology for other important, environmentally relevant species, opening possibilities for wider research. Rapid development of RNA-seq is likely to continue in the next few years, further improving the potential of this technology. For example, 3rd generation sequencing is likely to significantly improve the quality of *de novo* transcriptome assemblies by reducing transcript redundancy. The importance of properly considering experimental design and optimising data analysis strategy in RNA-seq experiments is a key conclusion from this work, but this can be a challenge for those new to this field. It is fundamental that, alongside the development of sequencing technology and bioinformatics tools, efforts are developed to improve the accessibility of RNA-seq to biologists. The rapid advancement of sequencing and bioinformatics offers a significant opportunity for environmental scientists to embrace these new technological possibilities and address fundamental questions that will contribute to the protection of ecosystems subject to multiple stressors.

CHAPTER 8

References

Below is a list of references cited in Chapter 1 (introduction) and Chapter 7 (general discussion) only, i.e. not those already cited in the data chapters (2 to 6).

Almroth, B. C., J. Sturve, A. Berglund, and L. Farlin. 2005. Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquatic Toxicology **73**:171-180.

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., AU - Mount, David R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrrano, J. A., Tietge, J. E., and Villeneuve, D. L. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environmental Toxicology and Chemistry, **29**: 730-741.

Aranda, A., and A. Pascual. 2001. Nuclear hormone receptors and gene expression. Physiological reviews **81**:1269-1304.

Bainbridge, M., R. Warren, M. Hirst, T. Romanuik, T. Zeng, A. Go, A. Delaney, M. Griffith, M. Hickenbotham, and V. Magrini. 2006. Analysis of the prostate cancer cell line LNCaP transcriptome using a sequencing-by-synthesis approach. BMC Genomics **7**:246-252.

Basu, N., A. Todgham, P. Ackerman, M. Bibeau, K. Nakano, P. Schulte, and G. K. Iwama. 2002. Heat shock protein genes and their functional significance in fish. Gene **295**:173-183.

Bayley, M., M. Junge, and E. Baatrup. 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquatic Toxicology **56**:227-239.

Bayley, M., P. F. Larsen, H. Bakgaard, and E. Baatrup. 2003. The effects of vinclozolin, an anti-androgenic fungicide, on male guppy secondary sex characters and reproductive success. Biology of Reproduction **69**:1951-1956.

Belpaeme, K., K. Delbeke, L. Zhu, and M. Kirsch-Volders. 1996. Cytogenetic studies of PCB77 on brown trout (Salmo trutta fario) using the micronucleus test and the alkaline comet assay. Mutagenesis **11**:485-492.

Bjerregaard, L. B., C. Lindholst, B. Korsgaard, and P. Bjerregaard. 2008. Sex hormone concentrations and gonad histology in brown trout (Salmo trutta) exposed to 17 beta-estradiol and bisphenol A. Ecotoxicology **17**:252-263.

Black, M. B., B. B. Parks, L. Pluta, T.-M. Chu, B. C. Allen, R. D. Wolfinger, and R. S. Thomas. 2013. Comparison of Microarrays and RNA-seq for Gene Expression Analyses of Dose-Response Experiments. Toxicological Sciences *In Press.*

Blanchoud, H., E. Moreau-Guigon, F. Farrugia, M. Chevreuil, and J. Mouchel. 2007. Contribution by urban and agricultural pesticide uses to water contamination at the scale of the Marne watershed. Science of The Total Environment **375**:168.

Borg, B. 1994. Androgens in teleost fishes. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology **109**:219-245.

Brion, F., C. R. Tyler, X. Palazzi, B. Laillet, J. M. Porcher, J. Garric, and P. Flammarion. 2004. Impacts of 17Î²-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo-larval-, juvenile- and adult-life stages in zebrafish (Danio rerio). Aquatic Toxicology **68**:193-217.

Brzozowski, A. M., A. C. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engstram, L. Ahman, G. L. Greene, J.k. Gustafsson, and M. Carlquist. 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature **389**:753-758.

Bullard, J., E. Purdom, K. Hansen, and S. Dudoit. 2010. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinformatics **11**:94-102.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology **25**:169-193.

Cannon, J., E. Place, A. Eve, C. Bradshaw, A. Sesay, N. Morrell, and J. Smith. 2012. Global analysis of the haematopoietic and endothelial transcriptome during zebrafish development. Mechanisms of development **130**:122-131.

Carney Almroth, B., A. Johansson, L. Farlin, and J. Sturve. 2010. Early-age changes in oxidative stress in brown trout, *Salmo trutta*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **155**:442-448.

Cheshenko, K., F. Pakdel, H. Segner, O. Kah, and R. I. Eggen. 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. General and Comparative Endocrinology **155**:31-62.

Corton, J. C., and P. J. Lapinskas. 2005. Peroxisome proliferator-activated receptors: Mediators of phthalate ester-induced effects in the male reproductive tract? Toxicol. Sci. **83**:4-17.

Couture, P.; Rajender Kumar, P. 2003. Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (Perca f lavescens). Aquatic Toxicology **64**: 107−120.

Cowper-Sal, R., X. Zhang, J. B. Wright, S. D. Bailey, M. D. Cole, J. Eeckhoute, J. H. Moore, and M. Lupien. 2012. Breast cancer risk-associated SNPs modulate the affinity of chromatin for FOXA1 and alter gene expression. Nature Genetics **44**:1191-1198.

Denny, J. S., M. A. Tapper, P. K. Schmieder, M. W. Hornung, K. M. Jensen, G. T. Ankley, and T. R. Henry. 2005. Comparison of relative binding affinities of endocrine active compounds to fathead minnow and rainbow trout estrogen receptors. Environmental Toxicology and Chemistry **24**:2948-2953.

Desbrow, C., E. J. Routledge, G. C. Brighty, J. P. Sumpter, and M. Waldock. 1998. Identification of estrogenic chemicals in STW effluent.1. Chemical fractionation and in vitro biological screening Environ. Sci. Technol. **32**:1549-1558.

Di Giulio, R. T., and J. N. Meyer. 2008. Reactive Oxygen Species and Oxidative Stress. In The Toxicology of Fishes. Eds. Di Giulio, R. T. and Hinton, D. E. Taylor and Francis.

Elliott, J. M. 1994. Quantitative ecology and the brown trout. Oxford University Press.

Environment Agency. 2004. Our nations' fisheries - The migratory and freshwater fisheries of England and Wales - a snapshot.

Erickson, R. J., J. W. Nichols, P. M. Cook, and G. Ankley. 2008. Bioavailability of Chemical Contaminants in Aquatic Systems. In The Toxicology of Fishes. Eds. Di Giulio, R. T. and Hinton, D. E. Taylor & Francis.

Escher, B. I., and J. L. Hermens. 2002. Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. Environmental Science & Technology **36**:4201-4217.

Filby, A., and C. Tyler. 2007. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. Bmc Molecular Biology **8**:10-17.

Filby, A. L., K. L. Thorpe, G. Maack, and C. R. Tyler. 2007. Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced feminization in fish. Aquatic Toxicology **81**:219-231.

Forman, H. J., and M. Torres. 2002. Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. American journal of respiratory and critical care medicine **166**:S4-S8.

Fraser, B. A., C. J. Weadick, I. Janowitz, F. H. Rodd, and K. A. Hughes. 2011. Sequencing and characterization of the guppy (Poecilia reticulata) transcriptome. BMC Genomics **12**:202-213.

Fulton, M. H., and P. B. Key. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Environmental Toxicology and Chemistry **20**:37-45.

Gale, S. A.; Smith, S. V.; Lim, R. P.; Jeffree, R. A.; Petocz, P. 2003. Insights into the mechanisms of copper tolerance of a population of black-banded rainbowfish (Melanotaenia nigrans) (Richardson) exposed to mine leachate, using 64/67Cu. Aquatic Toxicology **62:** 135−153.

Garcia-Reyero, N., R. J. Griffitt, L. Liu, K. J. Kroll, W. G. Farmerie, D. S. Barber, and N. D. Denslow. 2008. Construction of a robust microarray from a non-model species largemouth bass, Micropterus salmoides, using pyrosequencing technology. Journal of fish biology **72**:2354-2376.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, and Q. Zeng. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology **29**:644-652.

Gunnarsson, L., E. Kristiansson, L. Farlin, O. Nerman, and D. J. Larsson. 2007. Sensitive and robust gene expression changes in fish exposed to estrogen - “a microarray approach. BMC Genomics **8**:149-154.

Hahn, M. E., and E. V. Hestermann. 2008. Receptor-Mediated Mechanisms of Toxicity. In The Toxicology of Fishes. Eds. Di Giulio, R. T. and Hinton, D. E. Taylor and Francis.

Halliwell, B., and J. M. C. Gutteridge. 2007. Free Radicals in Biology and Medicine. Oxford University Press.

Hansen, B. H., Ã. A. Garmo, P. A. Olsvik, and R. A. Andersen. 2007. Gill metal binding and stress gene transcription in brown trout (Salmo trutta) exposed to metal environments: The effect of pre-exposure in natural populations. Environmental Toxicology and Chemistry **26**:944-953.

Hansen, B. H., S. Romma, L. I. R. Softeland, P. A. Olsvik, and R. A. Andersen. 2006. Induction and activity of oxidative stress-related proteins during waterborne Cu-exposure in brown trout (Salmo trutta). Chemosphere **65**:1707-1714.

Harries, J. E., D. A. Sheahan, S. Jobling, P. Matthiessen, P. Neall, E. J. Routledge, R. Rycroft, J. P. Sumpter, and T. Tylor. 1996. A survey of estrogenic activity in United Kingdom inland waters. Environmental Toxicology and Chemistry **15**:1993-2002.

Harris, C. A., P. Henttu, M. G. Parker, and J. P. Sumpter. 1995. The estrogenic activity of phthalate esters In Vitro. Environ. Health Perspect. **105**:802-811.

Hayes, J. D., and L. I. McLellan. 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free radical research **31**:273-300.

Hinton, D. E., S. W. Kullman, R. C. Hardman, D. C. Volz, P.-J. Chen, M. Carney, and D. C. Bencic. 2005. Resolving mechanisms of toxicity while pursuing ecotoxicological relevance? Marine Pollution Bulletin **51**:635-648.

Holbech, H., K. D. Schrader, M. L. Nielsen, N. Brande-Lavridsen, B. F. Holbech, and P. Bjerregaard. 2013. Estrogenic Effect of the Phytoestrogen Biochanin A in Zebrafish, *Danio rerio*, and Brown Trout, *Salmo trutta*. Aquatic Toxicology *In press*.

Jargensen, A., O. Andersen, P. Bjerregaard, and L. J. Rasmussen. 2007. Identification and characterisation of an androgen receptor from zebrafish *Danio rerio*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **146**:561-568.

Jobling, S., N. Beresford, M. Nolan, R. Rodgers-Gray, C. R. Tyler, and J. P. Sumpter. 2002a. Altered sexual maturation and gamete production in wild roach Rutilus rutilus living in rivers that receive treated sewage effluents. Biol. Reprod. **66**:515-524.

Jobling, S., R. W. Burn, K. Thorpe, R. Williams, and C. Tyler. 2009. Statistical Modeling Suggests that Antiandrogens in Effluents from Wastewater Treatment Works Contribute to Widespread Sexual Disruption in Fish Living in English Rivers. Environmental Health Perspectives **117**:797-802.

Jobling, S., S. Coey, W. G. Whitmore, D. E. Kime, K. J. W. Van Look, B. G. McAllister, N. Beresford, A. C. Henshaw, G. C. Brighty, C. R. Tyler, and J. P. Sumpter. 2002b. Wild Intersex Roach Rutilis rutilis have reduced fertility. Biol. Reprod. **67**:515-524.

Jobling, S., M. Nolan, C. R. Tyler, G. C. Brighty, and J. P. Sumpter. 1998. Widespread Sexual Disruption in Wild Fish. Environ. Sci. Technol. **32**:2498-2506.

Kalmar, B., and L. Greensmith. 2009. Induction of heat shock proteins for protection against oxidative stress. Advanced drug delivery reviews **61**:310-318.

Kapushesky, M., T. Adamusiak, T. Burdett, A. Culhane, A. Farne, A. Filippov, E. Holloway, A. Klebanov, N. Kryvych, and N. Kurbatova. 2012. Gene Expression Atlas update-a value-added database of microarray and sequencing-based functional genomics experiments. Nucleic Acids Research **40**:D1077-D1081.

