# Early exposure to atrazine in zebrafish: intergenerational effects and animal personality

**Simon D. Lamb**

A thesis submitted in partial fulfilment for the degree of

Master of Science in Zoology

University of Otago, Dunedin,

New Zealand

August, 2018

# Abstract

Animal personality (consistent inter-individual differences in behaviour over time and context) is important for an individual's fitness. Acute exposure studies in adults show that endocrine disrupting chemicals (EDCs) can deteriorate behavioural consistency and directionally alter personality phenotypes, which may decrease fitness. Early life development is sensitive to environmental stressors and exposure to EDCs may permanently alter phenotypes through to adulthood. In addition, the effects of environmental stressors may not be isolated to a single generation − offspring may indirectly be impacted, via non-genetic processes. Epigenetic mechanisms (e.g. DNA methylation) that help to regulate the genome, can become permanently altered via EDC exposure during early life and alterations have the potential to be inherited in the germ cells. The aims of this thesis were to investigate direct effects of the EDC, atrazine, on animal personality in the zebrafish (*Danio rerio*) and the indirect effects on personality in their offspring. Recent studies suggest that zebrafish inherit the methylome (total DNA-methylation marks across the genome) in an unchanged state from their fathers, suggesting a possible route for environmental specific information to be inherited. Hence, I focus on paternal effects in this thesis. In addition, I investigate the underlying basis of mRNA transcript number differences in $F_1$ zebrafish brain tissue, with the aim to determine whether observed changes in behaviour are underpinned by changes in gene expression. This is the first study to examine the effects of atrazine on personality, for both direct and indirect exposure.

Juvenile zebrafish were exposed to atrazine at typical environmental concentrations: 0.3, 3 or 30 part per billion (ppb) through sexual differentiation and their personality phenotypes were assayed (activity, aggression, boldness, anxiety and exploration) at adulthood. $F_1$ offspring were produced from atrazine exposed and control males, mated with unexposed females, creating full-sibling families. Progeny personality was tested at adulthood. Brain samples of a subset of $F_1$ progeny (based on anxiety and aggression phenotypes) were taken to determine mRNA transcript number of candidate genes involved in the regulation of the serotonergic system (*slc6a4a*, *slc6a4b*, *htr1Aa*, *htr1B*, *htr2B*), an underlying component involved in anxiety and aggressive-related behaviours, and a potential target of atrazine.

I found evidence that direct and paternal atrazine exposure significantly influences aspects of boldness, aggression, anxiety and exploration, though effects were not consistent across all doses. Changes in boldness were sex-dependent, specifically, $F_0$ female boldness was reduced in a dose-dependent manner and $F_0$ males exhibited a threshold increase in boldness. $F_1$ females exhibited a similar dose-dependent reduction in boldness but in the opposite direction to $F_0$ females and there was little change amongst $F_1$ males. There was some evidence of reduced aggression after direct exposure, but paternal atrazine exposure significantly reduced aggression in the 0.3ppb and 3ppb groups. No $F_0$ treatment differences in activity were observed, however, significant $F_1$ decreases in activity were observed during aggression testing in the 0.3ppb and 3ppb groups. Anxiety increased and exploration decreased significantly in the $F_0$ 0.3ppb group only, while in the $F_1$'s there was some evidence of alterations in anxiety and exploration, but not significantly so.

Paternal atrazine exposure significantly down-regulated *htr1Aa* mRNA expression in females and slightly up-regulated it in males; moreover, *htr1Aa* mRNA (and *slc6a4a* - but not significantly so) was found to correlate positively with anxiety levels in controls; however, this relationship was disrupted in the atrazine treatment group. The candidate genes tested here did not explain the significant reduction in aggression observed in the offspring of atrazine treated fathers, though both *slc6a4b* and *htr1B* mRNA correlated negatively with an aggressive personality phenotype.

In conclusion, early developmental exposure to environmental doses of atrazine resulted in persistent changes in personality phenotypes through to adulthood and effects were present in the offspring of atrazine exposed fathers. Moreover, some aspects of the serotonergic system were disrupted in the progeny. Overall, these results add to the ecological consequences of environmental contaminants, most importantly, that effects may be further propagated down the germ line.

# Acknowledgments

First and foremost I would like to thank my supervisor, Dr Sheri Johnson for taking me on as your student. I am grateful for the opportunity to work with you. Your patience, insight and ability to challenge me has greatly contributed to my learning. I would like to make special mention of Dr Anne Besson, for your support with zebrafish behavioural phenotyping, Jolyn Chia, for the long hours doing lab work and Luke Easton, who always makes time for a student in need. All of you were instrumental in helping me achieve this milestone.

Thank you to the Behavioural Ecology and Evolution lab group and my fellow zoology students and friends for keeping me going. Thank you to Dr Stephanie Godfrey for always having your door open when I needed statistical advice and Dr Jenny Jandt for your encouragement and continued enthusiasm. I would like to thank the Otago Zebrafish Facility, with special mention to Noel Jhinku and Ute Zellhuber-Mcmillan for your support in all things zebrafish.

I would like to thank my family and friends back home, with special acknowledgments to my parents, John and Shona Lamb and my sister, Olivia Lamb. It has been your unconditional support that has been the backbone of my time in Dunedin. Last but not least, I want to thank Andrea Barnaby for your continuous and unwavering support throughout this journey. I will be forever grateful.

# Contents

# List of Figures

# List of Tables

# Chapter 1

# General introduction

## 1.1 Animal personality

### 1.1.1 An overview

Animal personality is defined as consistent behavioural variation at the level of the individual that occurs across time and in different behavioural and environmental contexts (also previously known as temperament, emotionality or coping styles; Koolhaas et al. 1999, Sih et al. 2004a, Réale et al. 2007). Behavioural context describes an aspect of a behavioural function e.g. anti-predator, courtship, or dispersal. Environmental context or situation, describes the conditions experienced by an organism during a particular time point it is being measured under, such as levels of predator density (Sih et al. 2004a; Oers et al. 2005). Historically, behavioural ecology theory posited that behavioural variation of individuals within a given population of animals was due to random error (or non-adaptive variation) that lead towards a central tendency around an adaptive population mean (Dall 2004; Nettle et al. 2010). Today, however, consistent inter-individual differences in behaviour are readily observed across the animal kingdom from all levels of vertebrate systems (e.g. mammals, reptiles, fish birds and amphibians; Smith et al. 2008; Bell et al. 2009) and even to invertebrates (e.g. insects; Jandt et al. 2014).

Animal personality is typically characterised by five broad axes that encapsulate the response of an individual relative to other individuals. These axes being: boldness (also known as the boldness-shyness axis), exploration (also known as the exploration-avoidance axis), activity, aggressiveness and sociability (Réale et al. 2007). Boldness-shyness describes an individual's propensity to take risks (e.g. an animal's latency to emerge from a shelter, latency to approach a novel object or the duration spent in areas of high versus low risk; Wilson et al. 1994; Frost et al. 2007; Réale et al. 2007; Harris et al. 2010; Raoult et al. 2012).

Exploration describes the individual's propensity to seek out information of an unfamiliar space in a new environment (e.g. the duration, frequency or latency visiting distinct areas of a novel environment; Dingemanse et al. 2002; Réale et al. 2007). Activity is defined by the consistency of movement (or the consistency of the rate of movement) constrained by the costs of metabolism (Montiglio et al. 2010; Schuster et al. 2017). Aggressiveness is defined by the degree of antagonistic interactions with conspecifics (Réale et al. 2007) and can be measured by dyadic fights or by measuring interactions with a mirror image (Oliveira et al. 2005; Ariyomo et al. 2013a; Way et al. 2015). Finally, sociability is considered the propensity for an individual to seek out conspecifics (e.g. the cohesion of a group of animals; Miller et al. 2007; Réale et al. 2007). Recently, anxiety is considered to be an additional personality trait and is defined as anticipatory fear caused via uncertainty between a potential threat (that may not be specifically present, but is anticipated to occur), and lack of information needed to ascertain the actual risk of an action or situation (Blanchard et al. 1989; Belzung et al. 2007; Maximino et al. 2012). Anxiety may be measured by an animal's behavioural reaction in uncertain novel environment, such as erratic behaviour, freezing or residing in areas perceived to be of low risk (Egan et al. 2009; Maximino et al. 2012). These axes of variation may also co-vary together resulting in what is termed a behavioural syndrome. For example, on average, individuals that are more aggressive tend to also be bolder (Sih et al. 2004a).

Several aspects of ecology and evolution can be influenced by personality traits (see Wolf et al. 2012). Predator-prey interactions, for example, can shape personality traits and impact how individuals cope with predators. For instance, an aggressive-boldness syndrome in three spined stickleback (*Gasterosterus aculeatus*) is dependent on exposure to predation attempts (Bell et al. 2007) and pike (*Esox lucius*; an ambush predator) selectively target shyer roach (*Rutilus rutilus*), whereas Eurasian perch (*Perca fluviatilis*; an active forager) tend not to discriminate between behavioural phenotypes (Blake et al. 2018). Shyer three-spined sticklebacks also preferentially associate with smaller sized individuals and tend to have higher social interactions, whereas bolder fish interacted less and lacked discrete associations across their social network (Pike et al. 2008). Moreover, less sociable individuals are more likely to disperse from their population (Cote et al. 2007; Cote et al. 2010).

Animal personality can contribute to an individual's survival and life-time reproductive success (Smith et al. 2008; Réale et al. 2010; Sih et al. 2012; Wolf et al. 2012). Given the limited behavioural variation of individuals, fitness may shift according to changing contexts, such as predator density or food availability (Réale et al. 2003; Dingemanse et al. 2004). For instance, asocial common lizards (*Lacerta vivipara*) have higher fitness in low-density populations, but when population density increases, the fitness for asociality decreases (Cote et al. 2008). When environmental contexts remain relatively constant over time, variation

at the behavioural extremes may be more beneficial, for example, bolder individuals may trade-off lower survival for more immediate reproductive opportunities when predator abundance is high, whereas shyer individuals may forgo short-term reproduction in order to increase survival. In this situation, individuals at either ends of the boldness-shyness axis theoretically maintain higher life-time reproductive success than individuals that are intermediate in their behavioural phenotype (Smith et al. 2008; MacPherson et al. 2017).

### 1.1.2 Anthropogenic drivers of animal personality

Several factors are observed to contribute to an individual's personality, these being a combination of social-context (and social interaction), life-time experience, feedback from the environmental context, the early-life environment, and in some instances a heritable, genetic (and possibly non-genetic) component has been attributable (Drent et al. 2003; Frost et al. 2007; Chapman et al. 2010; Reddon 2011; Ariyomo et al. 2013a). These factors together influence the intertwined neural, genetic (including epigenetic) and endocrine processes which govern the expression of behaviour and therefore, the extent to which variation can exist within an individual. What is less understood is how environmental contaminants (that are able to interfere with these processes) may influence and shape an individual's consistency in behaviour along their axes of personality. Of particular interest and concern are endocrine (and neuro-endocrine) disrupting chemicals (EDCs), which interfere with key molecular mechanisms that underlie behaviour (i.e. hormones, gene expression and neural physiology: Vandenberg et al. 2012; Volkova et al. 2015a). These chemicals are predominantly implicated in the disruption of typical reproductive behaviours (i.e. courtship and parental behaviour, see review Söffker et al. 2012), but some studies have shown effects on animal personalities (Dzieweczynski 2011; Dzieweczynski et al. 2013; Dzieweczynski et al. 2014; Hebert et al. 2014; Dzieweczynski et al. 2016; Porseryd et al. 2017b). For example, adult male and female Siamese fighting fish (*Betta splendens*), acutely exposed to the oestrogen mimic 17$\alpha$-ethinylestradiol, exhibited reductions in boldness, aggression (males only), activity and the individual consistency of these traits. Moreover the behavioural syndrome between boldness and activity (in females) and boldness and aggression (in males) was disrupted (Dzieweczynski et al. 2014; Hebert et al. 2014).