Kelly, M. A., A. M. Reid, K. M. Quinn-Hosey, A. M. Fogarty, J. J. Roche, and C. A. Brougham. 2010. Investigation of the estrogenic risk to feral male brown trout (Salmo trutta) in the Shannon International River Basin District of Ireland. Ecotoxicology and Environmental Safety **73**:1658-1665.

Khan, I. A., and P. Thomas. 2001. Disruption of neuroendocrine control of luteinizing hormone secretion by Aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. Biology of Reproduction **64**:955-964.

Kidd, K. A., P. J. Blanchfield, K. H. Mills, V. P. Palace, R. E. Evans, J. M. Lazorchak, and R. W. Flick. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences **104**:8897-8903.

Kishi, K., E. Kitagawa, N. Onikura, A. Nakamura, and H. Iwahashi. 2006. Expression analysis of sex-specific and 17b-estradiol-responsive genes in the Japanese medaka, *Oryzias latipes*, using oligonucleotide microarrays. Genomics **88**:241-251.

Klemetsen, A., P. A. Amundsen, J. Dempson, B. Jonsson, N. Jonsson, M. O'connell, and E. Mortensen. 2003. Atlantic salmon Salmo salar L., brown trout Salmo trutta L. and Arctic charr Salvelinus alpinus (L.): a review of aspects of their life histories. Ecology of freshwater fish **12**:1-59.

Körner, O., E. L. M. Vermeirssen, and P. Burkhardt-Holm. 2005. Intersex in feral brown trout from Swiss midland rivers. Journal of fish biology **67**:1734-1740.

Laetz, C. A., D. H. Baldwin, T. K. Collier, V. Hebert, J. D. Stark, and N. L. Scholz. 2009. The synergistic toxicity of pesticide mixtures: implications for risk assessment and the conservation of endangered Pacific salmon. Environmental Health Perspectives **117**:348-354.

Lahnsteiner, F., B. Berger, M. Kletzl, and T. Weismann. 2005. Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, Salmo trutta f. fario. Aquatic Toxicology **75**:213-224.

Lange, A., Y. Katsu, R. Ichikawa, G. C. Paull, L. L. Chidgey, T. S. Coe, T. Iguchi, and C. R. Tyler. 2008. Altered Sexual Development in Roach (Rutilus rutilus) Exposed to Environmental Concentrations of the Pharmaceutical 17{alpha}-Ethinylestradiol and Associated Expression Dynamics of Aromatases and Estrogen Receptors. Toxicol. Sci. **106**:113-123.

Lettieri, T. 2006. Recent applications of DNA microarray technology to toxicology and ecotoxicology. Environmental Health Perspectives **114**:4-9.

Leusch, F., and D. L. MacLatchy. 2003. In vivo implants of β-sitosterol cause reductions of reactive cholesterol pools in mitochondria isolated from gonads of male goldfish (*Carassius auratus*). General and Comparative Endocrinology **134**:255-263.

Liu, S., Z. Zhou, J. Lu, F. Sun, S. Wang, H. Liu, Y. Jiang, H. Kucuktas, L. Kaltenboeck, and E. Peatman. 2011. Generation of genome-scale gene-associated SNPs in catfish for the construction of a high-density SNP array. BMC Genomics **12**:53-59.

Lu, B., Z. Zeng, and T. Shi. 2013. Comparative study of de novo assembly and genome-guided assembly strategies for transcriptome reconstruction based on RNA-Seq. Science China Life Sciences **56**:143-155.

Mardis, E. 2008. The impact of next-generation sequencing technology on genetics. Trends Genet **24**:133 - 141.

Martindale, J. L., and N. J. Holbrook. 2002. Cellular response to oxidative stress: Signaling for suicide and survival*. Journal of cellular physiology **192**:1-15.

McCarthy, D. J., Y. Chen, and G. K. Smyth. 2012. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Research **40**:4288-4297.

McDonnell, D. P. 2003. Mining the complexities of the estrogen signaling pathways for novel therapeutics. Endocrinology **144**:4237-4240.

McGettigan, P. A. 2013. Transcriptomics in the RNA-seq era. Current opinion in chemical biology **17**:4-11.

Mehinto, A. C., C. J. Martyniuk, D. J. Spade, and N. D. Denslow. 2012. Applications for next-generation sequencing in fish ecotoxicogenomics. Frontiers in genetics

Menuet, A., E. Pellegrini, I. Anglade, O. Blaise, V. Laudet, O. Kah, and F. Pakdel. 2002. Molecular characterization of three estrogen receptor forms in zebrafish:

binding characteristics, transactivation properties, and tissue distributions. Biology of Reproduction **66**:1881-1892.

Milla, S., S. Depiereux, and P. Kestemont. 2011. The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology **20**:305-319.

Moens, L. N., K. van der Ven, P. Van Remortel, J. Dela Favero, and W. De Coen. 2007. Gene expression analysis of estrogenic compounds in the liver of common carp (Cyprinus carpio) using a custom cDNA microarray. Journal of biochemical and molecular toxicology **21**:299-311.

Molony, B. 2001. Environmental requirements and tolerances of rainbow trout (Oncorhynchus mykiss) and brown trout (Salmo trutta) with special reference to Western Australia: a review. Fisheries Research Division.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature methods **5**:621-628.

Nagalakshmi, U., Z. Wang, K. Waern, C. Shou, D. Raha, M. Gerstein, and M. Snyder. 2008. The transcriptional landscape of the yeast genome defined by RNA sequencing. Science **320**:1344-1349.

Nagler, J., T. Cavileer, J. Sullivan, and D. Cyr. 2007. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERalpha2 and both ERbeta isoforms. Gene **392**:164-173.

Nash, J. P., D. E. Kime, L. T. M. Van der Ven, P. W. Wester, F. Brion, G. Maack, P. Stahlschmidt-Allner, and C. R. Tyler. 2004. Longterm exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish Environmental Health Perspectives **112**:1725-1733.

Nuwaysir, E. F., M. Bittner, J. Trent, J. C. Barrett, and C. A. Afshari. 1999. Microarrays and toxicology: the advent of toxicogenomics. Molecular carcinogenesis **24**:153-159.

Panter, G. H., R. S. Thompson, and J. P. Sumpter. 1998. Adverse reproductive effects in male fathead minnows (Pimephales promelas) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone. Aquatic Toxicology **42**:243-253.

Perkins, E. J., Chipman, J. K., Edwards, S., Habib, T., Falciani, F., Taylor, R., Van Aggelen, G., Vulpe, C., Antczak, P. and Loguinov, A. 2011. Reverse

engineering adverse outcome pathways. Environmental Toxicology and Chemistry, **30**: 22-38.

Pierron, F., E. Normandeau, M. Defo, P. Campbell, L. Bernatchez, and P. Couture. 2011. Effects of chronic metal exposure on wild fish populations revealed by high-throughput cDNA sequencing. Ecotoxicology **20**:1388-1399.

Pope, C. N. 1999. Organophosphorus pesticides: do they all have the same mechanism of toxicity? Journal of Toxicology and Environmental Health Part B: Critical Reviews **2**:161-181.

Racke, K. 2003. Release of pesticides into the environment and initial concentrations in soil, water, and plants. Pure and Applied Chemistry **75**:1905-1916.

Rand, G. M., P. Wells, and L. S. McCarty. 1995. Introduction to Aquatic Toxicology. In The Toxicology of Fishes. Eds. Di Giulio, R. T. and Hinton, D. E. Taylor & Francis.

Rapaport, F., R. Khanin, Y. Liang, M. Pirun, A. Krek, P. Zumbo, C. E. Mason, N. D. Socci, and D. Betel. 2013. Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. Genome biology: *In press*.

Ren, S., Z. Peng, J.-H. Mao, Y. Yu, C. Yin, X. Gao, Z. Cui, J. Zhang, K. Yi, and W. Xu. 2013. RNA-seq analysis of prostate cancer in the Chinese population identifies recurrent gene fusions, cancer-associated long noncoding RNAs and aberrant alternative splicings. Cell research **22**:806-821.

Rhee, J.-S., B.-M. Kim, R.-O. Kim, B.-S. Choi, I.-Y. Choi, Y.-M. Lee, and J.-S. Lee. 2011. Analysis of expressed sequence tags from the liver and ovary of the euryhaline hermaphroditic fish, Kryptolebias marmoratus. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics **6**:244-255.

Robertson, G., J. Schein, R. Chiu, R. Corbett, M. Field, S. D. Jackman, K. Mungall, S. Lee, H. M. Okada, and J. Q. Qian. 2010. De novo assembly and analysis of RNA-seq data. Nature methods **7**:909-912.

Rothberg, J. M., and J. H. Leamon. 2008. The development and impact of 454 sequencing. Nature Biotechnology **26**:1117-1124.

Russom, C. L., S. P. Bradbury, S. J. Broderius, D. E. Hammermeister, and R. A. Drummond. 1997. Predicting modes of toxic action from chemical structure: Acute toxicity in the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry **16**:948-967.

Sahu, P. P., Pandey, G., Sharma, N., Puranik, S., Muthamilarasan, M, and Prasad, M, 2013. Epigenetic mechanisms of plant stress responses and adaptation. Plant Cell reports **32:** 1151-1159.

Sandmann, T., M. C. Vogg, S. Owlarn, M. Boutros, and K. Bartscherer. 2011. The head-regeneration transcriptome of the planarian Schmidtea mediterranea. Genome biology **12**:R76.

Santos, E. M., J. S. Ball, T. D. Williams, H. Wu, F. Ortega, R. van Aerle, I. Katsiadaki, F. Falciani, M. R. Viant, J. K. Chipman, and C. R. Tyler. 2010. Identifying Health Impacts of Exposure to Copper Using Transcriptomics and Metabolomics in a Fish Model. Environmental Science & Technology **44**:820-826.

Schirmer, K., B. B. Fischer, D. J. Madureira, and S. Pillai. 2010. Transcriptomics in ecotoxicology. Analytical and Bioanalytical Chemistry **397**:917-923.

Schubert, S., A. Peter, R. Burki, R. Schonenberger, M. J. F. Suter, H. Segner, and P. Burkhardt-Holm. 2008. Sensitivity of brown trout reproduction to long-term estrogenic exposure. Aquatic Toxicology **90**:65-72.

Schultz, I. R., A. Skillman, J.-M. Nicolas, D. G. Cyr, and J. J. Nagler. 2003. Short-term exposure to 17α-ethynylestradiol decreases the fertility of sexually maturing male rainbow trout (Oncorhynchus mykiss). Environmental Toxicology and Chemistry **22**:1272-1280.

Schultz, T. W. 1989. Nonpolar narcosis: a review of the mechanism of action for baseline aquatic toxicity. Aquatic toxicology and hazard assessment **12**:104-109.

Schulz, M. H., D. R. Zerbino, M. Vingron, and E. Birney. 2012. Oases: Robust de novo RNA-seq assembly across the dynamic range of expression levels. Bioinformatics.

Sebire, M., Y. Allen, P. Bersuder, and I. Katsiadaki. 2008. The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. Aquatic Toxicology **90**:37-47.

Shah, S. P., A. Roth, R. Goya, A. Oloumi, G. Ha, Y. Zhao, G. Turashvili, J. Ding, K. Tse, and G. Haffari. 2012. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature **486**:395-399.

Shappell, N. W., K. H. Elder, and M. West. 2010. Estrogenicity and nutrient concentration of surface waters surrounding a large confinement dairy

operation using best management practices for land application of animal wastes. Environmental Science & Technology **44**:2365-2371.

Sharon, D., H. Tilgner, F. Grubert, and M. Snyder. 2013. A single-molecule long-read survey of the human transcriptome. Nat Biotech **31**:1009-1014.

Sheader, D. L., T. D. Williams, B. P. Lyons, and J. K. Chipman. 2006. Oxidative stress response of European flounder (Platichthys flesus) to cadmium determined by a custom cDNA microarray. Marine Environmental Research **62**:33-44.

Shore, L. S., and M. Shemesh. 2003. Naturally produced steroid hormones and their release into the environment. Pure and Applied Chemistry **75**:1859-1871.