To date, much work on how EDCs may affect personality has focused on acute adult exposures (e.g. Dzieweczynski 2011; Dzieweczynski et al. 2013; Dzieweczynski et al. 2014; Hebert et al. 2014; Dzieweczynski et al. 2016; with some exceptions, Volkova et al. 2015a; Volkova et al. 2015b; Porseryd et al. 2017a). What is less understood is how the early life environment in combination with EDCs may influence animal personalities through to adulthood. The early life environment refers to critical developmental periods of

an organisms life, i.e. the embryonic and juvenile period, when major development occurs (Burton et al. 2014). During this early developmental period, organisms have greater plasticity but are more susceptible to environmental pressures altering their course of development (reviewed by Jonsson et al. 2014). For example, guppies (*Poecilia reticulata*) raised with an unpredictable food supply became bolder, more exploratory, and tended to be less social at adulthood (Chapman et al. 2010). Early life exposures to EDCs routinely find that effects become either fixed, or manifest permanently later in life (Shenoy 2014; Volkova et al. 2015a; Wirbisky et al. 2015; Wirbisky et al. 2016c). However, little research on animal personalities has been done under an early life exposure framework (e.g. Volkova et al. 2015a).

### 1.1.3 Non-genetic inheritance and animal personality

Offspring phenotype is not solely derived from the genetic code it inherited from the gametes of its parents. Non-genetic inheritance describes the transmission of parental phenotypic or environmental variation to offspring that do not stem from the inheritance of the DNA sequence (Bonduriansky et al. 2012; O'Dea et al. 2016). Parental effects, a subset of non-genetic inheritance, shows that parents may mediate their offspring's phenotype through factors such as behaviour (e.g. nurturing behaviour) or physiology (e.g. the transfer of hormones and proteins into eggs; Badyaev et al. 2009; Curley et al. 2011; Sopinka et al. 2017). Moreover, there is some evidence that non-genetic inheritance may additionally attribute to animal personality (Reddon 2011). For instance, personality differences in magpie (*Pica pica*) offspring are predicted by laying order, which is also associated with altered hormone composition in eggs (Rokka et al. 2014). And hatching asynchrony by zebra finch (*Taeniopygia guttata*) mothers interacts with offspring sex to influence exploratory behaviour of offspring at adulthood (Mainwaring et al. 2013). In addition, Taylor et al. (2012) found evidence for maternal effects (as well as genetic effects) correlating with activity and aggression in North American red squirrels (*Tamiasciurus hudsonicus*). Recently, studies have found that personality differences associated with non-genetic inheritance may change with ontogeny. For instance, incubation temperature in bearded dragon (*Pogona vitticeps*) can influence boldness over the short term (but not long term; Siviter et al. 2017), and maternal effects in guppies are associated with risk-taking individual differences in juveniles, but the association is absent at adulthood (White et al. 2018). In general, non-genetic inheritance is an under-appreciated source for influencing animal personality, but also requires further research to validate its potential role in shaping personality traits.

Recent work is recognising that sperm can be a source contributing to non-genetic inheritance (see Immler 2018). Aside from inheritance of the paternal genome, sperm also carries epigenetic modifiers (e.g. DNA-methylation, non-coding RNAs and histone modifications; reviewed in Casas et al. 2014; Rando 2016).

Epigenetic modifiers are molecules that act as an interactive mechanism between environment and the genome by regulating gene expression (see review; Jablonka et al. 2009). These molecules regulate the genome as the environment shifts, but exposure to strong environmental stresses (especially during early life) may leave epigenetic marks that, in turn, permanently alter the phenotype of the adult (see reviews; Barker 2004; Jablonka et al. 2009). Studies are increasingly documenting non-genetic inheritance due to epigenetic effects (see review Jablonka et al. 2009; Curley et al. 2011; O'Dea et al. 2016). For example, mice raised with chronic and unpredictable maternal separation (between 1-14 days after birth, a high stress environment for new born pups) caused depressive-like behaviours and altered behavioural responses to aversive environments through to adulthood. The effects of this high stress and inconsistent nurturing environment during early life were also transferred to descendants, despite cross fostering and continuous maternal care during the offspring upbringing (Franklin et al. 2010). Dias et al. (2014) found that male adult mice subjected to odour fear conditioning produced descendants with increased behavioural sensitivity to the same odour and increased neuroanatomical structuring with the odorant receptor (that was specific to that particular odour). In addition, EDCs may be a potential source for producing maladaptive phenotypes through non-genetic inheritance mechanisms. Guppies exposed to 17*alpha*-ethinyl estradiol have a heightened anxiety like phenotype (observed through to two generations; Volkova et al. 2015a) and female $F_3$ descendants of mice embryonically exposed to the EDC, vinclozolin, prefer males without a history of exposure (Crews et al. 2007). Thus, EDCs may be a potential source for producing maladaptive phenotypes through non-genetic inheritance mechanisms.

Given the increased prevalence of EDCs in the environment (Colborn et al. 1993; Vos et al. 2000; Jenssen 2006; Diamanti-Kandarakis et al. 2009) it is important to understand the consequences of their contamination. Behaviour of the individual, through many interactions, may cascade up to the population level and dynamically affect population stability, persistence, structure or dispersal (Wolf et al. 2012; Araújo et al. 2018). Behaviour may therefore help to serve as preliminary guide to predict how populations might react to EDC exposure (Clotfelter et al. 2004; Araújo et al. 2018). Because animal personality is partly a function of the early life environment (among other factors), the sensitivity observed by organisms to EDCs during this period suggests that animal personality is likely to be affected (Jonsson et al. 2014). Furthermore, since non-genetic inheritance in general appears to be a mediator for altering the trajectories of offspring to suit the predicted local environment (based on the environment of the parent; Marshall et al. 2007; Burton et al. 2014), EDC induced pathological personality phenotypes may additionally be propagated indirectly to offspring. As animal personality contributes to fitness, non-genetic propagation of EDCs may also affect offspring fitness (Smith et al. 2008; Réale et al. 2010; Sih et al. 2012; Wolf et al. 2012). Overall, there is

a lack of research on animal personalities, EDCs and non-genetic inheritance; therefore, this thesis aims to better understand the relationship between these factors.

## 1.2 Thesis objectives and rationale

The aim of this thesis was to investigate non-genetic inheritance of animal personality under an EDC stressor in the zebrafish (*Danio rerio*; Figure 1.1). Zebrafish are an interesting model to investigate non-genetic inheritance because studies suggest that embryos inherit the methylome (total DNA-methylation marks across the genome) from their fathers, thereby suggesting a pathway for environmental signals to be inherited (Jiang et al. 2013; Potok et al. 2013). In addition, zebrafish have a short 3-4 month generation time (Kimmel et al. 1995) and their genetics are well understood (Norton et al. 2010; Howe et al. 2013). This species can act as a complementary model to mammalian studies (Norton et al. 2010), as well as providing a model for the potential effects of toxicants on other aquatic species. Researchers have established catalogues of complex zebrafish behaviours (Kalueff et al. 2013) and detailed insight into animal personality of zebrafish is becoming increasingly understood (e.g. Thomson 2017).

Figure 1.1: Example of an AB wildtype zebrafish female. Size of the animal is approximately ∼35mm.

I utilised the EDC, atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; Figure 1.2) as a non-genetic source of environmental variation. Atrazine is a common herbicide used to control broadleaf and weeds in a variety of crops, such as maize and sugarcane, via the disruption of photosynthetic pathways (Barr et al. 2007, Solomon et al. 2008). Previous research has demonstrated that atrazine can reduce mating behaviour (male-male aggression in a courtship context in guppies; Shenoy 2012; Shenoy 2014) and

alter some aspects of sociability (Schmidel et al. 2014). In mice, atrazine has been observed to increase anxiety-like behaviours after short term acute exposure (Lin et al. 2013) and after early life exposure as well (Belloni et al. 2011; Lin et al. 2014a). But no study has yet explored how atrazine may affect personality traits.

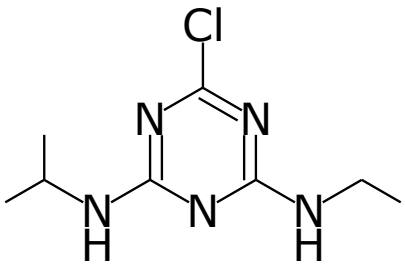


Figure 1.2: Atrazine chemical structure, modified from Graymore et al. 2001.

Atrazine is able to cross the blood brain barrier (Ross et al. 2009), but the mechanisms behind its action within the brain are not fully resolved. Developmental transcriptomic studies in zebrafish embryos at environmental doses highlight interference at the level of transcription of nervous system development and function, tissue development and organismal development through to adulthood (Wirbisky et al. 2015; Wirbisky et al. 2016a). Many studies in rodents implicate the dopaminergic system after adult or developmental exposure (Coban et al. 2007; Lin et al. 2013; Rodriguez et al. 2017). Recent studies in zebrafish and rodents also implicate disturbance of the serotonergic system (Lin et al. 2014a; Wirbisky et al. 2015) and by proxy, the hypothalamic-pituitary-adrenal/interrenal (Heisler et al. 2007; Fraites et al. 2009). A study of the male mice metabolome (composition of all metabolites in plasma) after acute exposure found disruption of several metabolic pathways such as tyrosine (precursor to dopamine), tryptophan (precursor to serotonin; 5-HT), linoleic acid and $\alpha$-linolenic acid (poly-unsaturated fatty acids, which play a role in normal brain development and can modulate expression of 5-HT and dopamine; Das 2013; Lin et al. 2014b). Atrazine has also been found to act transgenerationally on sperm biomarkers of disease in mice (McBirney et al. 2017) and can modify epigenetic molecules trangenerationally (Hao et al. 2016; Wirbisky et al. 2016b; Wirbisky-Hershberger et al. 2017). These studies indicate that sperm are able to carry epigenetic markers associated with atrazine exposure through the germ line.

The general frame work of this thesis is summarised in Figure 1.3 below. The experiments in Chapter 2 were specifically aimed at investigating to what extent the EDC, atrazine, may directly affect personality in adults and indirectly in offspring. Specifically, this study investigated the effect of atrazine on five personality traits: activity, anxiety, aggression, exploration and boldness. In Chapter 3, I investigate the underlying basis of

mRNA transcript number differences in $F_1$ zebrafish brain tissue, with the aim to combine observed changes in offspring behaviour with an epigenetic (assessed through gene expression) explanation of inheritance. I specifically targeted candidate genes involved in the regulation of the serotonergic system, the two serotonin (5-HT) transporters (*slc6a4a*, *slc6a4b*) and three 5-HT receptors (*htr1Aa*, *htr1B*, *htr2B*). These candidate genes are implicated as underlying components involved in anxiety and aggressive-related behaviours, and are also a potential target of atrazine. Lastly, Chapter 4 summarises and discusses the key findings of this work in the wider context of the literature.

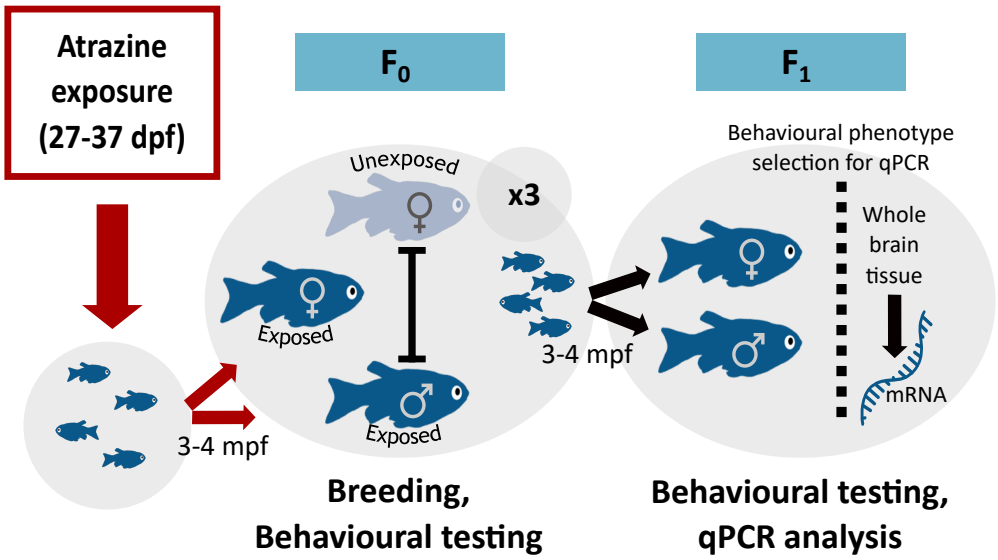


Figure 1.3: Experimental overview of thesis; first, juvenile zebrafish are exposed to atrazine from 27 to 37 days post fertilisation (dpf), then between 3-4 months post fertilisation (mpf), males are bred with unexposed females producing three full-sibling families. $F_0$ males and females then undergo behavioural testing. Between 3-4 mpf, $F_1$ fish have their behaviour tested the same way as the $F_0$'s. Whole brains of $F_1$'s are then tested for gene expression differences that correspond with differences in behaviour.