Simpson, J. T., K. Wong, S. D. Jackman, J. E. Schein, S. J. Jones, and Ä. n. Birol. 2009. ABySS: a parallel assembler for short read sequence data. Genome Research **19**:1117-1123.

Smith, K. E., S. N. Schmidt, N. Dom, R. Blust, M. Holmstrup, and P. Mayer. 2013a. Baseline Toxic Mixtures of Non-Toxic Chemicals:Solubility Addition Increases Exposure for Solid Hydrophobic Chemicals. Environmental Science & Technology **47**:2026-2033.

Smith, S., L. Bernatchez, and L. B. Beheregaray. 2013b. RNA-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species. BMC Genomics **14**:375-383.

Sohoni, P., C. R. Tyler, K. Hurd, J. Caunter, M. J. Hetheridge, T. Williams, C. Woods, M. Evans, R. Toy, M. Gargas, and J. P. Sumpter. 2001. Reproductive effects of long-term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. **35**:2917-2925.

Sumpter, J. P., and S. Jobling. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environmental Health Perspectives **103**:173.

Takeo, J., and S. Yamashita. 1999. Two distinct isoforms of cDNA encoding rainbow trout androgen receptors. Journal of Biological Chemistry **274**:5674-5680.

Thilagam, H., S. Gopalakrishnan, H.-D. Qu, J. Bo, and K.-J. Wang. 2011. 17b estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass. Ecotoxicology **19**:1258-1267.

Thomas, P. 2008. The Endocrine System. In The Toxicology of Fishes. Eds. Di Giulio, R. T. and Hinton, D. E. Taylor and Francis.

Thomas, P., Y. Zhu, and M. Pace. 2002. Progestin membrane receptors involved in the meiotic maturation of teleost oocytes: a review with some new findings. Steroids **67**:511-517.

Thorpe, K. L., R. I. Cummings, T. H. Hutchinson, M. Scholze, G. Brighty, J. P. Sumpter, and C. R. Tyler. 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environmental Science & Technology **37**:1142-1149.

Thorpe, K. L., T. H. Hutchinson, M. J. Hetheridge, M. Scholze, J. P. Sumpter, and C. R. Tyler. 2001. Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (Oncorhynchus mykiss). Environmental Science & Technology **35**:2476-2481.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn, and L. Pachter. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols **7**:562-578.

Trapnell, C., B. A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M. J. Van Baren, S. L. Salzberg, B. J. Wold, and L. Pachter. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology **28**:511-515.

Uren-Webster, T. M., C. Lewis, A. L. Filby, G. C. Paull, and E. M. Santos. 2010. Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish. Aquatic Toxicology ***99***:360-369.

van Aerle, R., N. Pounds, T. H. Hutchinson, S. Maddix, and C. R. Tyler. 2002. Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow Pimephales promelas. Ecotoxicology **11**:423-434.

Vera, J. C., C. W. Wheat, H. W. Fescemyer, M. J. Frilander, D. L. Crawford, I. Hanski, and J. H. Marden. 2008. Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. Molecular ecology **17**:1636-1647.

Vijay, N., J. W. Poelstra, A. Kanstner, and J. B. Wolf. 2013. Challenges and strategies in transcriptome assembly and differential gene expression quantification. A comprehensive in silico assessment of RNA-seq experiments. Molecular ecology **22**:620-634.

Walker, P. A., P. Kille, A. Hurley, N. R. Bury, and C. Hogstrand. 2008. An in vitro method to assess toxicity of waterborne metals to fish. Toxicology and Applied Pharmacology **230**:67-77.

Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews Genetics **10**:57-63.

Wezel, A. P. v., and A. Opperhuizen. 1995. Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity, mechanisms, and membrane burdens. CRC Critical Reviews in Toxicology **25**:255-279.

Whitehead, A., B. Dubansky, C. Bodinier, T. I. Garcia, S. Miles, C. Pilley, V. Raghunathan, J. L. Roach, N. Walker, and R. B. Walter. 2012. Genomic and physiological footprint of the Deepwater Horizon oil spill on resident marsh fishes. Proceedings of the National Academy of Sciences **109**:20298-20302.

Williams, T. D., H. Wu, E. M. Santos, J. Ball, I. Katsiadaki, M. M. Brown, P. Baker, F. Ortega, F. Falciani, and J. A. Craft. 2009. Hepatic transcriptomic and metabolomic responses in the stickleback (Gasterosteus aculeatus) exposed to environmentally relevant concentrations of dibenzanthracene. Environmental Science & Technology **43**:6341-6348.

Wysujack, K., L. Greenberg, E. Bergman, and I. Olsson. 2009. The role of the environment in partial migration: food availability affects the adoption of a migratory tactic in brown trout Salmo trutta. Ecology of freshwater fish **18**:52.

Xie, Y., G. Wu, J. Tang, R. Luo, J. Patterson, S. Liu, W. Huang, G. He, S. Gu, and S. Li. 2013. SOAPdenovo-Trans: De novo transcriptome assembly with short RNA-Seq reads. arXiv preprint arXiv:1305.6760.

Xie, L.; Klerks, P. L. 2004. Changes in cadmium accumulation as a mechanism for cadmium resistance in the least killifish Heterandria formosa. Aquatic Toxicology **66:** 73−81.

Young, G., M. Kusakabe, I. Nakamura, L. P.M., and F. W. Goetz. 2005. Gonadal steroidogenesis in teleost fish. *In* Hormones and their receptors in fish reproduction. (Molecular aspects of fish and marine biology; v.4). Eds. Melamed, P, Sherwood, N.

Zerbino, D. R., and E. Birney. 2008. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Research **18**:821-829.

Zhang, K., E.-B. Yang, W.-Y. Tang, K. P. Wong, and P. Mack. 1997. Inhibition of glutathione reductase by plant polyphenols. Biochemical Pharmacology **54**:1047-1053.

Zohar, Y., J. A. Munoz-Cueto, A. Elizur, and O. Kah. 2010. Neuroendocrinology of reproduction in teleost fish. General and Comparative Endocrinology **165**:438.

CHAPTER 9

Appendix

Aquatic Toxicology 99 (2010) 360–369

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Mechanisms of toxicity of di(2-ethylhexyl) phthalate on the reproductive health of male zebrafish

Tamsyn M. Uren-Webster, Ceri Lewis, Amy L. Filby, Gregory C. Paull, Eduarda M. Santos*

Hatherly Laboratories, School of Biosciences, University of Exeter, Prince of Wales Road, Exeter, Devon EX4 4PS, UK

ARTICLE INFO

Article history:
Received 22 February 2010
Received in revised form 17 May 2010
Accepted 19 May 2010

Keywords:
Estrogen
DEHP
Danio rerio
PPAR
Peroxisome proliferation
Testes

ABSTRACT

Phthalates are ubiquitous in the aquatic environment and are known to adversely affect male reproductive health in mammals through interactions with multiple receptor systems. However, little is known about the risks they pose to fish. This project investigated the effects of di(2-ethylhexyl) phthalate (DEHP), the most commonly used phthalate, on the reproductive health of male zebrafish (*Danio rerio*). Males were treated with 0.5, 50 and 5000 mg DEHP kg^{-1} (body weight) for a period of 10 days via intraperitoneal injection. The effects of the exposure were assessed by analysing fertilisation success, testis histology, sperm DNA integrity and transcript profiles of the liver and testis. A significant increase in the hepatosomatic index and levels of hepatic vitellogenin transcript were observed following exposure to 5000 mg DEHP kg^{-1}. Exposure to 5000 mg DEHP kg^{-1} also resulted in a reduction in fertilisation success of oocytes spawned by untreated females. However, survival and development of the resulting embryos were unaffected by all treatments, and no evidence of DEHP-induced sperm DNA damage was observed. Exposure to 50 and 5000 mg DEHP kg^{-1} caused alterations in the proportion of germ cells at specific stages of spermatogenesis in the testis, including a reduction in the proportion of spermatozoa and an increase in the proportion of spermatocytes, suggesting that DEHP may inhibit the progression of meiosis. In parallel, exposure to 5000 mg DEHP kg^{-1} increased the levels of two peroxisome proliferator-activated receptor (PPAR) responsive genes (*acyl-coenzyme A oxidase 1* (*acox1*) and *enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase* (*ehhadh*). These data demonstrated that exposure to high concentrations of DEHP disrupts spermatogenesis in adult zebrafish with a consequent decrease in their ability to fertilise oocytes spawned by untreated females. Furthermore, our data suggest that the adverse effects caused by exposure to DEHP are likely to occur preferentially via PPAR signalling pathways in the testis and oestrogen signalling pathways in the liver. We found no evidence of adverse effects on zebrafish reproductive health following exposure to the concentrations occurring in most aquatic systems, indicating that DEHP alone may not be a causative agent of the reproductive abnormalities seen in wildlife, at least as a result of short-term exposures.

1. Introduction

Phthalates are extensively used as plasticisers in many mass-produced products including food packaging, toys, electrical equipment, medical devices, paints and cosmetics (Jobling et al., 1995; Bauer and Herrmann, 1997). Global production is now over 4 million tonnes per year with the most widely used phthalate, di(2-ethylhexyl) phthalate (DEHP), accounting for at least a quarter of this production (Bauer and Herrmann, 1997; Petrovic et al., 2001). Phthalates are not chemically bound to plastic materials, and so are easily leached into the environment with time and use (Bauer and Herrmann, 1997). Phthalates also enter surface waters via waste-water treatment works effluents and from the atmosphere via plastic manufacture and burning (Jobling et al., 1995; Bauer and Herrmann, 1997; Staples et al., 1997). Although phthalates readily undergo microbial and abiotic degradation, and are therefore not persistent in the aquatic environment (Staples et al., 1997), continual release of large volumes means they are found very widely and often at substantial concentrations. DEHP is the most widespread phthalate in the aquatic environment which reflects its highest rate of production. The reported concentrations of DEHP are up to 100 $\mu g\,L^{-1}$ in surface waters and 200 mg kg^{-1} (wet weight) in sediments, although hotspots of contamination occur in heavily industrialised areas (Petrovic et al., 2001; Fromme et al., 2002). Fish are exposed to phthalates present in the water column and sediment, and also via their diet, and the concentration of DEHP in wild freshwater fish tissue ranges widely. For example, a comprehensive survey of DEHP in fish in Austrian rivers found concentrations ranging up to 1 mg kg^{-1} (wet weight) in most cases, and the maximum value measured

* Corresponding author. Tel.: +44 01392 264607; fax: +44 01392 263700.
E-mail address: e.santos@exeter.ac.uk (E.M. Santos).

0166-445X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2010.05.015

was 2.6 mg kg^{-1} in carp (*Cyprinus carpio*) (European Commission, 2003).

Phthalates are generally classed as oestrogenic chemicals due to their ability to bind and activate oestrogen receptors (ER) *in vitro* (Harris et al., 1995; Jobling et al., 1995; Takeuchi et al., 2005). However, *in vivo* studies have consistently reported that phthalates are extremely weak oestrogens in fish. For example, a concentration of 1500 mg DEHP kg^{-1} dosed via the diet was required to induce a small incidence of intersex in juvenile salmon (*Salmo salar*) (Norrgren et al., 1999; Norman et al., 2007), and 500 mg kg^{-1} (dosed via intraperitoneal injection) of butylbenzyl phthalate (BBP) was required to induce a 3-fold increase in vitellogenin (VTG) in male rainbow trout (*Oncorhynchus mykiss*) (Christiansen et al., 2000). Other studies have found no evidence that phthalates induce any oestrogenic effects at the concentrations tested (Harries et al., 2000; Metcalfe et al., 2001; Patyna et al., 2006).

Phthalates have been widely reported to disrupt the male reproductive system in fish. Dibutyl phthalate (DBP) and DEHP disrupted the activity of various enzymes involved in the metabolism and synthesis of testosterone in carp *in vitro* (Thibaut and Porte, 2004). A high concentration of diethyl phthalate (DEP) (>1 mg L^{-1}) induced testicular atrophy in carp (Barse et al., 2007). Recently, exposure to an environmentally relevant concentration of BBP (6 μg L^{-1}) was found to induce changes in sperm motility, an important component of male reproductive health, in the zebrafish (*Danio rerio*) (Oehlmann et al., 2009). The exact mechanisms by which phthalates induce these effects are not clear, and may involve interaction with multiple receptor systems including those responsible for mediating the effects of sex steroids and peroxisome proliferator-activated receptors (PPARs) (Lampen et al., 2003; Takeuchi et al., 2005).

Mammalian studies have provided considerable evidence that phthalates induce a range of reproductive effects in males including disruption of reproductive development, alteration of steroid hormone balance, testicular lesions and atrophy, disruption of spermatogenesis and infertility (Sharpe et al., 1995; Bhattacharya et al., 2005; Corton and Lapinskas, 2005; Howdeshell et al., 2008). These effects are distinct from an oestrogenic mechanism of action, but are also considered to be independent of the androgen receptor (AR) (Miura et al., 2007; Onorato et al., 2008; Pant et al., 2008). It is unlikely that one exclusive mechanism is responsible for the complex effects seen, but activation of PPARs is widely recognised as an important mechanism by which phthalates induce some of these effects (Bhattacharya et al., 2005; Corton and Lapinskas, 2005). Specifically, disruption of spermatogenesis by phthalates in mammals has been attributed to disturbance of oxidative balance in the testis, and this has been suggested to occur via PPAR activation (Kasahara et al., 2002; Corton and Lapinskas, 2005; Onorato et al., 2008), potentially resulting in lower sperm quality/quantity, fertilisation ability and embryo survival (Park et al., 2002; Agarwal and Said, 2005; Pant et al., 2008).

There is a significant lack of research investigating the effects of phthalates on the reproductive health of fish, particularly considering the multiple mechanisms of action of these environmentally relevant compounds. This information is essential to establish the mechanisms of toxicity of phthalates in lower vertebrates and will help to provide a more comprehensive understanding of the potential threat phthalates pose to the reproductive health of fish in the environment. In this study, we investigated the effects of exposure to a range of concentrations of DEHP, including those occurring in the aquatic environment, on the reproductive health of male zebrafish (*D. rerio*). Males were exposed via intraperitoneal injection and allowed to breed with untreated females for a period of 10 days. The effects of the exposure on spermatogenesis, fertilisation success and embryo survival were investigated, together with measurements of transcript profiles for 13 genes, to elucidate the pathways of disruption mediating the effects seen.

2. Materials and methods

2.1. Fish maintenance

Adult zebrafish (wild-type WIK strain, originally from the Max Planck Institute, Tubingen, Germany) were bred and maintained in the specialist zebrafish aquaria facility at the University of Exeter in 140 L mixed sex stock tanks before experimentation. The aquarium water supply was reverse-osmosis treated tap water reconstituted with analytical grade salts to produce a standardised synthetic freshwater, as described in Paull et al. (2008), the temperature was maintained at 28 ± 1 °C and the photoperiod was set at 12:12 h light:dark with a 30 min gradual transition period at dawn and dusk. Fish were fed with freshly hatched *Artemia nauplii* every morning and Tetramin tropical flake food (Tetra; Melle, Germany) every afternoon to satiation.

Sexually mature males and females were identified visually and allocated into colonies of two male and two female fish (2 × 2 colonies) which were allowed to breed naturally in 15 L glass aquaria. Each aquaria was aerated and supplied with a water flow rate of 48 L day^{-1}. In small colonies of zebrafish, dominance hierarchies typically develop between males, and aggression from dominant males can cause stress of subordinate males (Paull et al., 2008). To avoid this, in this study males and females in each colony were evenly matched in size to limit the chance of dominant individuals becoming overly aggressive. Spawning occurred daily at dawn, with all males and females within each tank breeding as a group, using artificial weed placed at the base of the tank as a spawning substrate. Eggs were collected from each tank 1 h post-fertilisation (hpf) before morning feeding, transferred to Petri dishes and maintained in tank water. Immediately after collection, eggs were washed with tank water to remove waste food and faeces and dead eggs were removed, while the remaining eggs were left to incubate in tank water at 28 °C. The number of fertilised and unfertilised eggs was determined at 3 hpf by visual inspection using light microscopy (Kyowa Optical SDZ PL, Kyowa Optical, Kanagawa, Japan). The unfertilised eggs were removed, while the fertilised eggs were left to incubate until 24 hpf, under the same conditions. Embryo mortalities were assessed and removed at 6, 8 and 24 hpf. Embryos were regularly monitored at 24 hpf, using light microscopy, to assess if normal development was occurring.

2.2. Pre-exposure

At the start of the experiment, 18 colonies were set up and allowed to acclimate for a period of 5 days. Following this initial acclimation, egg number, fertilisation success and embryo survival at 6, 8 and 24 hpf were monitored daily during a pre-exposure period of 10 days. Colony egg production tends to follow a number of patterns including consistent daily spawning, regular intermittent peaks in spawning, and irregular spawning including several days without spawning (Paull et al., 2008). The aim of this pre-exposure period was to select 16 colonies with consistent egg production and fertilisation success for use in the DEHP exposure.

2.3. Exposure experiment

Although exposure via water or diet is advocated as the most environmentally relevant route of phthalate exposure (Patyna et al., 2006; Norman et al., 2007), intraperitoneal injection was used to deliver DEHP in this study because it allowed males to be targeted, and the effects of DEHP on male reproduction to be specifically investigated, in isolation from possible effects on females. Additionally, dietary exposure does not guarantee that an equal dose is delivered to each fish, and there are significant practical difficulties in maintaining the required exposure concentration of DEHP

in water because of its low solubility in water and rapid degradation (Oehlmann et al., 2009). In this study, fish were injected with three concentrations of DEHP: an environmentally relevant dose of 0.5 mg DEHP kg^{-1} (body weight) which is within the range of values of DEHP measured in wild fish tissue reported in the literature (European Commission, 2003), an intermediate dose of 50 mg DEHP kg^{-1}, and an extremely high dose of 5000 mg DEHP kg^{-1} that is very unlikely to ever occur in the environment, but that was investigated to provide an opportunity to assess the mechanisms of phthalate toxicity in fish.

The amount of DEHP (Sigma–Aldrich, Poole, UK) injected into each male fish for each treatment was calculated based on an estimated average body mass of 0.5 g (based on measurements of a subset of fish). Male fish were injected with 10 μL of a carrier alone (olive oil; Sigma–Aldrich), or the same volume of carrier containing the appropriate amount of DEHP. All chemical solutions were freshly prepared immediately prior to injection.

The 16 colonies that spawned most consistently during the pre-exposure period were randomly allocated to the three DEHP treatment groups and to the control group (4 colonies per treatment). Males were injected into the intraperitoneal cavity using a 0.5 mL syringe (Monoject, Sherwood Davis and Geck, Missouri, U.S.) on day 1 and 5 of the experimental period. The number of fertilised embryos at 3 hpf, and the embryo survival at 6, 8 and 24 hpf, were assessed daily during the exposure period, as described above.

2.4. Fish sampling

On day 10 of the exposure period, all male fish were humanely sacrificed by a lethal dose of benzocaine (concentration 0.5 g L^{-1}; Sigma–Aldrich) in accordance with UK Home Office regulations. Wet weight and fork length were measured and used to calculate the condition factor (k = (wet weight (g) × 100)/(fork length $(cm)^3$)). The testes were dissected and one testis from each fish was divided equally for histological analysis and for assessment of sperm DNA damage (via a Comet assay). The remaining testis was snap frozen in liquid nitrogen and stored at −80 °C until analysis of transcript profiles. The liver was dissected and weighed (for determination of the hepatosomatic index; HSI = (liver weight (mg)/total weight (mg)) × 100)), and immediately snap frozen in liquid nitrogen and stored at −80 °C until analysis of transcript profiles.

2.5. DNA damage in germ cells

Evidence from mammalian and human studies indicates that phthalates induce oxidative stress in the testis and can cause subsequent adverse effects on sperm DNA integrity (Kasahara et al., 2002; Hauser et al., 2007; Pant et al., 2008). In this study, we hypothesised that exposure to DEHP might cause alterations in DNA integrity in the germ cells and assessed this by measuring DNA damage of testis cells using the Comet assay, at the end of the exposure. Fresh testis tissue was mixed with 100 μL aquarium water and gently disrupted to release the sperm. 10 μL of the cell suspension was then mixed with 1% low melting point agarose solution, warmed to 37 °C, then spread on slides coated with 1% high melting point agarose solution. The slides were covered and cooled to 4 °C for 10 min. The slides were then placed in a lysis solution (0.25 M NaCl, 0.1 M EDTA, 10 μM Trizma Base, 10% DMSO, 1% Triton X-100 (All Sigma–Aldrich), pH 10) for 1 h at 4 °C before incubation in proteinase k (Sigma–Aldrich) for 1 h at 35 °C. Cells were then denatured in an alkaline (pH 13) electrophoresis buffer (0.3 M NaOH, 1 mM EDTA (Sigma–Aldrich)) for 40 min before an electric current (25 V, 300 mA) was applied for 30 min. Cells were washed in neutralising buffer (0.4 M Trizma Base, pH 7.5) before staining with 20 μL of 20 mg L^{-1} ethidium bromide solution (Sigma–Aldrich). This alkaline Comet assay assessed both single- and double-stranded DNA breakage. Each sample was viewed with a fluorescence microscope (420–490 excitation filter; 520 nm emission filter) and the % tail DNA of 100 cells from each sample were quantified (Kinetic COMET software) and used as a measure of DNA damage.

2.6. Histological analysis

Immediately after extraction, half of one testis for each individual fish was fixed in Bouin's solution (Sigma–Aldrich) for 4 h, then washed and stored in 70% ethanol prior to histological analysis. Samples were dehydrated in 70–100% industrial methylated spirits (IMS) and ethanol (both Fisher Scientific, Loughborough, UK) using a Shandon (Citadel 2000) tissue processor, and then embedded in paraffin wax (Sigma–Aldrich). The embedded samples were cut into 5 μm sections (Leica RM 2125RTF, Leica Microsystems, Nussloch, Germany), floated in 30% IMS in a water bath at 45 °C, and then laid onto glass slides and dried on a 40 °C heat tray overnight. Sections were stained with Harris' non-acidified haematoxylin and Eosin Y (both Thermoshandon, Pittsburg, U.S.) and treated with Histomount (National Diagnostics, Hull, UK). Analysis was conducted with a light microscope (Zeiss Axioskop 40, Carl Zeiss, Oberkochen, Germany) connected to an Olympus DP70 camera (Olympus Optical) using analySIS image processing 3.2 software (Soft Imaging System, Munster, Germany). The percentage of cells at various stages of spermatogenesis (spermatogonia, spermatocytes, spermatids, and spermatozoa), as described by Maack and Segner (2003), was assessed by overlaying a grid onto the image generated and recording the cell type at each of the 100 intersections. The percentage of cells at each stage was calculated from two sections per fish.

2.7. Transcript profiling

Real-time quantitative PCR (RT-QPCR) was used to quantify the expression of 13 target genes: *oestrogen receptor 1*, *2a* and *2b* (*esr1*, *esr2a*, *esr2b*) and *androgen receptor* (*ar*), the nuclear receptors mediating sex steroid function; *cytochrome P450 11b* (*cyp11b*) and *cytochrome P450 17a* (*cyp17a*) which are involved in testosterone biosynthesis; *vitellogenin* (*vtg*), a egg yolk protein readily induced by oestrogens; *anti-Mullerian hormone* (*amh*), *septin 4* (*sept4*) and *cyclin g2* (*ccng2*) which are all over-expressed in the testis (Santos et al., 2007); and *acyl-coenzyme A oxidase 1* (*acox1*), *lipoprotein lipase* (*lpl*) and *enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase* (*ehhadh*) which are all regulated via the activation of PPAR receptors and involved in lipid metabolism (Mandard et al., 2004).

Primers for each target gene were designed with Beacon Designer 3.0 software (Premier Biosoft International, Paulo Alto, CA), purchased from MWG-Biotech (Ebersburg, Germany) and optimised for RT-QPCR as described previously (Filby and Tyler, 2005). Specificity of primer sets throughout the range of detection was confirmed by the observation of single amplification products of the expected size and T_m. All assays were quantitative, with standard curve (mean threshold cycle (C_t) vs. log cDNA dilution) slopes of between −2.856 and −3.722, translating to high efficiencies (E; $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2001) of 1.84–2.24. Over the detection range, the linear correlation (R^2) between the mean C_t and the logarithm of the cDNA dilution was >0.98 in each case. The sequences, PCR product sizes, annealing temperatures and PCR efficiencies for each primer pair are shown in Table 1.