# Chapter 2

# Intergenerational effects of early life atrazine exposure on zebrafish behaviour

## 2.1 Introduction

Environmental contamination by anthropogenic pollutants is higher than any time in history, and continues to be a growing concern for wildlife, human health and the ecosystem (Vos et al. 2000; Noyes et al. 2015; Wilcox et al. 2016). One group of chemicals that have increased in interest and notoriety within the last decade are endocrine disrupting chemicals (EDCs; Colborn et al. 1993; Vos et al. 2000; Jenssen 2006; Diamanti-Kandarakis et al. 2009). These chemicals interfere with the endocrine system by blocking or mimicking hormone receptors (among a plethora of other complex interactions, see reviews Hotchkiss et al. 2008; Diamanti-Kandarakis et al. 2009; Vandenberg et al. 2012), leading to a range of negative effects ranging from disruption of morphology, behaviour, physiology and reproduction. Endocrine disrupting chemicals resemble aspects of how hormones function at minute concentrations and have the ability to exert different effects at varying levels (Vandenberg et al. 2012). As a result, EDCs frequently exhibit unusual dose-response relationships, (e.g. non-monotonic response Lin et al. 2014b; Vandenberg 2014), and can yield different effects depending on sex, age and exposure duration (Gioiosa et al. 2013; Palanza et al. 2016; see review by Söffker et al. 2012).

The direct effects of EDCs on behaviour has largely focused on the more immediate, reproductive behaviours

(and has been limited to a few EDCs; Söffker et al. 2012), such as reproductive success, sexual displays, nesting behaviours and competition between individuals for mates (e.g. Brian et al. 2006; Saaristo et al. 2010; see review Söffker et al. 2012). However, several recent studies have demonstrated that anthropogenic stressors, including EDCs, can also negatively affect animal personalities under acute exposures (Dzieweczynski et al. 2014; Dzieweczynski et al. 2016; Grunst et al. 2018). Animal personality is characterised as behaviours produced at the level of the individual, which are consistent over time and across different environmental contexts (Dall et al. 2004; Sih et al. 2004b; Réale et al. 2007). These traits include broad patterns of behaviour: anxiety, boldness-shyness, exploration, activity, sociability and aggressiveness (Sih et al. 2004b; Réale et al. 2007). Personality traits have been observed across a variety of taxa (e.g. insects, reptiles, birds, fish, amphibians and mammals; Bell et al. 2009). Personality traits are ecologically significant and can indirectly have major influence over life-time reproductive success, survival of individuals and social interactions (see review Smith et al. 2008). For example, male sleepy lizards (*Tiliqua rugosa*) that are less aggressive tend to associate with more females than more aggressive males (Godfrey et al. 2012). Likewise, bolder homing pigeons (*Columba livia*) are more likely to occupy higher ranks in their leadership hierarchy of the flock, influencing the direction of the flock's collective movement (Sasaki et al. 2018).

Interestingly, the effects of EDC exposure are not consistent across an organisms lifespan. During critical windows of early development (i.e. during early life, embryonic or juvenile development), the environment imparts a stronger pressure on organisms and increases the likelihood of altering developmental trajectories in a range of endpoints such as morphology, physiology and behaviour to mediate environmental pressure (Madsen et al. 2000; reviewed by Jonsson et al. 2014). In addition, developmental plasticity attenuates with age, such that environmentally influenced trajectories tend to become permanent (see Jonsson et al. 2014). For example, guppies raised with an unpredictable food supply became bolder, more exploratory, and tended to be less social at adulthood (Chapman et al. 2010). The plastic ability to respond is particularly adaptive if the early life environment predicts the later life environment, but may be detrimental if a miss-match between phenotype and environment occurs (e.g. Fleming et al. 1997; Visser 2008; Reed et al. 2010). In addition, the lasting impact of strong environmental stressors may be more severe or become pathological later in life. For instance, continuous maternal separation in rodents rapidly augments the stress axis to pathological levels, resulting in depressive, anxiety-like behaviours and changes to neural functioning through to adulthood (Nishi et al. 2013; reviewed by Nishi et al. 2014). In a similar way, exposure to EDCs during early life risks the development of pathological phenotypes, that have the potential to be permanent (Shenoy 2014; Porseryd et al. 2017a).

As well as considering direct effects of EDCs over a single life-time, offspring may indirectly be impacted by

exposure through their parents (Anway et al. 2005; Burton et al. 2014). Non-genetic inheritance is defined as the transmission of traits to offspring. These traits are components of either the parental phenotype or environmental variation, but are not attributable to the inheritance of DNA sequence (Bonduriansky et al. 2012). Presumably this is a proximate mechanism to adjust offspring phenotypes to expected local environments (Burton et al. 2014; O'Dea et al. 2016). A variety of natural environmental stressors including, but not limited to, parental behaviour and social stress have been documented to alter phenotypes in their descendants (see review O'Dea et al. 2016). Studies increasingly find the effects of EDCs can be propagated through generations (see reviews Skinner et al. 2011; O'Dea et al. 2016). For instance, zebrafish developmentally exposed to 17*alpha*-ethinyl estradiol and their offspring exhibit heightened anxiety-like phenotypes and increased shoaling intensity (Volkova et al. 2015b). In another study, $F_3$ descendants of mice embryonically exposed to vinclozolin during sexual differentiation had an altered (and sex dependent) anxiety phenotype (increased in females and decreased in males; Skinner et al. 2008). Intergenerational phenotypes in general may be produced via a range of non-genetic mechanisms. Maternal effects are particularly well documented (e.g. maternal glucocorticoids influencing offspring fitness; Sopinka et al. 2017), and to some extent so too are paternal effects (e.g. paternal care; Curley et al. 2011), but increasingly, studies are documenting non-genetic inheritance due to epigenetic effects (see reviews Jablonka et al. 2009, Curley et al. 2011, and O'Dea et al. 2016 for examples). Epigenetic inheritance, where the patterning of epigenetic mechanisms (such as DNA-methylation or histone modifications) across the genome (i.e. the epigenome) is inherited, may be a causal mechanism underlying changes associated with parental or ancestral EDC exposure (Skinner et al. 2008; Skinner et al. 2010; Skinner et al. 2011; Wolstenholme et al. 2012). For instance, atrazine exposure induced multiple, transgenerational diseases in rat testes that were associated with sperm DNA-methylation mutations McBirney et al. 2017). Variation in gene expression is typically associated with behavioural variation (Alter et al. 2008; Jöngren et al. 2010; Theodoridi et al. 2017). As epigenetic mechanisms mediate between the genetic background and the internal/external environment, epigenetic mechanisms may be important for personality differences (Riyahi et al. 2015; Verhulst et al. 2016; Bierbach et al. 2017). For example, DNA-methylation variation at the dopamine receptor D4 gene in great tit (*Parus major*) was associated with personality difference in exploration (Verhulst et al. 2016). Though, more studies are needed to understand the role of how epigenetics (and other factors such as neuroendocrine factors) contributes to animal personality (Trillmich et al. 2018).

In the present study, I investigated if early life exposure to the neuroendocrine disruptor atrazine (also a common herbicide; see Chapter 1) at environmentally relevant doses affects the personality traits (activity, aggression, exploration, boldness and anxiety) of adult zebrafish and their $F_1$ progeny. Zebrafish make a

good model system for behavioural testing as methods are well developed from the toxicology and behaviour literature (Egan et al. 2009; Maximino et al. 2010) and have since been extensively appropriated into personality research (e.g. Martins et al. 2014; Way et al. 2015; Thomson 2017). Furthermore, zebrafish are a model for research into testing non-genetic inheritance (Wang et al. 2017) and zebrafish males exhibit interesting epigenetic mechanisms dissimilar to other known organisms. After conception by the egg and sperm, studies suggest that the pattern of methylation across the genome (i.e. the methylome) is stably inherited via the father's sperm whereas the mother's methylome is quickly degraded and 're-programmed' (Jiang et al. 2013; Potok et al. 2013), suggesting a possible route for environmental signals to be inherited.

Only a handful of studies have investigated behavioural changes at maturity as result of developmental exposure (e.g. Belloni et al. 2011; Bardullas et al. 2013; Lin et al. 2014a; Shenoy 2014) and no studies, to my knowledge, have investigated specific personality changes due to exposure of atrazine (either acutely or during development). I hypothesised that exposure to atrazine would affect the personality of directly exposed zebrafish and their $F_1$ progeny, through indirect paternal effects. I predicted that anxiety levels would be increased and that the effect would be stronger in females than males due to the sex specific, neurological effects of atrazine on the serotonergic system from transcriptomic studies (Wirbisky et al. 2015). I predicted that an increase in anxiety would result in an inverse decrease in boldness because these two traits tend to be negatively correlated with one another (Thomson 2017). Furthermore, I predicted that aggression levels would be negatively affected by atrazine exposure based on the studies by Shenoy (2012; 2014), whom found decreased aggression levels in the guppy during a mating context. Exploration is closely related to boldness (Thomson 2017), therefore I predicted that I would observe a decrease in exploration. Overall, activity was expected to be higher based on larval and gestational studies exposed to atrazine at environmental doses in fish and rodents respectively (Carmen Alvarez et al. 2005; Lin et al. 2014a) − though how activity levels might translate to adulthood was unknown.

## 2.2 Methods

### 2.2.1 Experimental design

To investigate if early life exposure to atrazine affects the personality of adult zebrafish and their $F_1$ progeny, I exposed $F_0$ juvenile zebrafish to three environmentally relevant concentrations, 0.3ppb, 3ppb, and 30ppb, of atrazine during sexual differentiation (27-37 days post fertilisation (dpf); Takahashi 1977; Uchida et al. 2002; Lee 2015). For an ecological perspective, the concentrations used in this study are typical environmental ranges and were selected based on previous use (e.g. Hayes et al. 2002; Weber et al.

2013; Wirbisky et al. 2015). A random sample of three $F_0$ males per treatment were bred to produce $F_1$ full-sibling families (Figure 2.1). Both $F_0$ and $F_1$ fish were phenotyped at ~3 months of age using three behavioural tests: a novel arena test, a novel object test and a mirror assay (see details below). Brains of $F_1$ fish were further analysed for changes in gene expression of genes involved in the serotonergic system (Chapter 3). All procedures were approved by the University of Otago Animal Ethics Committee, protocol 44/16.

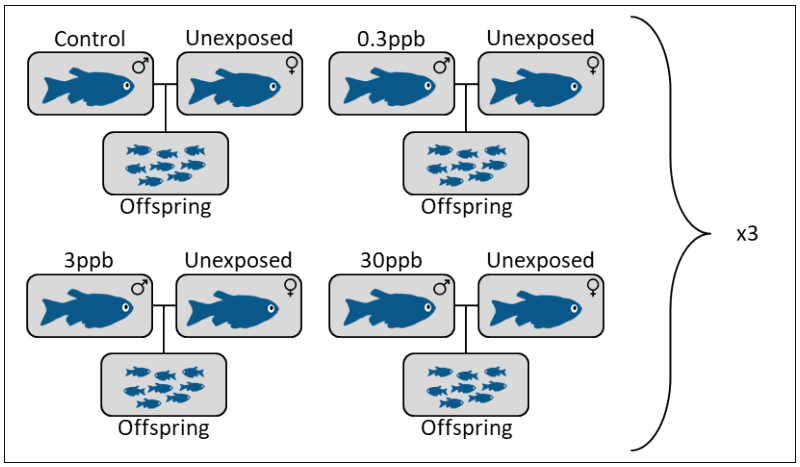


Figure 2.1: Design for assessing the effects of atrazine on $F_1$ progeny. A male from each treatment is bred with an unexposed female creating a full-sibling family. There were three families per treatment produced for behavioural phenotyping, 12 full-sibling families in total.