RNA was extracted from testis and liver samples from individual male fish (n = 6–8 per treatment group) using TRI reagent (Sigma–Aldrich) according to the manufacturer's instructions. RNA concentration was estimated from absorbance at 260 nm and purity assessed from the 260 nm/280 nm absorbance ratio using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies,

Table 1
Technical information on the RT-QPCR assays employed.

Target gene		Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Ta (°C)	PCR efficiency
Ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	95.0%
Estrogen receptor 1	*esr1*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	104.0%
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	85.6%
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	118.0%
Vitellogenin	*vtg*	AGCAGCAGCAGTCGTAAC	CAATGATGGTGGCAGTCTTAG	148	57.5	84.0%
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	96.8%
Acyl-coenzyme A oxidase 1	*acox1*	ACAGCAGAGCAAGAGTAACG	TGAAGGGCATAAAGCAGAGC	177	60.0	104.0%
Enoyl-coenzyme A, hydratase/ 3-hydroxyacyl coenzyme A dehydrogenase	*ehhadh*	GGCAGGCAGATGGTGATTG	GGAGAACTTGAGGAGATTGAGG	84	60.0	105.0%
Lipoprotein lipase	*lpl*	CGCAGGAGCAGCAAGATG	GTTCAAAGTAGGCATAATGTAGGG	186	60.5	105.3%
Septin	*sept4*	GTGATGGGTGGTGTGTTTC	GAACGGTAGAAGTGGAGAATC	101	58.0	94.4%
Anti-Mullerian hormone	*amh*	TGTCTCAACCATCGTCTTCAG	CAGTCAATCCATCCATCCAAAC	124	61.0	123.9%
Cyclin G2	*ccng2*	TGGTTTGGAGCATTATTAGAGTC	GAGAGCGAGAGTGAGGATTC	144	59.5	103.7%
Cytochrome P450 11b	*cyp11b*	ACGCAGACACAGCAAAGG	CAGACGAGGACACCATCAC	95	59.0	96.0%
Cytochrome P450 17a	*cyp17a*	CGACAGTAAGATTGGGAAAGAAAG	GATGAGGAGCGGAGAAACAG	118	60.5	115.5%

Wilmington, USA). cDNA was synthesised according to manufacturer's instructions from 1 μg of total RNA treated with RQ1 DNase (Promega, Southampton, UK) using random hexamers (MWG-Biotech) and M-MLV reverse transcriptase (Promega). cDNA was diluted (1:2) then RT-QPCR was performed using an iCycler iQ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) with SYBR Green chemistry as described by Filby and Tyler (2005). A template-minus negative control was run in triplicate on each plate to verify the absence of cDNA contamination. Efficiency-corrected relative expression levels were determined as in (Filby and Tyler, 2005) by normalizing to the well established 'housekeeping' gene ribosomal protein l8 (*rpl8*), whose expression is unaffected by oestrogen exposure (Filby et al., 2007), which was measured in each sample. For some transcripts, amplification of the specific fragments was not detected in some samples and those data points were excluded from the analysis. All outliers in transcript expression data for each gene were identified and removed according to Chauvenet's criterion (Chauvenet, 1863), then new mean and standard deviation values were calculated before statistical analysis.

2.8. Statistical analysis

All data was analysed using SigmaStat (version 2, Jandel Scientific Software, U.S.). When data met the assumptions of normal distribution and equal variance required for the application of parametric tests, comparisons between treatment groups were performed using one-way analysis of variance (ANOVA), followed by the Fisher LSD post hoc test. For data that failed to meet these assumptions, the Kruskal–Wallis one-way ANOVA on ranks was employed followed by the appropriate post hoc test (Dunn's method or Tukey test). Additionally, for transcript profiles, *t*-tests, or Mann–Whitney rank sum test when data was not normally distributed or did not have equal variance, were employed to determine differences between the control group and each DEHP treatment group. The *P* values were adjusted for multiple tests using Bonferroni's corrections. Results were considered statistically significant at $P < 0.05$. Throughout this paper data are presented as mean ± standard error of the mean.

3. Results

3.1. Morphometric parameters

During the course of the experiment, there were five mortalities amongst male fish. The deaths occurred randomly across treatment groups, and all were within 24 h of an injection, suggesting that they occurred as result of the injection process. There were no colonies where both males died. During the experiment, there were no obvious alterations in swimming or feeding behaviour in any of the treatment groups.

The weight of all surviving male fish was 0.44 ± 0.13 g and length was 34.04 ± 0.38 mm ($n = 27$). There was no significant difference in condition factor between fish in any of the treatment groups (Fig. 1). The HSI of the male fish in the 5000 mg DEHP kg^{-1} group was significantly higher than that of fish in both the olive oil control and 0.5 mg DEHP kg^{-1} groups (Fig. 1).

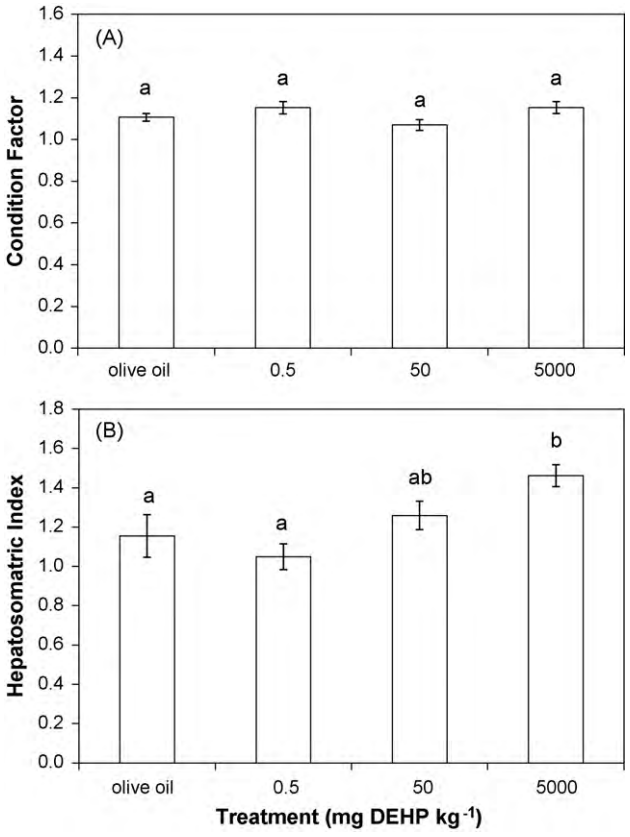


Fig. 1. Morphometric parameters in male fish following exposure to DEHP for 10 days ($n = 6$–8 per treatment group): A – condition factor and B – hepatosomatic index. Different letters indicate significant differences between treatment groups ($P < 0.05$).

3.2. Egg production and fertilisation success during the pre-exposure period

During the 10-day pre-exposure period, egg production and fertilisation in all colonies followed the patterns reported in Paull et al. (2008) for breeding zebrafish under our laboratory conditions. The majority of colonies spawned on a daily basis. Based on these data, the 2 colonies showing the lowest rate of egg production and/or the most inconsistent spawning patterns were removed from the experiment. The remaining 16 colonies selected for use in the exposure experiment spawned regularly and had consistent fertilisation success, allowing us to determine the ability of the sperm produced by the treated males to fertilise the eggs spawned by untreated females.

3.3. Effects of exposure to DEHP on fertilisation success and embryo survival

The mean number of eggs spawned by each colony per day, over the 10-day exposure period, was 53 ± 8.31; 45 ± 4.63; 27 ± 5.16; and 65 ± 8.23 for the olive oil control, 0.5, 50 and 5000 mg DEHP kg^{-1} groups, respectively. Egg output in the 50 mg DEHP kg^{-1} group was significantly lower than in the 5000 mg DEHP kg^{-1} group, but it was not significantly different from the olive oil control group. This result was in line with the egg production for the same colonies during the pre-exposure period and reflected normal variation in zebrafish spawning patterns (Paull et al., 2008). Females were not subjected to any treatment in this study, and therefore this variability did not reflect any effects of the chemical on female reproduction.

The fertilisation success for colonies where males were injected with olive oil alone was $82.71 \pm 1.88\%$. Exposure of males to 5000 mg DEHP kg^{-1} resulted in a decrease in fertilisation success compared to all other treatment groups when considering the 10-day exposure period as a whole ($P < 0.001$), but there was no change in any other treatment group (Fig. 2). Further analysis of the data showed that in the first 5 days of the exposure there was no significant difference in fertilisation success between any of the treatment groups, but the fertilisation success decreased significantly during the second 5-day period in colonies where males were exposed to 5000 DEHP kg^{-1} ($P < 0.001$). Correspondingly, the fertilisation success in colonies where males were exposed to 5000 mg DEHP kg^{-1}

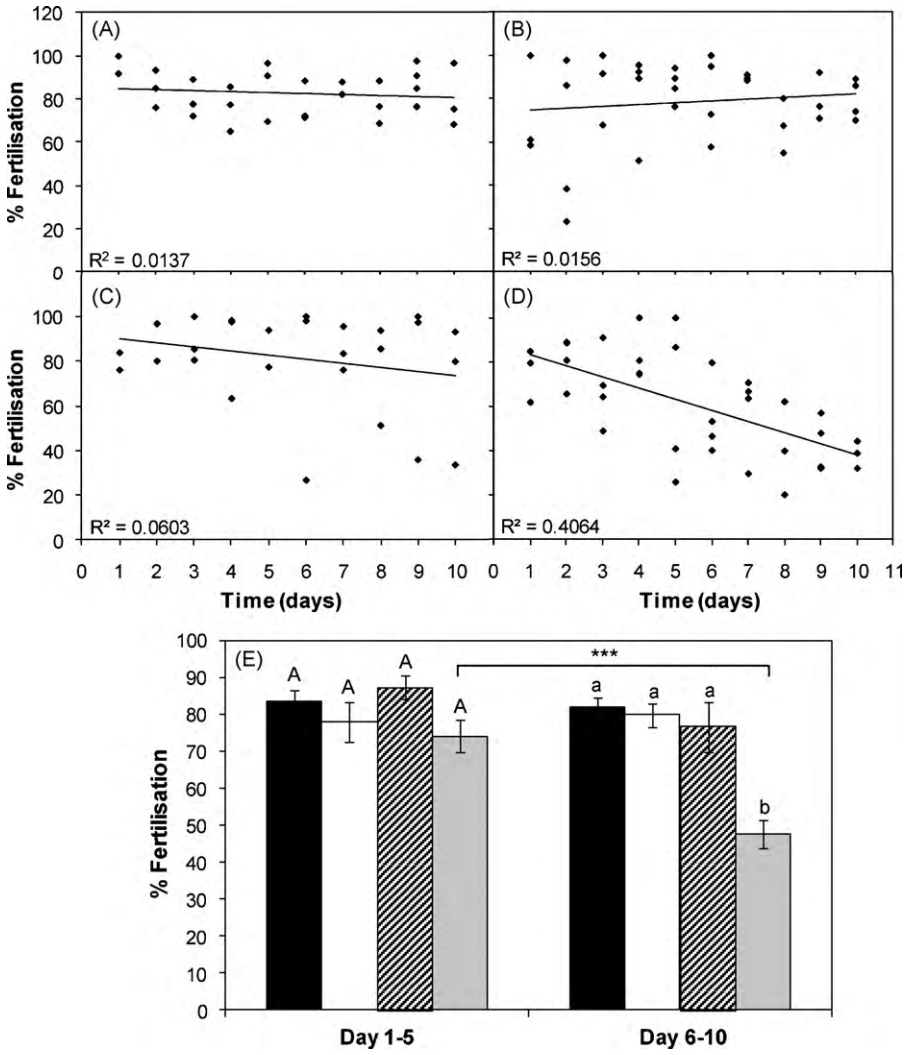


Fig. 2. Fertilisation success over time for colonies where males were injected with olive oil alone (A), or 0.5 (B), 50 (C) and 5000 (D) mg DEHP kg^{-1} (each symbol represents the daily % fertilisation for a single colony; $n = 4$ colonies per treatment group) E – the mean percentage of fertilisation for each treatment group on days 1–5 and days 6–10. Black bars represent olive oil, clear bars represent 0.5 mg DEHP kg^{-1}, hatched bars represent 50 mg DEHP kg^{-1} and grey bars represent 5000 mg DEHP kg^{-1}. Different upper case letters indicate significant differences between treatment groups for days 1–5, and different lower case letters indicate significant differences between treatment groups for days 6–10 ($P < 0.05$). Asterisks indicate a significant difference between time periods for the same treatment group ($^{***}P < 0.001$).

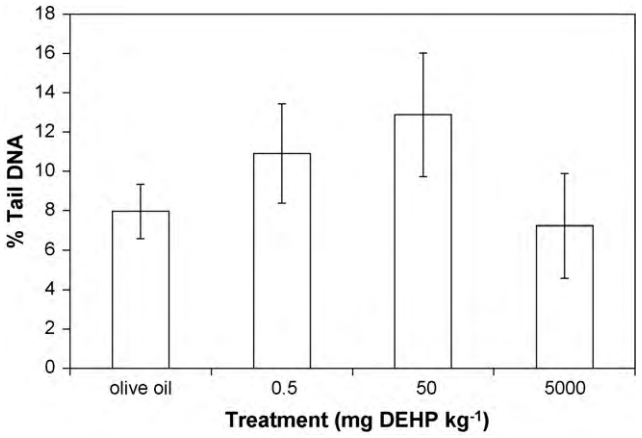


Fig. 3. DNA damage (expressed as % of DNA in the Comet tails) in germ cells extracted from the testes of all males included in this study (n = 6–8 males per treatment group).

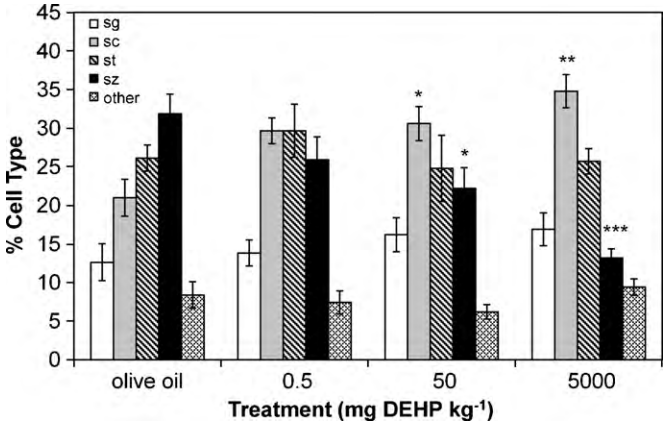


Fig. 4. Proportion of cells at each stage of spermatogenesis (spermatogonia (sg); spermatocytes (sc); spermatids (st); spermatozoa (sz) and other) in the testes of zebrafish in each of the treatment groups (n = 6–8 males per treatment group). Asterisks indicate significant differences compared to the control group, for each cell ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

was significantly lower during days 6–10 compared to days 1–5 ($P < 0.001$), but there were no significant differences between these two time periods for any of the other treatment groups (Fig. 2).

The percentage of embryo survival for all colonies at 6, 8 and 24 hpf was 99.30 ± 0.26, 98.68 ± 0.39 and 96.60 ± 0.47%, respectively. There was no significant difference in embryo survival at 6, 8 or 24 hpf between any of the treatment groups. In addition, no abnormal embryo development was observed at 24 hpf.

3.4. DNA damage in germ cells

The DNA damage measured in this study was based on the mean percentage of DNA in the tail (tail intensity) for the 100 germ cells scored, as reported by Lewis and Galloway (2009). This is a measure of double- and single-strand breaks. The percentage tail DNA ranged between 7.24 ± 2.65 and 12.87 ± 3.14 across all treatments and there was no significant difference in DNA damage between treatment groups (Fig. 3).

3.5. Histological analysis

Histological analysis of the gonads confirmed that all the fish visually identified as males, and subjected to treatments, were sexually mature males. There was a significantly lower proportion of spermatozoa in the testes of males injected with 50 mg DEHP kg^{-1} ($P < 0.05$) and with 5000 mg DEHP kg^{-1} ($P < 0.001$) compared to those injected with olive oil alone. Conversely, there were significantly more spermatocytes in the testes of fish injected with 50 mg DEHP kg^{-1} ($P < 0.05$) and 5000 mg DEHP kg^{-1} ($P < 0.01$) compared to those injected with olive oil alone (Fig. 4).

3.6. Transcript profiling

The transcripts quantified in the liver were *esr1*, *esr2a*, *esr2b*, *ar*, *vtg*, *acox1*, *lpl* and *ehhadh*. Injection of 5000 mg DEHP kg^{-1} induced a significant increase in the concentration of *vtg* compared with injections with olive oil alone ($P < 0.05$) (Fig. 5A).

In the testis, the transcripts quantified were *esr2b*, *acox1*, *lpl*, *ehhadh*, *cyp11b*, *cyp17a*, *amh*, *sept4* and *ccng2*. Treatment with 5000 mg DEHP kg^{-1} resulted in a significant induction in the expression of *acox1* compared with those injected with olive oil alone ($P < 0.01$) and also a significant increase in the expression of *ehhadh* compared with those injected with 50 mg DEHP kg^{-1} ($P < 0.05$) (Fig. 5B).

4. Discussion

4.1. Effects of exposure to DEHP in the liver

There was a significant increase in HSI in fish injected with 5000 mg DEHP kg^{-1}, compared to fish injected with olive oil alone, despite the short-term duration of this study. This finding aligns with mammalian studies where increased liver weight following exposure to phthalates has been reported. For example, David et al. (1999) observed significantly elevated male rat and mouse liver weights following dietary exposure to 2500 and 3000 mg DEHP kg^{-1}, respectively. This effect has been attributed to an increase in the size and number of liver peroxisomes, which typically characterises PPAR activation (Reddy et al., 1980). Similarly, a number of studies in fish have found that phthalates induce effects consistent with PPAR activation. These include increased liver peroxisome proliferation in the absence of VTG induction (Ortiz-Zarragoitia and Cajaraville, 2005; Ortiz-Zarragoitia et al., 2006), and increased HSI (Barse et al., 2007), suggesting that these effects are independent of an oestrogenic mechanism of action. In order to investigate the potential for PPAR-regulated pathways to mediate the effects of the exposure to DEHP in the liver, in our study, we analysed the transcript profiles for three PPAR-regulated genes (*acox1*, *ehhadh* and *lpl*) in this organ. *acox1* has been the most common PPARα target gene used as an indicator of exposure to peroxisome proliferators (PPs), including phthalates, in mammalian studies (e.g. Bility et al., 2004). In fish, levels of the peroxisomal enzyme ACOX have been successfully used as an indicator of PPARα activation (Ortiz-Zarragoitia and Cajaraville, 2005; Ortiz-Zarragoitia et al., 2006), but expression analysis of the gene has never been conducted. *ehhadah* is a well established PPARα target gene and *lpl* is regulated by both PPARα and PPARγ activation (Mandard et al., 2004; Nakachi et al., 2008), but these genes have been less extensively used in mammals as markers of exposure to peroxisome proliferators, and never in fish. We found no evidence of increased hepatic expression of any of these three genes for any of the concentrations tested. This does not exclude the potential for PPAR receptors to mediate some of the hepatic response of male zebrafish to DEHP, but indicates that this may not be the main mechanism of toxicity of this phthalate in the liver of male zebrafish during this short-term exposure.

In addition to the PPAR-regulated genes, we analysed the transcript profiles of five genes involved in oestrogen and androgen signalling pathways (*esr1*, *esr2a*, *esr2b*, *vtg* and *ar*), to search for evidence of the involvement of these signalling pathways in the

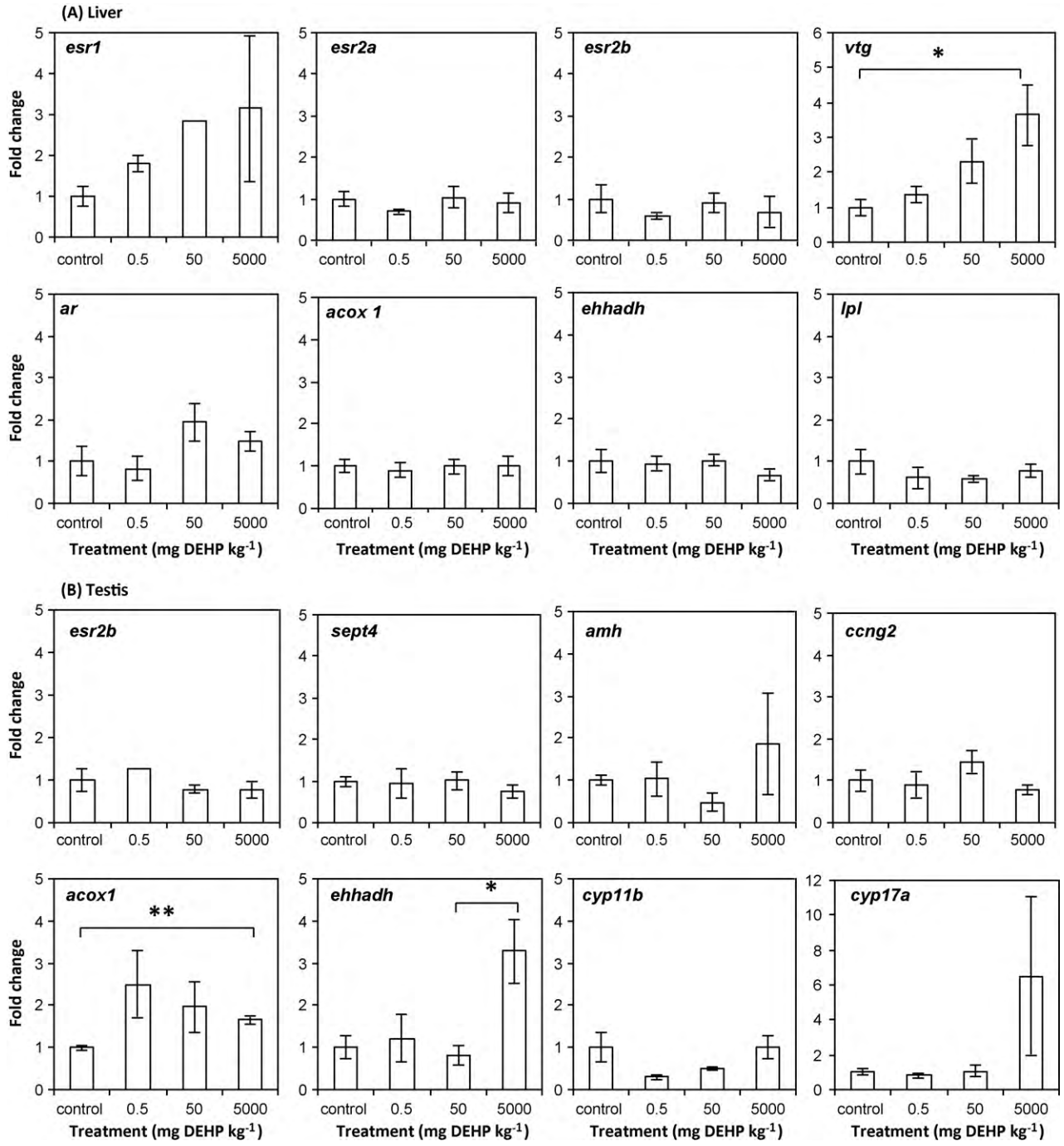


Fig. 5. Transcript profiles for selected genes in the liver (A) and testes (B). Data are presented as fold change relative to the transcript expression in the control group. Relative mRNA concentration was calculated as ratio of target gene mRNA/*rpl8* mRNA. For each treatment, data was collected for 6–8 fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay, were excluded from the analysis. Asterisks represent significant differences between treatment groups ($^{*}P < 0.05$, $^{**}P < 0.01$).

response of male zebrafish to DEHP. Our data showed that injections of 5000 mg DEHP kg^{-1} caused an induction of *vtg* transcripts, and, similarly, an increasing trend was observed for *esr1*. These results demonstrate that DEHP activates oestrogen-mediated signalling pathways in the liver at high concentrations of DEHP, but not at the environmentally relevant concentrations. These data are in agreement with previous results for fish where phthalate concentrations of 500 mg BBP kg^{-1} (body weight) (Christiansen et al., 2000) and 100 μg DEP L^{-1} (Barse et al., 2007) induced an increase in plasma VTG, but environmentally relevant concentrations did not (Harries et al., 2000; Ortiz-Zarragoitia et al., 2006; Patyna et al., 2006; Oehlmann et al., 2009). The auto-regulatory effect of oestrogens on their own receptors generally results in an increase in hepatic expression of *esr1* in male fish in response to oestrogen treatment, but not as dramatically as for *vtg* (Filby et al., 2007). Here, a similar trend was observed, but this was not statistically significant. This finding for *esr1*, together with the relatively modest induction of *vtg*, reflects the weak oestrogenicity of DEHP. The lack of change in transcription of *esr2a* and *esr2b* was in agreement with previous studies reporting that these transcripts were not altered in the liver of male fathead minnow following exposure to natural and synthetic oestrogens (Filby and Tyler, 2005; Filby et al., 2007).