### 2.2.2 Zebrafish husbandry

Breeding, husbandry and atrazine exposure took place within the Otago Zebrafish Facility (OZF). The OZF is a temperature controlled facility with a 14 hr (0800-2200 hr) dawn-dusk light cycle. The conductivity, temperature and pH were maintained at range of 390-458µS, 25.2-26.1 °C and 7-7.8 pH, respectively. Phenotyping was performed in the Zoology Department. Room temperature was controlled at 25 °C and had a 13.5 hr (0700-2030 hr) light cycle with 30 min of simulated dawn and dusk at the start and end of each day.

$F_0$ embryos were produced by group spawning wildtype zebrafish (strain AB; 24 females; date of birth (DOB); 22/3/16 and 34 males; DOB; 26/2/16) using a Techniplast iSpawn Breeding System. Embryos were collected 30 min to 1 hour post fertilisation (hpf) and incubated in petri dishes (90mm diameter) with E3 media (Cold-Spring-Harbor-Protocols 2008) at 28.4°C until 4 dpf. Dead or unfertilised embryos were removed from petri dishes once every day and E3 media was replaced every day until hatching. After four

days, hatched fry were moved into tanks with AquaOne, 5 parts per trillion (ppt) Aquaria Salt solution (synthetic sea salt), at a density of about 12 fry per litre. From 4-10 dpf zebrafish larvae were fed ad libitum, twice daily with dry food ZM000 (ZM systems; morning and afternoon) and once daily with rotifer (*Brachionus* spp) at midday. At 10 dpf water flow was turned on. After 10 dpf, fry were fed ad libitum, twice daily with dry food mixture of ZM000 and ZM100 (morning and afternoon), and once daily with and live Artemia (*Artemia salina*; midday).

### 2.2.3 Atrazine exposure

Atrazine was purchased from Sigma-Aldrich (Auckland, New Zealand; CAS 1912-24-9), and a stock solution was prepared three days before exposures started (23/02/17) by dissolving 5mg of atrazine powder in 200mL of purified water to achieve solubility. Ten day exposures to atrazine took place in 44mm x 600mL glass beakers. Eight beakers of 500mL system water were prepared; two served as controls and replicated atrazine concentrations of 0.3ppb, 3ppb or 30ppb were administered to the other six beakers. Forty zebrafish fry (27dpf) were randomly assigned to each of the eight beakers. The atrazine exposure regime lasted 10 days (26/02/17 through to the 8/03/17). This 10 day exposure regime was chosen because it covered the early-mid period of sexual differentiation in zebrafish (Takahashi 1977; Uchida et al. 2002; Lee 2015), where the probability of epigenetic information transfer is hypothesised to be high (based on studies of sexual differentiation and epigenetic inheritance in mammals; it is unknown whether this period is equivalent in zebrafish; Hackett et al. 2013). The juvenile period was also chosen to examine if the sensitivity window (where effects become permanent) of atrazine exposure extends beyond traditional embryonic (or prenatal) exposure regimes, resulting in behavioural changes at adulthood (e.g. Belloni et al. 2011; Shenoy 2014). Fry were fed once a day with ZM100 dry food during the exposure regime and water was changed every day (from day 2 until day 10); thus, concentrations of atrazine were also renewed every day. At the end of 10 days, fry were moved to new tanks in a Techniplast toxicology filtration system, which filtered water every 30min. After three days, fry were re-transferred onto the main OZF system and left to grow.

### 2.2.4 *In vitro* fertilisation, to produce $F_1$ progeny

At sexual maturity, ∼3 months post fertilisation (mpf; 106−141 dpf), three males per treatment were sampled to produce three $F_1$ full-sibling families per treatment (Figure 2.1) using *in vitro fertilisation* (IVF) with untreated AB females (DOB=13/05/16). Breeding was done by setting up two males per treatment with an untreated female in breeding boxes (length=18.5cm, width=11cm, depth=8.5cm, water depth=6cm), with the sexes separated by a divider. This setup enables the pheromones of males to contact

the female, synchronising their spawning behaviour and stimulating female zebrafish to release oocytes in preparation for mating (Hisaoka et al. 1962; Gerlach 2006). Breeding boxes were setup in the afternoon and left overnight. The following morning IVF was performed using a modified protocol (see Appendix I; Johnson et al. 2018). Data on survival at 24 hpf and hatching at 48-96 hpf was also collected (see Appendix II). Embryos were transferred into petri dishes (90mm diameter) with E3 media and left to grow, following the same protocol described above.

### 2.2.5 Personality assays employed: descriptions and rationale

There are several ways in which to measure personality, but it must be suited to the system in question. Below I describe the three methods employed in this thesis for delineating personality phenotypes between zebrafish individuals.

The novel arena test also known as the novel tank diving test is a routinely used assay to test changes in levels of anxiety in response to pharmacological agents, as well as other behavioural measures, such as the activity profile, exploration and boldness (Egan et al. 2009; Maximino et al. 2010). Zebrafish, when exposed to an unfamiliar environment, tend to dive to the bottom of the test arena and tend to remain in the lowest portion (bottom dwelling or freezing), avoiding the higher portions of the water column, and after a few minutes the fish usually begin to explore the other areas of the tank (Egan et al. 2009; Maximino et al. 2010). Bottom dwelling is a common measure of anxiety and correlates negatively with the neurotransmitter serotonin (5-HT; Maximino et al. 2012). In fish, exploration may be tested by the amount of time an animal spends in different areas of the tank (Thomson 2017). Boldness in the novel arena test is based on the fish's risk-taking behaviour in relation to areas of higher risk such as entering or spending more time in areas of higher risk. For instance, spending more time in portions of the water column closer to the surface would constitute a greater boldness phenotype (Maximino et al. 2010).

The novel object test is adapted from rodent studies (Ennaceur et al. 1988) and is considered a complementary assay to the novel arena test to examine differences in boldness and anxiety in fish (Wright et al. 2006a; Ogwang 2017; Maximino et al. 2010). When exposed to a novel object (after an acclimation period within a novel environment), zebrafish show a tendency to avoid the foreign object, exhibit bottom dwelling behaviour or freezing, reduce their activity profile, and after a certain amount of time the zebrafish will begin to occasionally inspect it (Wright et al. 2003; Wright et al. 2006a). Approaching within 1-1.5 body lengths of the novel object is considered a measure of boldness (Wright et al. 2003; Wright et al. 2006a), and is interpreted as a predator inspection behaviour (Wright et al. 2003; Wright et al. 2006a; Ogwang 2017; Maximino et al. 2010).

The mirror test is typically used in assessing an individual's level of aggression (e.g. Ariyomo et al. 2012; Ariyomo et al. 2013a; Way et al. 2015). The mirror test is an alternative to dyadic fights (fights between two individuals) as both assays elicit similar antagonistic responses to one another. The fish are unable to recognise their reflection and are thought to perceive the mirror image as an intruder (Oliveira et al. 2005; Way et al. 2015). Zebrafish naturally form hierarchies and both males and females will be aggressive to one another to maintain dominance and monopolise resources (Spence et al. 2008). The mirror produces a size matched individual removing the variation of the size of an opponent. Mirror images also differ from dyadic fights on a molecular and hormonal level. A dyadic fight always results in either a winner or loser, influencing the level of whole body androgens (11-ketotestosterone and testosterone) and cortisol which in turn leads to transcription changes (depending on the outcome), whereas a fight between a mirror opponent does not resolve, allowing insight into individual level of antagonistic behaviour without the likely confounding effects of transcription and hormonal changes (Oliveira et al. 2005; Oliveira et al. 2016; Teles et al. 2016). This method was adapted from Ariyomo et al. (2013b) and Way et al. (2015), and though only capturing a smaller portion of the repertoire of zebrafish aggressive behaviour (see Oliveira et al. 2011; Kalueff et al. 2013), the mirror test was considered over a dyadic fights for logistical reasons, to limit possible confounding changes in gene expression and hormones (Oliveira et al. 2005; Oliveira et al. 2016; Teles et al. 2016) and because the correlation between an individual's behaviour in a flat mirror assay and a dyadic fight is moderately high (Ariyomo et al. 2013b; Way et al. 2015).

### 2.2.6 Behavioural phenotyping: experimental protocols

Prior to behavioural assays, all fish were transferred from the OZF to the animal rearing facility in the Otago Zoology Department. Zebrafish were left to acclimate to the new facility for 7 days before behaviour tests began. Fish were fed as described above, except on the day of behavioural phenotyping, where fish were fed after filming finished. The $F_0$ fish were transferred at 113dpf and the $F_1$'s at 110-131dpf.

The novel arena (Figure 2.2), the novel object (Figure 2.3) and the mirror tests (Figure 2.4). The novel arena (Figure 2.2), the novel object (Figure 2.3) and the mirror tests (Figure 2.4) were run consecutively in the same tank with each assay lasting 10min, with three fish assayed simultaneously in three separate tanks (length=30cm, width=15.5cm, height=27cm filled with 8L of system water to a depth of 19.5 cm). White coloured white-board film was fixed to the back, bottom and side of each tank wall to limit light and reflection during filming. A flat mirror (vertical length = 19cm, width=15cm) was fixed to the outside wall of each tank (mirror placed on the left side of one tank, and the right side of the other two tanks). During the novel arena and novel object tests, the mirrors were covered with a removable, hard plastic opaque

barrier. Tanks were lighted from 30cm above the tanks with one 240V 48LED aluminium light strip and from behind using three Godox LED170 lights (31-35.5cm away) to provide diffusing light to increase fish contrast during filming. Fish were filmed with a Basler acA1300-60/gc GigE camera with a 4.4-11mm lens placed about 112cm away from the row of tanks and live-tracked using EthoVision XT behavioural tracking software version 11.5 (Noldus et al. 2001).

Previous work in our lab has shown that the repeatability (the intra-class correlation values, i.e. animal personality) of zebrafish behaviours is dependent on an initial exposure to the assay regime (including exposure to the novel arena and novel object) and thereafter, in any subsequent behavioural assays, the exhibited behaviour during the trial is typical of the individual's personality phenotype (Thomson 2017). In light of these findings, a 'tank experience' was provided after 7 days of acclimation. The repeatability of behaviours exhibited during the mirror tests is not dependent on an initial exposure (see Appendix IV), but was included to maintain consistency with the other behavioural tests.

**Novel arena test**

The first test, 'novel arena' (Figure 2.2), consisted of carefully netting the fish from their home tank into the novel, empty tank. Live-tracking with EthoVision started 10-30sec after the fish were placed in the tanks. Within EthoVision, the novel arena was divided into 12 square zones (four across, three down, approximately 5x5cm) to measure exploration. The total distance moved (cm), velocity (cm/s), the frequency of transitions into the top third, time spent moving/ not moving and the cumulative duration (sec) in each zone was recorded. The time spent within each third of water column was calculated by the sum of time spent in each zone of the same level. The latency to enter the top zone was taken as the first moment that a fish entered into any one of the four zones in the top third. Exploration was calculated as the standard deviation of time spent in each of the 12 zones (i.e. an explorative fish would have a standard deviation closer to zero, meaning a fish spent an equal amount of time in all 12 zones, a high standard deviation indicates the fish was less explorative, preferring to spend the majority of the assay in a few zones; Thomson 2017).