The oestrogenic response seen following injection of 5000 mg DEHP kg^{-1} is likely to have contributed to the increased HSI observed in those fish, given that the process of vitellogenesis is generally associated with significant increases in the size of the liver (Harris et al., 2001).

4.2. Effects of exposure to DEHP in the testis

4.2.1. Effects of exposure to DEHP on fertilisation success and embryo survival

The 5000 mg DEHP kg^{-1} injections caused a marked reduction in fertilisation success during the exposure period. This reduction was time-dependent, with the fertilisation success decreasing over time, suggesting that an early stage of spermatogenesis may have been disrupted leading to a reduction in the quality and/or quantity of mature sperm released. Fish rapidly metabolise and excrete DEHP (Barron et al., 1995), therefore this progressive reduction in the ability of treated males to fertilise oocytes spawned by untreated females is likely to be associated with the time needed for the progression of the germ cells through spermatogenesis, rather than an increase in DEHP or its metabolites in the body caused by the second injection. The DEHP injections did not affect embryo survival to 24 hpf, and no abnormal development was observed at the end of this period. In parallel, there was no increase in DNA damage of the germ cells in the testis as a result of the exposure to DEHP at any of the concentrations tested. Phthalates are known to induce oxidative stress in the testis (Kasahara et al., 2002) and developing sperm are particularly susceptible to DNA damage because they lack buffering systems and DNA repair mechanisms (Agarwal and Said, 2005; Lewis and Galloway, 2009). Several correlative studies have linked phthalate metabolites in human urine with adverse effects on a number of sperm quality parameters, in particular motility and DNA integrity, and subsequently reduced fertility (Hauser et al., 2007; Pant et al., 2008) but the existing data are often not clear (Hauser et al., 2006). In fish, phthalates have also been shown to disrupt sperm motility (Oehlmann et al., 2009). Our data suggest that DEHP compromised reproduction in zebrafish through mechanisms other than an increase in DNA damage in germ cells. Further studies should focus on the analysis of sperm quality (density and motility) and male spawning behaviour to establish their importance as potential mechanisms via which DEHP reduced the ability of treated males to fertilise the oocytes spawned by untreated females.

4.2.2. Effects of exposure to DEHP on spermatogenesis

Spermatogenesis is cystic in fish and non-cystic in mammals and other amniotes, but in all other aspects is very similar in all vertebrates (Leal et al., 2009; Schulz et al., 2009). Briefly, spermatogenesis starts with a mitotic phase whereby small numbers of undifferentiated, diploid spermatogonia undergo differentiation (type A spermatogonia) and rapid proliferation (type B spermatogonia). The mitotic phase is followed by a meiotic stage; primary spermatocytes, formed during the final mitotic division, undergo a first meiotic division to produce secondary spermatocytes, then a second meiotic division results in the formation of haploid spermatids. Finally, the process of spermiation occurs whereby spermatids undergo further differentiation, before contact between the Sertoli and germ cells is terminated and motile, condensed spermatozoa are released into the testes tubular lumen (Schulz et al., 2009). Our data showed that injections of 5000 mg DEHP kg^{-1} caused a marked reduction in the proportion of spermatozoa in the testes of treated fish compared with all of the other treatment groups. This reduction in sperm production is likely to have contributed towards the decreased fertilisation success following this treatment. Furthermore, injection of 50 mg DEHP kg^{-1} resulted in significantly fewer spermatozoa in the testes of those males compared with those injected with olive oil alone. In this group, the fertilisation success also followed a decreasing trend over the course of the experiment, albeit it was not statistically significant. Here it is important to consider that under laboratory conditions sperm and oocytes are released into a relatively small volume of water, whereas in the wild sperm and oocytes are released into much larger volumes. This may explain why the reduction in the production of sperm in this group did not impact on fertilisation success, but does not eliminate the possibility that such an effect may occur in the environment.

Our results are in agreement with a considerable amount of evidence from the mammalian literature showing that phthalates, particularly DEHP, disrupt the process of spermatogenesis, resulting in the production of fewer spermatozoa. It is well established that phthalates disrupt oxidative balance in the testes, in both the somatic cells and developing sperm cells, by increasing the production of reactive oxygen species (ROS) and decreasing protective antioxidant production (Kasahara et al., 2002; Park et al., 2002; Miura et al., 2007; Onorato et al., 2008). It has been hypothesised that endogenous PPAR ligands play a role in maintaining normal testis oxidative balance. Therefore, phthalates may cause oxidative stress within the testis by disrupting the PPAR-mediated regulation of oxidative balance (Kasahara et al., 2002; Corton and Lapinskas, 2005), and this may contribute to the decreased production of spermatozoa observed in the present study. The monoester metabolites of phthalates, for example MEHP, bind and activate PPARs much more strongly than the parent diester compounds (Lampen et al., 2003; Bility et al., 2004). This is in contrast to the activation of steroid receptors, including the ER, which are only activated by diester phthalates (Harris et al., 1995; Takeuchi et al., 2005). These monoester metabolites were shown to be the active agents in inducing oxidative stress in the testis (Kasahara et al., 2002; Miura et al., 2007; Onorato et al., 2008). This suggests that oxidative stress within the testis, and resulting effects on developing sperm cells, is most likely mediated through PPAR activation, rather than through the activation/repression of sex steroid hormone pathways. We investigated this hypothesis by analysing the transcript profiles of PPAR-regulated genes (*acox1*, *ehhadh* and *lpl*) and genes involved in sex steroid synthesis (*cyp17a* and *cyp11b)* and signalling pathways (*esr2b* and *amh*) in the testis of the same individual fish. Gonadal *acox1* mRNA was elevated following injection with 5000 mg DEHP kg^{-1} compared with the olive oil control, and *ehhadh* was significantly higher in fish treated with 5000 mg DEHP kg^{-1} than with 50 mg DEHP kg^{-1}. No changes in transcript profiles were observed for any other genes across all treatment groups. The disruption of spermatogenesis observed in this study is, therefore, consistent with a putative PPAR-mediated mechanism of action, but this does not rule out the possibility that other mechanisms of toxicity may also contribute to the effects seen.

The histological data further support the hypothesis that DEHP inhibits the progression of spermatogenesis, potentially by causing an arrest of meiosis. PPAR signalling is known to play a role in the regulation of the cell cycle, and exogenous PPAR ligands can cause arrest of the cell cycle (e.g. Desvergne and Wahli, 1999). For example, DEHP was shown to alter the expression of proteins involved in regulating the cell cycle in rat testis via PPARy activation (Ryu et al., 2007). Progression through the cell cycle is prevented when the supply of molecules vital for specific checkpoints is disrupted (Desvergne and Wahli, 1999). We found an increased proportion of spermatocytes in the testes of males injected with 50 mg DEHP kg^{-1} and 5000 mg DEHP kg^{-1}, compared with the testes of males injected with the olive oil alone. This suggests that DEHP may have inhibited the transition into meiosis. The duration of the meiotic and spermiogenic phases of spermatogenesis (from spermatocytes to spermatozoa) in zebrafish is approximately 6 days (Leal et al., 2009) which corresponds with the timing of when the decreased

fertilisation success was observed in fish treated with 5000 mg DEHP kg^{-1} (approximately 5–6 days after the initiation of the exposure). This delay further supports the hypothesis that DEHP caused an arrest in spermatogenesis at the onset of meiosis. In order to investigate the molecular mechanisms underpinning the arrest in spermatogenesis observed, we measured the transcript profiles of *sept4* and *ccng2* in the testis. *sept4* plays an important role in the functional development of sperm in mice (Kissel et al., 2005). In the zebrafish, *sept4* has been shown to be most highly expressed in (primary and secondary) spermatocytes, indicating that this gene plays an important role in early spermatogenesis (Sreenivasan et al., 2008). *ccng2* is essential in mediating progression through the cell cycle checkpoints (Jensen et al., 1999). However, there were no significant alterations in transcript levels of either of these genes resulting from DEHP exposure. A more comprehensive analysis of genes involved in regulating apoptosis and the cell cycle is required in order to gain a better mechanistic understanding how DEHP disrupted the process of spermatogenesis in this study.

Oestrogenic chemicals, similarly, disrupt spermatogenesis in fish and reduce the production of spermatozoa (Sohoni et al., 2001). They cause this disruption mainly by inhibiting the synthesis of androgens which are vital for the proliferation and differentiation of spermatogonia, specifically the transition between type A and B spermatogonia. A typical oestrogenic effect on fish spermatogenesis involves the inhibition of mitosis and accumulation of (type A) spermatogonia (de Waal et al., 2009). This is clearly distinct from the accumulation of spermatocytes, not spermatogonia, and suspected inhibition of meiosis observed in the present study, further supporting the hypothesis that DEHP did not induce these effects via an oestrogenic mechanism of action. This is supported by the molecular data obtained in this study that showed no evidence of alteration in the transcript profiles for genes that have previously been shown to be responsive to oestrogen, including *esr2b*, *cyp17a* and *cyp11b*, which are involved in androgen synthesis, and *amh* which is involved in sexual differentiation (Baron et al., 2005; Filby et al., 2007; Schulz et al., 2007). In addition, alterations in the levels of these transcripts have previously been associated with exposure to model anti-androgens such as flutamide, which antagonistically binds the AR (Filby et al., 2007). The lack of change in these transcripts fits with previous data suggesting that, although DEHP may induce demasculinised phenotypes, it does so independently of the AR (Miura et al., 2007; Onorato et al., 2008; Pant et al., 2008). It should be noted, however, that the present dataset was restricted to a relatively limited number of transcripts and more studies need to be conducted to fully elucidate the pathways involved in the disruption of testicular function in fish by DEHP.

5. Conclusions

The present study demonstrated the potential of DEHP to disrupt spermatogenesis in adult zebrafish at concentrations above 50 mg kg^{-1} (body weight), with a resulting decline in their ability to fertilise untreated oocytes following treatment with 5000 mg kg^{-1}. Our data suggest that these adverse effects induced by DEHP exposure may occur preferentially (but not exclusively) via PPAR signalling pathways in the testis and oestrogen signalling pathways in the liver, demonstrating the importance of both of these pathways in the toxicology of this compound in lower vertebrates. We found no evidence that exposure to environmentally relevant concentrations of DEHP adversely affects the reproductive health of male zebrafish, indicating that DEHP alone is unlikely to be responsible for the reproductive abnormalities seen in wildlife, at least as a result of short-term exposures. However, the evidence of disruption caused by higher concentrations of DEHP suggests that the potential contribution of this chemical to reproductive disruption in both humans and wildlife may not be ruled out, in particular when acting in combination with other chemicals sharing similar mechanisms of action. Furthermore, similarly to that occurring in mammals, early life stages may be more sensitive to the effects of exposure to DEHP compared to mature fish. Further research is required to elucidate the long term effects of exposure to phthalates, both when acting alone and as part of complex mixtures, in fish populations inhabiting contaminated environments.

Acknowledgments

The authors would like to acknowledge Toby Coe for advice on the statistical analyses, Lisa Bickley for help with the histology, Chris Green and Mark Puttick for their contribution towards developing the experimental system, and Charles Tyler for critical discussions and comments on an earlier version of this manuscript. This work was funded in part by the Royal Society (RG081237). TUW received funding from the Aquatic Biology and Resource Management M.Sc. bursary fund within the School of Biosciences, University of Exeter, and from a NERC postgraduate studentship to EMS. CL was funded by NERC (NE/G014728/1).

References

Agarwal, A., Said, T.M., 2005. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int. 95, 503–507.
Baron, D., Fostier, A., Breton, B., Guiguen, Y., 2005. Androgen and estrogen treatments alter steady state messengers RNA (mRNA) levels of testicular steroidogenic enzymes in the rainbow trout, (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 71, 471–479.
Barron, M.G., Albro, P.W., Hayton, W.L., 1995. Biotransformation of di(2-ethylhexyl) phthalate by rainbow trout. Environ. Toxicol. Chem. 14, 873–876.
Barse, A.V., Chakrabarti, T., Ghosh, T.K., Pal, A.K., Jadhao, S.B., 2007. Endocrine disruption and metabolic changes following exposure of *Cyprinus carpio* to diethyl phthalate. Pestic. Biochem. Physiol. 88, 36–42.
Bauer, M.J., Herrmann, R., 1997. Estimation of the environmental contamination by phthalic acid esters leaching from household wastes. Sci. Total Environ. 208, 49–57.
Bhattacharya, N., Dufour, J.M., Vo, M., Okita, J., Okita, R., Kim, K.H., 2005. Differential effects of phthalates on the testis and the liver. Biol. Reprod. 72, 745–754.
Bility, M.T., Thompson, J.T., McKee, R.H., David, R.M., Butala, J.H., Heuvel, J.P.V., Peters, J.M., 2004. Activation of mouse and human peroxisome proliferator activated receptors (PPARs) by phthalate monoesters. Toxicol. Sci. 82, 170–182.
Chauvenet, W., 1863. A Manual of Spherical and Practical Astronomy. Lippincott, Philadelphia.
Christiansen, L.B., Pedersen, K.L., Pedersen, S.N., Korsgaard, B., Bjerregaard, P., 2000. In vivo comparison of xenoestrogens using rainbow trout vitellogenin induction as a screening system. Environ. Toxicol. Chem. 19, 1867–1874.
Corton, J.C., Lapinskas, P.J., 2005. Peroxisome proliferator-activated receptors: mediators of phthalate ester-induced effects in the male reproductive tract? Toxicol. Sci. 83, 4–17.
David, R.M., Moore, M.R., Cifone, M.A., Finney, D.C., Guest, D., 1999. Chronic peroxisome proliferation and hepatomegaly associated with the hepatocellular tumorigenesis of di(2-ethylhexyl)phthalate and the effects of recovery. Toxicol. Sci. 50, 195–205.
de Waal, P., Leal, M., Garcia-Lopez, A., Liarte, S., de Jonge, H., Hinfray, N., Brion, F., Schulz, R., Bogerd, J., 2009. Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis. J. Endocrinol. 202, 287–297.
Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20, 649–688.
European Commission, 2003. EU Risk Assessment Report for 1,2-Benzenedicarboxylic Acid, Di-C9-11-Branched Alkyl Esters, C10 Rich and Di-"Isodecyl" Phthalate (DIDP), vol. 36. European Chemicals Bureau.
Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquat. Toxicol. 81, 219–231.
Filby, A.L., Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (*Pimephales promelas*). Biol. Reprod. 73, 648.
Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., Wenzel, A., 2002. Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 36, 1429–1438.
Harries, J.E., Runnalls, T., Hill, E., Harris, C.A., Maddix, S., Sumpter, J.P., Tyler, C.R., 2000. Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 34, 3003–3011.
Harris, C.A., Henttu, P., Parker, M.G., Sumpter, J.P., 1995. The estrogenic activity of phthalate esters *in vitro*. Environ. Health Perspect. 105, 802–811.

Harris, C.A., Santos, E.M., Janbakhsh, A., Pottinger, T.G., Tyler, C.R., Sumpter, J.P., 2001. Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female rainbow trout. Environ. Sci. Technol. 35, 2909–2916.

Hauser, R., Meeker, J.D., Duty, S., Silva, M.J., Calafat, A.M., 2006. Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. Epidemiology 17, 682.

Hauser, R., Meeker, J.D., Singh, N.P., Silva, M.J., Ryan, L., Duty, S., Calafat, A.M., 2007. DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. Hum. Reprod. 22, 688.

Howdeshell, K.L., Wilson, V.S., Furr, J., Lambright, C.R., Rider, C.V., Blystone, C.R., Hotchkiss, A.K., Gray, L.E., 2008. A mixture of five phthalate esters inhibits fetal testicular testosterone production in the Sprague–Dawley rat in a cumulative, dose-additive manner. Toxicol. Sci. 105, 153–165.

Jensen, M.R., Audolfsson, T., Keck, C.L., Zimonjic, D.B., Thorgeirsson, S.S., 1999. Gene structure and chromosomal localization of mouse cyclin G2 (Ccng2). Gene 230, 171–180.

Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P., 1995. A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ. Health Perspect. 103, 582–587.

Kasahara, E., Sato, E.F., Miyoshi, M., Konaka, R., Hiramoto, K., Sasaki, J., Tokuda, M., Nakano, Y., Inque, M., 2002. Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. Biochem. J. 365, 849–856.

Kissel, H., Georgescu, M.M., Larisch, S., Manova, K., Hunnicutt, G.R., Steller, H., 2005. The Sept4 septin locus is required for sperm terminal differentiation in mice. Dev. Cell 8, 353–364.

Lampen, A., Zimnik, S., Nau, H., 2003. Teratogenic phthalate esters and metabolites activate the nuclear receptors PPARs and induce differentiation of F9 cells. Toxicol. Appl. Pharmacol. 188, 14–23.

Leal, M.C., Cardoso, E.R., Nobrega, R.H., Batlouni, S.R., Bogerd, J., Franca, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod. 81, 177–187.

Lewis, C., Galloway, T., 2009. Reproductive consequences of paternal genotoxin exposure in marine invertebrates. Environ. Sci. Technol. 43, 928–933.

Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. J. Fish Biol. 62, 895–906.

Mandard, S., Müller, M., Kersten, S., 2004. Peroxisome proliferator-activated receptor α target genes. Cell. Mol. Life Sci. 61, 393–416.

Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughs, R.J., Croley, T.R., March, R.E., Potter, T., 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 20, 297–308.

Miura, Y., Naito, M., Ablake, M., Terayama, H., Yi, S.-Q., Qu, N., Cheng, L.-X., Suna, S., Jitsunari, F., Itoh, M., 2007. Short-term effects of di-(2-ethylhexyl) phthalate on testes, liver, kidneys and pancreas in mice. Asian J. Androl. 9, 199–205.

Nakachi, Y., Yagi, K., Nikaido, I., Bono, H., Tonouchi, M., Schönbach, C., Okazaki, Y., 2008. Identification of novel PPAR target genes by integrated analysis of ChIP-on-chip and microarray expression data during adipocyte differentiation. Biochem. Biophys. Res. Commun. 372, 362–366.

Norman, A., Borjeson, H., David, F., Tienpont, B., Norrgren, L., 2007. Studies of uptake, elimination and late effects in Atlantic salmon (*Salmo salar*) dietary exposed to di-2-ethylhexyl phthalate (DEHP) during early life. Arch. Environ. Contam. Toxicol. 52, 235–242.

Norrgren, L., Blom, A., Andersson, P.L., Borjenson, H., Larsson, D.G.J., Olsson, P.E., 1999. Effects of potential xenoestrogens (DEHP, nonylphenol and PCB) on sexual differentiation in juvenile Atlantic salmon (*Salmo salar*). Aquat. Ecosyst. Health Manage. 2, 311–317.

Oehlmann, J., Schulte-Oehlmann, U., Kloas, W., Jagnytsch, O., Lutz, I., Kusk, K.O., Wollenberger, L., Santos, E.M., Paull, G.C., Van Look, K.J.W., 2009. A critical analysis of the biological impacts of plasticizers on wildlife. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 364, 2047–2062.

Onorato, T.M., Brown, P.W., Morris, P.L., 2008. Mono-(2-ethylhexyl) phthalate increases spermatocyte mitochondrial peroxiredoxin 3 and cyclooxygenase 2. J. Androl. 29, 293–303.

Ortiz-Zarragoitia, M., Cajaraville, M.P., 2005. Effects of selected xenoestrogens on liver peroxisomes, vitellogenin levels and spermatogenic cell proliferation in male zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 141, 133–144.

Ortiz-Zarragoitia, M., Trant, J.M., Cajaraville, M.P., 2006. Effects of dibutylphthalate and ethynylestradiol on liver peroxisomes, reproduction, and development of zebrafish (*Danio rerio*). Environ. Toxicol. 25, 2394–2404.

Pant, N., Shukla, M., Kumar Patel, D., Shukla, Y., Mathur, N., Kumar Gupta, Y., Saxena, D.K., 2008. Correlation of phthalate exposures with semen quality. Toxicol. Appl. Pharmacol. 231, 112–116.

Park, J.D., Habeebu, S.S.M., Klaassen, C.D., 2002. Testicular toxicity of di-(2-ethylhexyl)phthalate in young Sprague–Dawley rats. Toxicology 171, 105–115.

Patyna, P.J., Brown, R.P., Davi, R.A., Letinski, D.J., Thomas, P.E., Cooper, K.R., Parkerton, T.F., 2006. Hazard evaluation of diisononyl phthalate and diisodecyl phthalate in a Japanese medaka multigenerational assay. Ecotoxicol. Environ. Saf. 65, 36–47.

Paull, G.C., Van Look, K.J.W., Santos, E.M., Filby, A.L., Gray, D.M., Nash, J.P., Tyler, C.R., 2008. Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. Aquat. Toxicol. 87, 115–126.

Petrovic, M., Eljarrat, E., López de Alda, M.J., Barceló, D., 2001. Analysis and environmental levels of endocrine-disrupting compounds in freshwater sediments. Trends Anal. Chem. 20, 637–648.

Rasmussen, R., 2001. Quantification on the LightCycler. Springer Press, Heidelberg, pp. 21–34.

Reddy, J.K., Azarnoff, D.L., Hignite, C.E., 1980. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature 283, 397–398.

Ryu, J.Y., Whang, J., Park, H., Im, J.Y., Kim, J., Ahn, M.Y., Lee, J., Kim, H.S., Lee, B.M., Yoo, S.D., Kwack, S.J., Oh, J.H., Park, K.L., Han, S.Y., Kim, S.H., 2007. Di(2-ethylhexyl) phthalate induces apoptosis through peroxisome proliferators-activated receptor-gamma and ERK 1/2 activation in testis of Sprague–Dawley rats. J. Toxicol. Environ. Health Part A 70, 1296–1303.

Santos, E.M., Workman, V.L., Paull, G.C., Filby, A.L., Van Look, K.J.W., Killie, P., Tyler, C.R., 2007. Molecular basis of sex and reproductive status in breeding zebrafish. Physiol. Genom. 30, 111–122.

Schulz, R.d.W., Bogerd, J., Male, R., Ball, J., Fenske, M., Olsen, L.C., Tyler, C.R., 2007. Estrogen-induced alterations in amh and dmrt1 expression signal for disruption in male sexual development in the zebrafish. Environ. Sci. Technol. 41, 6305–6310.

Schulz, R.W., de França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2009. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Sharpe, R.M., Fisher, J.S., Millar, M.M., Jobling, S., Sumpter, J.P., 1995. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ. Health Perspect. 103, 1136–1143.

Sohoni, P., Tyler, C.R., Hurd, K., Caunter, J., Hetheridge, M.J., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M., Sumpter, J.P., 2001. reproductive effects of long-term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). Environ. Sci. Technol. 35, 2917–2925.

Sreenivasan, R., Cai, M., Bartfai, R., Wang, X., Christoffels, A., Orban, L., 2008. Transcriptomic analyses reveal novel genes with sexually dimorphic expression in the zebrafish gonad and brain. PLoS ONE 3, e1791.

Staples, C.A., Peterson, D.R., Parkerton, T.F., Adams, W.J., 1997. The environmental fate of phthalate esters: a literature review. Chemosphere 35, 667–749.

Takeuchi, S., Iida, M., Kobayashi, S., Jin, K., Matsuda, T., Kojima, H., 2005. Differential effects of phthalate esters on transcriptional activities via human estrogen receptors [alpha] and [beta], and androgen receptor. Toxicology 210, 223–233.

Thibaut, R., Porte, C., 2004. Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. J. Steroid Biochem. Mol. Biol. 92, 485–494.