**Novel object test**

After filming the novel arena, the novel object test was conducted (Figure 2.3). An orange rubber bung (measuring 3.2cm long, 4.3cm wide at the bottom and 3.7cm wide at the top) attached to plastic fishing wire was placed slowly into the middle of the tank, hanging in the middle of the arena (at an approximate depth of 6.9cm below the surface of the water). Live-tracking started 10-30sec after the rubber bung was positioned, once the water settled. Within EthoVision, the novel object arena was divided into an upper

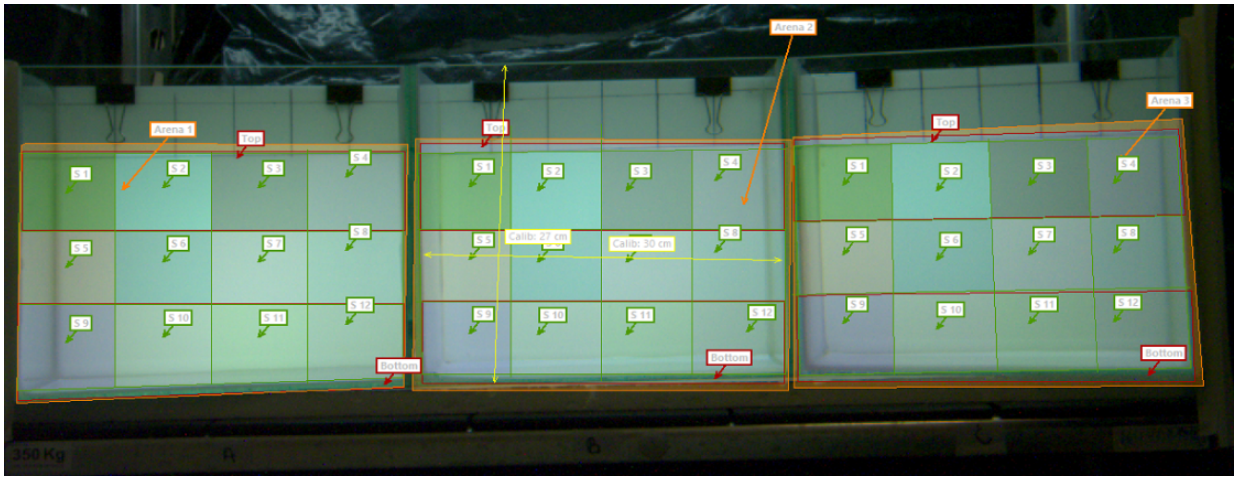


Figure 2.2: The settings for quantifying behaviours during the novel arena test. Within EthoVision XT, a four by three square grid is superimposed over the three test tanks. Each differently coloured square indicates a different zone (of ∼5cm wide and long).

and lower zone and a zone around the novel object. The total distance moved (cm), the mean velocity (cm/s), whether or not the fish entered the novel object zone (0,1), and the cumulative duration (sec) in the bottom half of the tank was recorded.

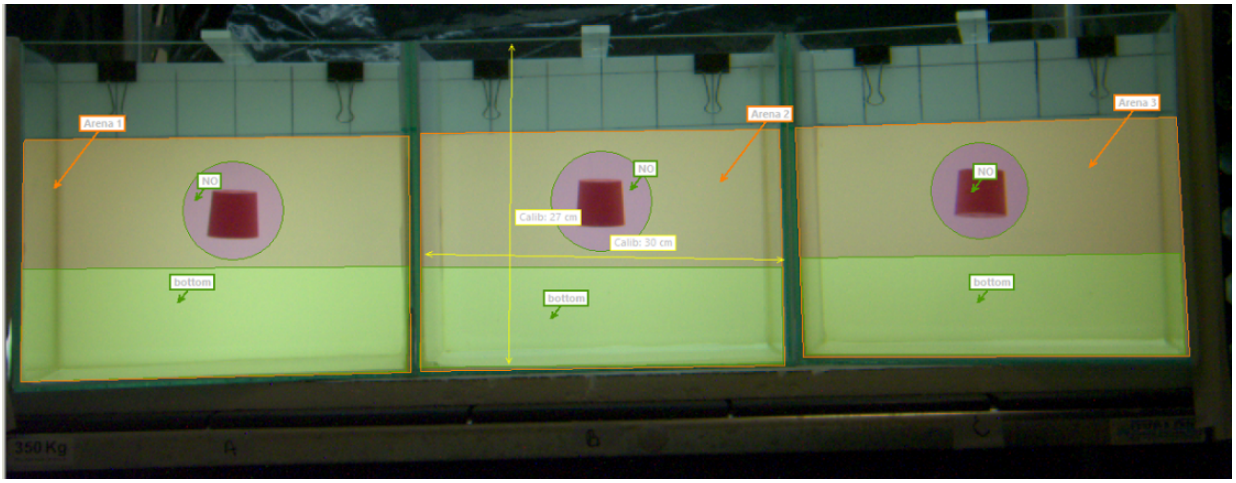


Figure 2.3: The settings for quantifying behaviours during the novel object test. Within EthoVision XT, a circular purple zone, measuring ∼1.5 body lengths is superimposed over the novel object that hangs from the top of the tank. A green zone superimposed onto the tank indicates the bottom zone.

### Mirror test

After filming the novel object assay, the mirror test was conducted (Figure 2.4). The novel object and opaque barrier were removed, and live-tracking started 10-30sec once the water had settled. The total

distance moved (cm), velocity (cm/s), the frequency of times the fish entered into mirror zone and the time spent interacting with the mirror (sec) was recorded.

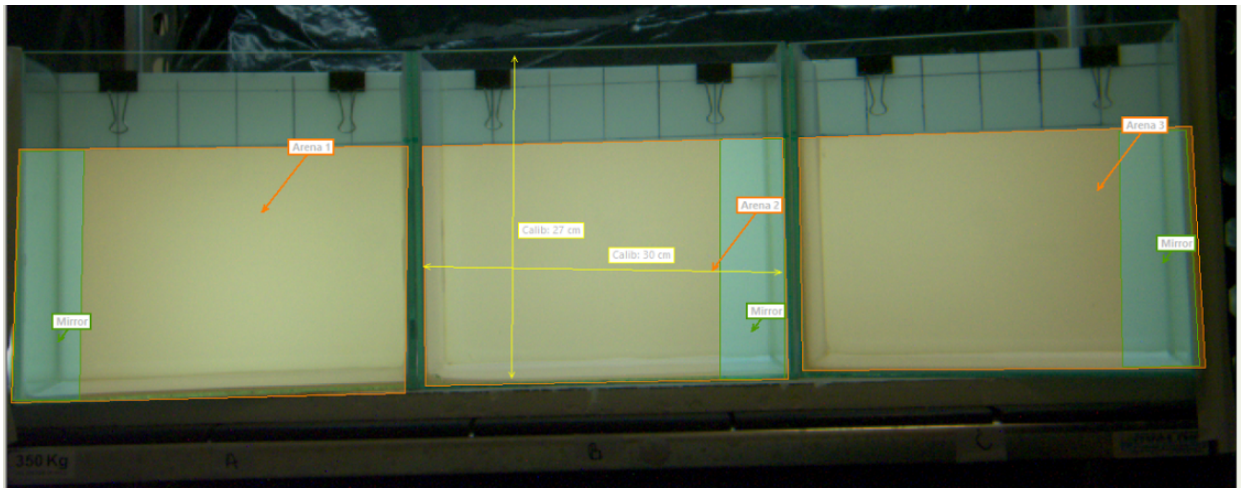


Figure 2.4: The settings for quantifying behaviours during the mirror test. Within EthoVision XT, a blue zone measuring ~5cm wide, along the side of the tank indicates the mirror zone, where the flat mirror was located.

**Faulty tracking**

Tracking was occasionally lost when fish velocity was lower than 1.5cm/s or stopped moving and settled on the bottom of the tank, this was noted and was corrected for post assay. Tracking continued if the fish increased speed or discontinued resting. Other tracking errors that occurred during filming were manually identified and fixed using EthoVision's 'integrated vision function', and were either corrected or the video was re-analysed using different detection settings. During the behavioural testing of the $F_1$ generation, there were eight instances during the novel object assay where tracking was unable to be accurately established due to experimental error. All cases were therefore excluded from subsequent analyses; three controls (two male, one female), one 0.3ppb male, three 3ppb fish (one male and two females) and one 30ppb female.

### 2.2.7 Statistical analysis

All statistical analyses were conducted in R, version 3.5.0 (R Core Team 2018). All analyses described below contained the two main predictors, treatment (i.e. controls, 0.3ppb, 3ppb, 30ppb) and sex (male and female) and an interaction term. The interaction term was removed if non-significant and the data was re-analysed without it. All $F_0$ behavioural measures were analysed with linear models (LMs) or generalised linear models (GLMs). All $F_1$ behavioural measures were analysed with linear mixed effects models (LMMs) or

with generalised linear mixed effects models (GLMMs) using the package '*lme4*' version 1.1-1.3 (Bates et al. 2015), with family ID included as a random effect to account for genetic influences (with some exceptions, see Appendix V). Specific details on each behavioural measure analysed can be found in Appendix V.

For all models, results are presented with estimates and 95% confidence intervals (CI). Confidence intervals were calculated using the '*confint*' function. For reference, 95% CI that do not include zero are statistically significant. The full model output, including parameter coefficients (back transformed where appropriate), parameter standard error (SE), test statistics and p-values are reported in Appendix VIII. P-values for LMMs and GLMMs were calculated using the '*lmerTest*' package (Kuznetsova et al. 2017). A subset of the behavioural data is presented in text, other measures can be found in Appendix VI. See Appendix III for specific sample sizes used for each assay, per treatment and sex (sample size ranges are given in figure legends). Descriptive statistics are reported as means ± SE in Appendix VI.

Lastly, in some instances of $F_1$ analyses, family level variation was suspected of influencing the model outputs, indicating a genetic component of behaviour. Therefore, likelihood ratio tests (LRT) were used to compare models with and without the random effect. The variance and standard deviation (SD) from the LMM or GLMM model output are reported in text when they occurred (models summarised in Appendix IX, Table A9.1). Family variation was also visually compared across treatments to investigate if atrazine altered the amount of variation present at the family level (Appendix IX).

## 2.3 Results

### 2.3.1 Anxiety

Changes in anxiety occurred in the lowest $F_0$ treatment (0.3ppb) in the novel arena assay. $F_0$ fish from the 0.3ppb treatment spent significantly longer in the bottom zone of the novel arena compared to controls (Est. 83.03 [1.18, 164.87 CI]; Figure 2.5 A), with no other treatment or sex differences occurring. In the $F_1$'s there was significant sex difference; males spent less time in the bottom zone (Est. $-86.78$ [$-128.30$, $-45.25$ CI]) than females (Figure 2.5 B). $F_1$ controls (both males and females), on average spent less time in the bottom zone than all other treatment groups (Figure 2.5 B), however no significant treatment effects were observed owing to the large variance produced at the family level (Appendix IX). The family level variance was 11425 sec with a SD of 106.9 sec and explained ($\chi^2 = 33.6$, df = 1, p <0.001) a significant portion of variation present in the LMM model (Appendix IX). Nonetheless, there appears to be a suggestive trend of more time spent in the bottom zone with fish from treated males.

### 2.3.2 Exploration

In the novel arena assay, $F_0$ fish from the 0.3ppb treatment were significantly less exploratory than controls (Est. 15.12 [2.94, 27.30 CI]; Figure 2.5 C), but no other treatment effects were observed. Sex differences were marginally non-significant, with males tending to be more exploratory than females (Est. −7.705 [−16.17, 0.76]). In the $F_1$'s there was no difference amongst treatments in exploratory behaviour, but there was a significant sex difference observed, with males being more exploratory than females (Est. −12.43 [−20.77, −4.09 CI]; Figure 2.5 D).

### 2.3.3 Activity

There was a significant sex difference in activity observed in both generation during the novel arena assay, with males travelling a greater total distance than females ($F_0$ males Est. 532.19 [198.37, 866.02 CI]; $F_1$ males Est. 1255.64 [892.14, 1619.14 CI]); however, no treatment differences were observed in either generation in the total distance moved (Figure 2.6 A and B).

During the novel object assay, males from both generations travelled a significantly greater total distance than females ($F_0$ males Est. 631.47 [284.32, 978.62 CI]; $F_1$ males Est. 1257.18 [917.76, 1596.60 CI]). But no treatment differences were observed in either generation (Figure 2.6 C and D).

During the mirror test assay, males travelled a significantly greater total distance than females ($F_0$ males Est. 700.55 [400.53, 1000.57 CI]; $F_1$ males Est. 689.30 [393.78, 984.89 CI]). In the $F_0$'s there was no treatment effect observed (Figure 2.6 E). However, activity levels were significantly different amongst $F_1$ fish from the atrazine treatment compared to controls. $F_1$ fish from both 0.3pbb (Est. −110.63 [−175.35, −45.91]) and 30ppb (Est. −95.98 [−160.24, −31.72]) travelled a significantly less total distance than controls (Figure 2.7 F). $F_1$ 3ppb fish also travelled less than controls, but this observation was marginally non-significant (Est. −56.32 [−120.25, 7.61] Figure 2.7 F).

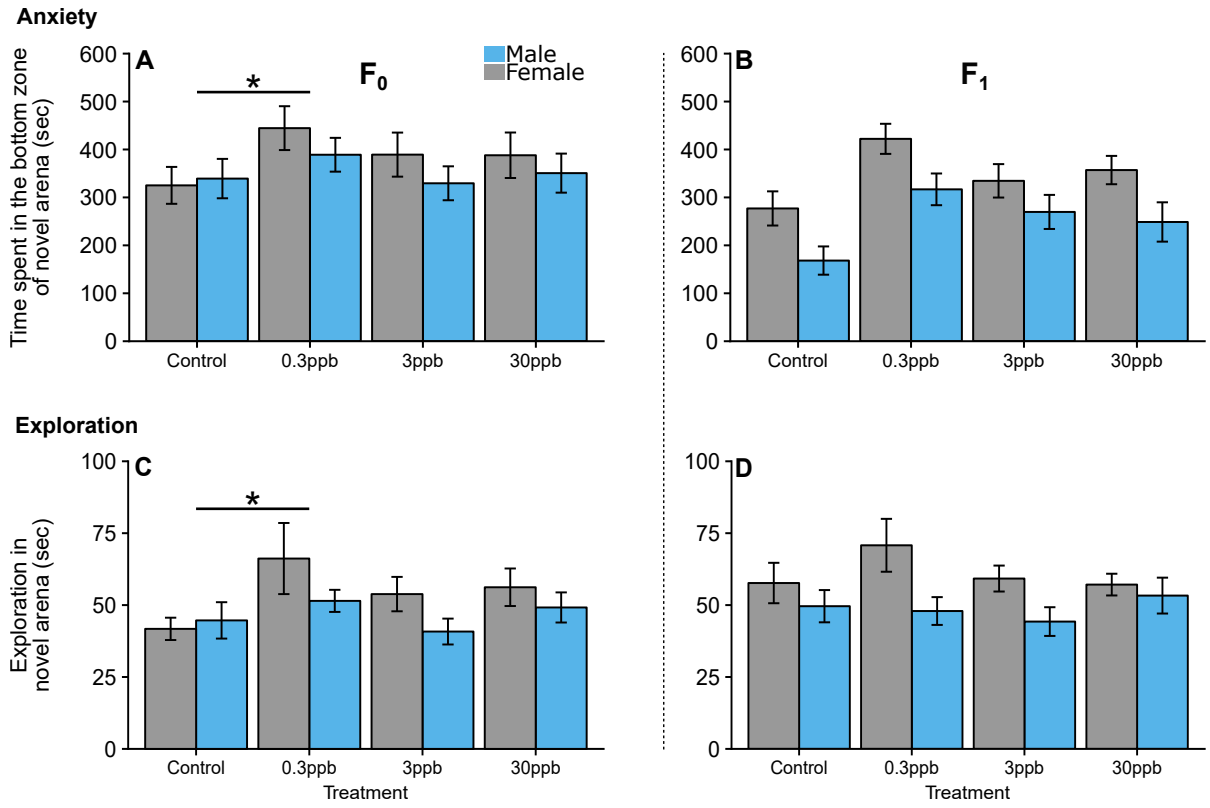


Figure 2.5: Measures of anxiety and exploration of atrazine exposed $F_0$ fish (left) and $F_1$ offspring from exposed males (right). (A) and (B) the time spent in the bottom zone of the novel arena (sec) during the novel arena test; (C) and (D) the standard deviation of exploration (sec) during the novel arena test. Bars represent means, with error bars representing standard errors of the mean. An asterisk indicates a significant difference between controls and atrazine treatment ($p < 0.05$). For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=18-30. Total sample size for $F_0$'s were n=93; $F_1$'s were n=190, with n=3 families per treatment.

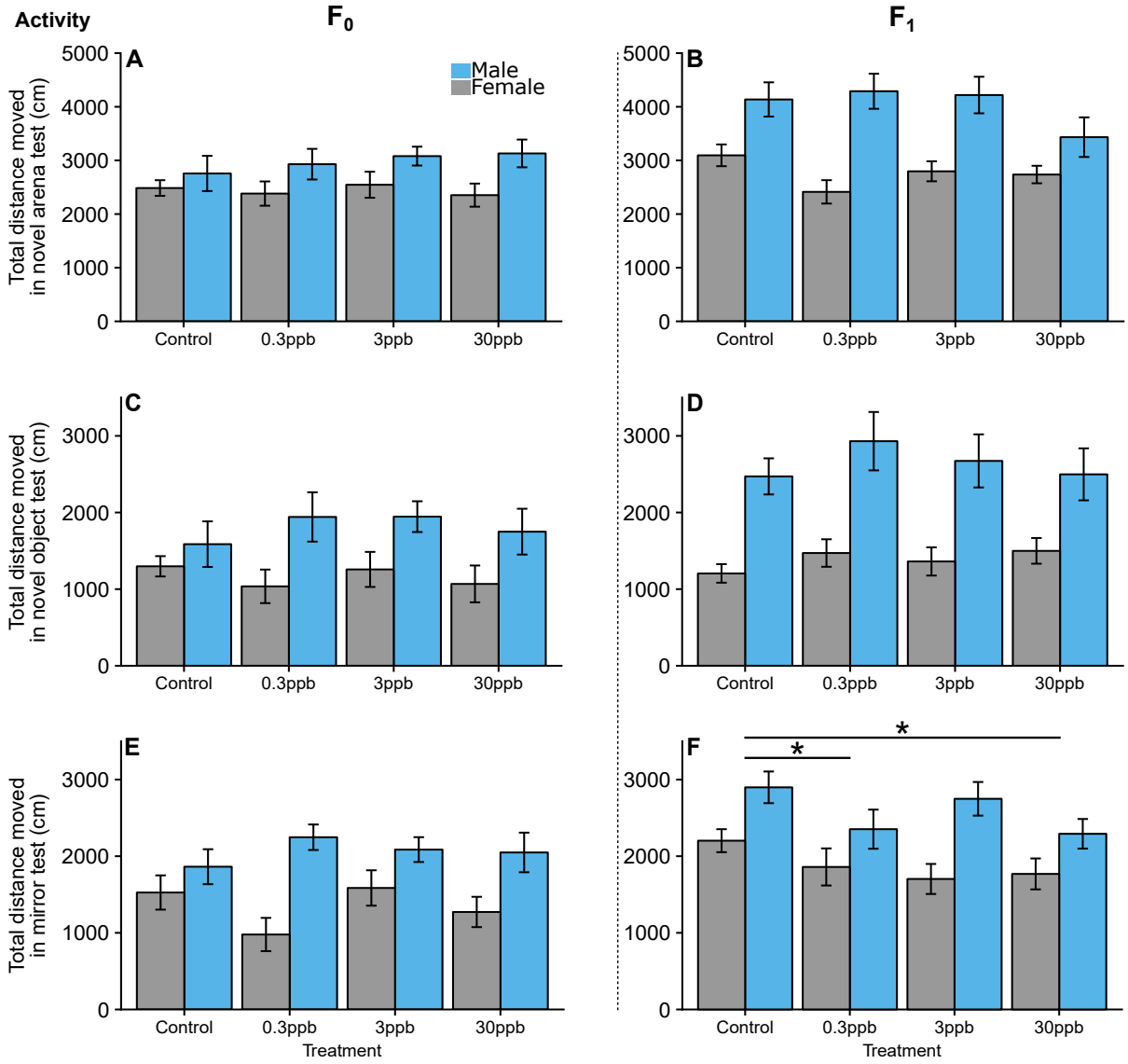


Figure 2.6: Measures of activity of atrazine exposed $F_0$ fish (left) and $F_1$ offspring from exposed males (right). The total distance moved (cm); (A) and (B) during the novel arena test; (C) and (D) during the novel object test; (E) and (F) during the mirror test. Bars represent means, with error bars representing standard errors of the mean. An asterisk indicates a significant difference between controls and atrazine treatment ($p < 0.05$). For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=18-30. Total sample size for $F_0$'s were n=93; $F_1$'s were n=190, with n=3 families per treatment.

### 2.3.4 Boldness

Developmental atrazine exposure altered measures of boldness in both the $F_0$ and $F_1$ generation in the novel arena assay (Figure 2.7 A and B). Direct $F_0$ atrazine exposure significantly increased latency in the 30ppb concentration (Est. 2.36 [1.17, 6.96 CI]) and non-significantly increased latency in the 3ppb treatment (Est. 2.36 [−1.03, 5.75 CI]; Figure 2.7 A), but no significant treatment differences were observed in the 0.3ppb treatment (Figure 2.7 A). Sex itself did not influence overall latency in the $F_0$'s, however several treatment:sex interactions were observed. $F_0$ males from both the 3ppb (Est. −3.88 [−13.46, −1.12 CI]) and the 30ppb (Est. −5.39 [−18.73, −1.55 CI]) treatment showed a significantly lower latency in comparison with females from the same treatment when compared to controls. Differences between males and females from the 0.3ppb treatment were marginally non-significant (Est. −3.54 [−13.20, 1.02 CI]). On average, $F_0$ female latency increased with increasing atrazine concentration, whereas treated $F_0$ males exhibited lower latency than controls, but then plateaued regardless of the atrazine concentration (Figure 2.7 A).

In the $F_1$ generation, all treatments exhibited significantly higher latency compared to controls (0.3ppb Est. 6.16 [2.01, 18.73 CI]; 3ppb Est. 4.07 [1.34, 12.30 CI]; 30ppb Est. 3.71 [1.22, 11.25 CI]; Figure 2.7 B), but no interactive effects were observed. Males, regardless of treatment took significantly less time to enter the top zone compared to females (Est. −2.3 [−2.32, −2.27 CI]). On average, $F_1$ female latency was highest in the lowest treatment (0.3ppb atrazine) and then declined with increased atrazine concentration. Control $F_1$ females displayed the lowest latency on average compared to all other females (Figure 2.7 B). The variance produced at the family level was 1.85 sec with a SD of 1.36 and significantly influenced the model ($\chi^2$ = 157.4, df = 1, p <0.001; Appendix IX).

In both generations there was no treatment effect observed in whether or not an individual approached the novel object (Figure 2.7 C and D). In the $F_0$'s there was no sex effect observed, whereas in the $F_1$ generation, males approached the novel object significantly more so than females did (Est. 0.87 [0.76, 0.93 CI]; Figure 2.7 D).

### 2.3.5 Aggression

There was a non-significant trend of reduced time spent interacting with the mirror with increasing atrazine concentration amongst the $F_0$ males, however no specific treatment effects nor sex differences were observed (Figure 2.7 E). Significant treatment effects were observed amongst the $F_1$'s, where $F_1$ fish from the 0.3ppb (Est. −110.63 [−45.91, −3.35 CI]) and 30ppb (Est. −95.98 [−31.72, −2.93 CI]; Figure 2.7 F) treatments spent significantly less time in the mirror zone compared to controls. Fish from the 3ppb treatment also

spent less time in the mirror zone but this was marginally non-significant (Est. −56.32 [−120.25, 7.61 CI]; Figure 2.7 F). There was also a clear sex difference in the $F_1$'s, with males spending significantly more time in the mirror zone than females (Est. 74.42 [27.96, 120.89 CI]).

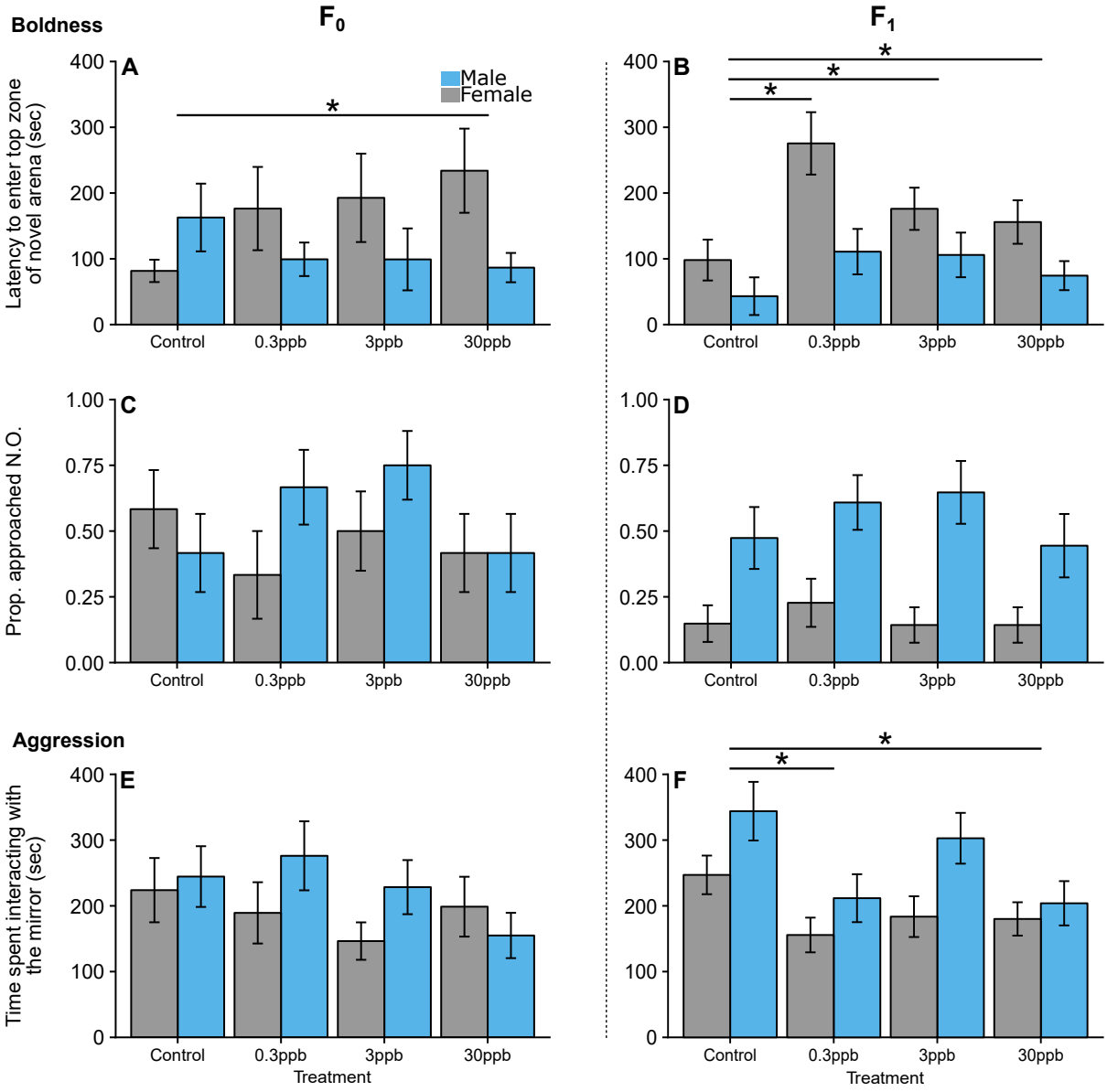


Figure 2.7: Measures of boldness and aggression on atrazine exposed $F_0$ fish (left) and $F_1$ offspring from exposed males (right). (A) and (B) the latency to enter the top zone during the novel arena test (sec); (C) and (D) the proportion of fish that approached the novel object; (E) and (F) the time spent interacting with the mirror (sec). Bars represent means, with error bars representing standard errors of the mean. An asterisk indicates a significant difference between controls and atrazine treatment ($p < 0.05$). For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=18-30. Total sample size for $F_0$'s were n=93; $F_1$'s n=182-190, with n=3 families per treatment.

## 2.4 Discussion

### 2.4.1 Revisiting aims

The aims of this study were two-fold: first, to identify if environmentally relevant concentrations of atrazine exposure during zebrafish juvenile development altered the personality phenotype in adulthood, and secondly, if the offspring of exposed males also showed an altered phenotype. I predicted that direct and indirect exposure to atrazine would increase anxiety levels and decrease boldness, aggression and exploration, and potentially alter activity levels in both generations. Furthermore, I predicted that increases in anxiety would be female biased.

Overall, several behavioural measures were altered under juvenile atrazine treatment in adulthood and manifested in the adult offspring, though not all effects were consistent across all doses. Several significant changes to behaviour occurred in either generation. Changes to the latency to enter the top zone of the novel arena (boldness) were particularly altered. $F_0$ females exhibited increased latency with increasing concentration and $F_0$ males exhibited decreased latency in a threshold-like pattern compared to controls. In the following generation, $F_1$ females exhibited a significant increase in latency that decreased with increasing concentration, but there was little change amongst $F_1$ males compared to controls. The time spent interacting with the mirror (aggression), and the total distance moved during the mirror test (activity) were significantly reduced amongst the $F_1$ progeny in the 0.3ppb and 30ppb treatment groups, with some evidence for reduced aggression but no evidence for altered activity in the mirror test amongst the $F_0$'s. Lastly, the time spent in the bottom zone (anxiety), and the time spent exploring the novel arena (exploration) were significantly reduced in the $F_0$ 0.3ppb group only, while in the $F_1$'s there was some evidence of alterations in anxiety and exploration. These results suggest that atrazine disrupts aspects of anxiety, boldness, aggression and exploratory personality phenotypes at environmentally relevant concentrations, and these effects can be transferred via sperm to the following generation.

### 2.4.2 Effects of atrazine exposure on boldness, anxiety and exploration

One of the strongest effects of the atrazine treatment was observed in the latency to enter the top zone (latency) of the novel arena (a measure of boldness; Figure 2.7 A and B). Changes in latency occurred in different directions depending on the sex, with phenotypes more pronounced in females (in both generations) than males. $F_0$ females exhibited increased latency with increasing atrazine concentration compared to controls, whereas $F_0$ males displayed reduced latency across all treatments compared to controls. This phenotype was inconsistent between generations, female $F_1$ offspring from atrazine treated males exhibited

decreased latency with increased atrazine concentration (though all higher than control females).

In an ecological setting, entering the top portion of the water column where little vegetation occurs likely carries a higher risk of avian predation and may increase conspicuousness of the individual to fish predators (Werner et al. 1983; Rypel et al. 2007; Pink et al. 2018). Avian predation is not well documented in wild zebrafish, but populations tend to live in tandem with the common kingfisher (*Alcedo atthis*) and the Indian pond heron (*Ardeola grayii*), which are hypothesised to be the main avian predators (Spence et al. 2006). When confronted by an avian predator simulation above the tank (e.g. a bird silhouette or a black dot increasing in size, simulating an approaching bird), defensive behaviours such as bottom dwelling are triggered (Luca et al. 2012a; Luca et al. 2012b). The costs of entering the top portion of the water column may be offset by access to resources such as surface dwelling invertebrates, which make up the largest portion of wild zebrafish diet (Pitcher et al. 1988; McClure et al. 2006; Arunachalam et al. 2013; but also see; Spence et al. 2007). If the highest portion is perceived to carry a higher risk, then changes in latency observed from atrazine treated fish might reflect a distortion in an individuals ability to either assess risk, or a change in propensity to take risks regardless of the risk entailed. Based on this data, it is difficult to tease apart these two components as they are theoretically both tied to one another, though the consequences of either remain the same. The lack of difference (amongst the $F_0$'s) in the time spent in the top zone or in the frequency to enter the top zone might indicate a stronger hypothesis for the latter. However, the non-significant reduction amongst the treated $F_1$'s might suggest more weight for the former. Changes in perceived risk is not the only factor impacting survival that needs to be considered (see General Discussion).

In addition to changes in boldness, developmental atrazine exposure altered zebrafish anxiety and exploratory phenotypes in the lowest dose (Figure 2.5 A and C). Previous findings have suggested that atrazine exposure produces an anxiety-like behaviour in fish (Steinberg et al. 1995) and in rodents (Lin et al. 2013; Walters et al. 2015). Changes in exploration profiles have also been observed in rodents (Belloni et al. 2011) as well as in insects (Figueira et al. 2017), but not so in fish (Schmidel et al. 2014).

Risk assessment of the environment is crucial during foraging and exploration (Pitcher et al. 1988). Individuals that under assess risk, or are generally bolder, increase their susceptibility to predation (Smith et al. 2008; MacPherson et al. 2017). Whereas over assessment or more anxious individuals may increase long-term survival, but may place costs on other functions, such as the ability to forage (Wolf et al. 2007; Biro et al. 2008b). Furthermore, in a given contexts, individuals that are less willing to explore their environments may be at risk of starvation if resources are generally scarce, limit the ability to find suitable mates, or to seek out pertinent information relating to survival if new threats arise (e.g. increased predator

prevalence or territorial intruders; MacPherson et al. 2017). Populations naturally contain an assortment of individuals that display risk-taking or risk averse like personality phenotypes (Bierbach et al. 2017), with each strategy hypothesised to be maintained by life-history trade-offs (i.e. between early vs later life reproduction; Schuett et al. 2015). Populations may also differ in the extent of phenotypes produced, depending on factors such as differences in predation rates or food and mate availability, and may change across space and through time if these factors also change (Bierbach et al. 2015; Barbosa et al. 2018b). It stands to reason that the consequences of EDCs influencing risk assessment, anxiety or exploratory behaviour are wide reaching at the individual level by influencing their probability of survival (MacPherson et al. 2017), and may also extend to the population level by pushing groups of exposed individuals in one direction or possibly pushing each sex in differing or converging directions (or by disrupting signals; Senior et al. 2014; White et al. 2017). For instance, diminished behavioural variation commonly found in exploited fishing populations, due to the tendency of bolder individuals to take bait, have led to a skewed distribution of behavioural phenotypes comprising a greater proportion of anxious individuals (Biro et al. 2008a).

### 2.4.3 Possible mechanisms behind altered boldness, exploration and anxiety

Mechanistic explanations behind this distorted boldness, exploratory and anxious phenotype are difficult to pinpoint, but several neurotransmitter systems have been implicated in atrazine exposure across taxa (fish, mammals and insects), including the cholinergic (Schmidel et al. 2014), dopaminergic (Walters et al. 2015; Figueira et al. 2017) and serotenergic (Wirbisky et al. 2015) systems. A cell culture study found that atrazine causes neuronal damage and augments neurotransmitter release (Peña-Contreras et al. 2016). In particular, these aforementioned studies have found greater distortion towards females than males. For example, Wirbisky et al. (2015) found that there was a significant reduction in neurotransmitter turnover of serotonin (5-HT; i.e. the rate of 5-HT synthesis, indicative of reduced serotonergic activity), in adult zebrafish females exposed as embryos, but not in males. Brain transcriptome studies also implicate several genes involved in the serotonergic pathway, steroidogenesis and neurotransmission within the hypothalamus-pituitary-interenal and hypothalamus-pituitary-thyroid axes (Weber et al. 2013; Wirbisky et al. 2015; 2016a; 2016c).

Another possible reason for a distortion in risk assessment might be a result of atrazine's immmunotoxic action. Atrazine, as well as a neuroendocrine disruptor, is also immunotoxic acting via the disregulation of specific cytokine genes, leading to an immunosupression profile (Devos et al. 2003; Wang et al. 2011; Kirsten et al. 2017). Studies in mammals and now recently in fish have shown a relationship between immune system function and neural activity that reflects changes in behaviour (Moon et al. 2015; Filiano

et al. 2016; Kirsten et al. 2018). Cytokines are a broad group of protein molecules functioning within the immune system (for review see; Dinarello 2007).

Studies have shown that cytokine expression is related to individual behavioural patterns (e.g. anxiety-like behaviour in mice; Moon et al. 2015; Kirsten et al. 2018). Moreover, cytokines appear to be involved or associated with sociality, social status (through contests of aggression), defensive behaviours and exploratory behaviour (among others; Moon et al. 2015; Filby et al. 2010a; Kirsten et al. 2018). Fish with an immune suppression cytokine profile exhibit alterations in these aspects of behaviour (Moon et al. 2015; Kirsten et al. 2018). Atrazine at high concentrations (1000ppb) has been shown to disrupt defensive behaviours which include shoaling (Schmidel et al. 2014) as well as reduce male-male competition, and interactions with other conspecifics (Shenoy 2014). It is also hypothesised that immune-suppressed fish might lead to an erroneous ability to assess risk (Kirsten et al. 2018). Given that neural disruption is frequently observed at these same concentrations used in this study (Weber et al. 2013; Wirbisky et al. 2015; Wirbisky et al. 2016a; Wirbisky et al. 2016c), it stands to reason that immunodisruption could also occur, and that these systems together may influence behaviour.

### 2.4.4 Diminished aggression response

Juvenile exposure to atrazine did not result in a statistically clear reduction in time spent interacting with the mirror; however, there appeared to be a suggestive negative trend as concentration increased amongst the $F_0$ males (Figure 2.7 E). Interestingly, the suggestive trend observed in the $F_0$ males was significantly pronounced in the $F_1$ males, though not in the similar dose-dependent manner suggesting a non-monotonic effect (Figure 2.7 F). Furthermore, the reduction in mirror interaction in the $F_1$ males was paralleled with reductions in their activity levels (Figure 2.6 F). Previous studies have found impairment in reproductive behaviours after direct atrazine exposure, in particular with reductions in male-male aggression and sexual displays (Hayes et al. 2002; Hayes et al. 2010; Shenoy 2012; Shenoy 2014); however, this is the first study to document altered aggression as a function of an individual's personality, due to paternal atrazine exposure.

Mechanisms behind this change in aggression via atrazine exposure are conflicting throughout the literature. Primarily, it is hypothesised that atrazine influences levels of androgens, testosterone and 11-ketotestosterone (11-KT; in fish), which are important for mediating aggression (Bell 2001). Atrazine exposure is hypothesised to increase the expression of aromatase, an enzyme that converts testosterone to oestrogen (Hecker et al. 2005; Fan et al. 2007; Hayes et al. 2010; Fa et al. 2013). Atrazine has been shown to lower testosterone levels in mammals (Trentacoste et al. 2001; Gely-Pernot et al. 2015), amphibians (Hecker et al. 2005) and

lower both testosterone and 11-KT in goldfish (Spanò et al. 2004), though this finding is not always consistent (Wirbisky et al. 2016a). In addition, the effects through aromatase expression are less clear, especially in fish when compared to other vertebrates (Papoulias et al. 2014).

In the wild, aggression in zebrafish is important for establishing dominance hierarchies, which in the wild enables the monopolisation of foraging resources and the maintenance of territories for spawning sites (Spence et al. 2008). In addition, more aggressive zebrafish males exhibit higher reproductive success (Ariyomo et al. 2012). The consequences of reduced aggression may therefore limit the ability to compete for access to foraging sites and limit the ability to maintain territories. Aggression is also important in females, as either sex can establish a dominance hierarchy (Grant et al. 1992, Spence et al. 2008). Males and females also tend to exhibit similar levels of aggression (Ariyomo et al. 2013a; Ariyomo et al. 2013b), though this was not observed in this study. In this study, $F_0$ female aggression levels tended to decrease with increasing concentration, though $F_0$ 30ppb females appeared to be more aggressive than their male counterparts. In the $F_1$'s, control females exhibited the highest levels of aggression compared to other $F_1$ females. Previous works have identified similar reduced aggression responses from other EDCs: chronic BPA (Wang et al. 2015), ethinyloestradiol (Bell 2001) and nonylphenol (Xia et al. 2010).

### 2.4.5 Potential of non-genetic environmental inheritance

The data shown here demonstrates that paternal exposure to atrazine (at environmental doses) has the potential to produce intergenerational effects on behaviour. Indeed, as similar (or dose dependent) changes in boldness and aggression (and to some extent, anxiety and exploration) occurred across differing concentrations, there is a stronger implication that effects were transferred via exposed sires. Moreover, it appears that intergenerational behavioural changes associated with atrazine are sex dependent.

In general, the process of producing a phenotype in zebrafish offspring is a complex combination of the inherited genetics from both parents, maternal factors deposited in the egg (Ariyomo et al. 2013a), experience during life and possibly environmentally induced epigenetic factors derived from paternal ancestors (e.g. the global methylome pattern; Jiang et al. 2013; Potok et al. 2013). Recent work has shown that direct atrazine exposure can interfere with the epigenetic machinery itself (e.g. regulation and activity of *dmnt* expression and maintenance DNMT respectively), and by altering global DNA-methylation levels (Wirbisky-Hershberger et al. 2017). However an epigenetic inheritance explanation requires transgenerational observations i.e. the effect must be observed in the first generation where the germ line was not developmentally exposed (Szyf 2015; Jacobs et al. 2017). As this study only investigated intergenerational effects (i.e from $F_0$ to $F_1$), it is not possible to discern whether the effect of the atrazine on the $F_1$'s was

an effect of the 'experience' of exposure to atrazine of the $F_0$'s (i.e. the distortion to neural pathways, leading to an alteration in behaviour, that then lead to a change in the germ line) or whether it was the direct exposure of the $F_0$'s developing germ cells (the future cells of $F_1$'s), that gave rise to the changes in behavioural phenotypes observed in the $F_1$'s. Nonetheless, these results suggest that the transgenerational effects on behaviour may be possible. Previous research has shown that atrazine (at high doses) can induce transgenerational effects of disease in mice (Hao et al. 2016; McBirney et al. 2017), and indeed it appears that disease phenotypes can be exacerbated with each generation (McBirney et al. 2017). Timing and logistical reasons did not allow trangenerational effects to be tested for, but should an interesting prospect for future studies.

Differences in behaviours observed between each generation (e.g. changes in boldness Figure 2.7 A and B), are also likely to be a symptom of atrazine causing a germ line epigenetic effect (that would be passed to their progeny) in combination with a direct effect on other (somatic) tissues in the $F_0$'s. Whereas in the $F_1$'s (and beyond), epigenetic changes in somatic cells derive their origin from the germ cells (Jacobs et al. 2017). This effect is more readily witnessed with increasing generation distances, i.e. $F_1$ to $F_3$, rather than intergenerationally. For instance, bisphenol-A exposure of mice *in utero* lead to dampened social interactions in $F_1$ juveniles, but juvenile social interactions significantly increased in the $F_2$ - $F_4$ generations (Wolstenholme et al. 2012). Furthermore, as males were randomly selected for breeding, it is not possible to discern whether or not the phenotype of the offspring were similar to the father.

### 2.4.6 Genetic influence

Several behavioural measures showed signs of a strong genetic component, i.e. there were large amounts of family level variation present (Appendix IX). Environmental inheritance studies utilise families (the inclusion of individuals from the same parents) to limit the genetic variability and to emphasise an epigenetic effect if it was passed on. If an inherited effect was present in the $F_1$'s, the effect should supersede the variation between families of the same treatment. In this respect, the lack of the number of families was a limitation in this study. In some cases an inherited effect was observed despite the presence of family level variation, such as in the latency to enter the top zone (Appendix IX, Table A9.1). However, there were several endpoints where the genetic component superseded an environmental inheritance effect, for instance in the time spent in the bottom zone of the novel arena and novel object assays (Appendix IX). Overall, $F_1$ offspring from atrazine treated fathers showed more variation at the family level between each treatment level (0.3ppb, 3ppb and 30ppb), than the family level variation observed between $F_1$ control fish and $F_1$ treated fish. However, there was no indication that atrazine treatment influenced the level of variation, due to a lack of

a patterning in rank orders. Therefore the variation present at the family level is likely more attributable to genetic differences, rather than atrazine (Appendix IX, Table A9.1).

### 2.4.7 Conclusion

In conclusion, the aims of this study were to determine if direct and paternal exposure to atrazine altered personality phenotype in adulthood of zebrafish (*Danio rerio*). I found evidence for changes in boldness, aggression, anxiety and exploratory personality phenotypes due to direct and paternal atrazine exposure. Furthermore some behavioural changes exhibited a sex-specific interaction (e.g. stronger effects in females for changes in boldness), while the majority of behavioural effects did not (e.g. aggression, anxiety and exploration). Personality traits can be associated with fitness costs and benefits (Smith et al. 2008; MacPherson et al. 2017), therefore understanding how common environmental contaminants may shape behavioural phenotypes might be potentially informative to understand consequences up to the population level. The findings from this study contribute to the growing body of literature that finds that EDCs have the potential to affect non-reproductive behaviours and effects can be propagated beyond the generation that was directly exposed, suggesting potential transgenerational effects. From the basis of behavioural alterations observed in this study, the following chapter (Chapter 3) investigates mRNA expression differences in the brains of $F_1$ progeny. Specifically, genes involved in regulating the serotonergic, which are key genes thought to be underpinning aspects of anxiety and aggression.

# Chapter 3

# The influence of atrazine on the serotonergic system in $F_1$ progeny

## 3.1 Introduction

Endocrine disrupting chemicals are reported to induce a variety of aberrant behaviours (see reviews Clotfelter et al. 2004; Söffker et al. 2012). How EDCs mechanistically exert their effects (e.g. in the brain) that in turn, alter behaviour of an organism is one of the fundamental challenges of EDC research (see review Nesan et al. 2018). Any complex behaviour, including animal personality, is understood to be underpinned by a combination of molecular mechanisms such as changes in gene expression, hormones, neurotransmitter levels and molecular machinery that mediates between these inputs (e.g. Whitfield et al. 2003; Filby et al. 2010b; Thörnqvist et al. 2015; Wiese et al. 2018). Alterations in gene expression (that then leads to changes in behaviour) from EDC exposure may be a result of how an EDC interacts with hormone receptors, interferes with hormone metabolism, alters structuring of the brain during developmental or possibly by altering the epigenetic machinery that controls gene expression (see review Nesan et al. 2018). For example, one specific effect of the EDC, bisphenol-A (BPA; see review Nesan et al. 2018), causes hyperactivity in larval zebrafish, via an induced up-regulation of aromatase transcripts (the aromatase enzyme converts androgens to oestrogens) mediated by agonism of androgen receptors in the developing hypothalamus, resulting in an unexpected early neurogenesis (Kinch et al. 2015; Le Fol et al. 2017).

As discussed in the previous chapter, non-genetic factors may play a role in facilitating behavioural phenotypes through to the following generation (see Chapter 2), and as a consequence, may influence the underlying genetic components that reflect changes in expression of the behavioural phenotype (Burton

et al. 2014; Dias et al. 2014). In the previous chapter I showed that the offspring of males exposed to the neuro-endocrine disruptor, atrazine (at concentrations 0.3ppb, 3ppb and 30ppb) during juvenile development exhibited reduced aggression (0.3ppb and 30ppb significantly reduced), and also showed some evidence that paternal atrazine may also affect anxiety (see Chapter 2). Aggression plays a crucial role across the animal kingdom (Bell et al. 2009) and is characterised as antagonistic interactions between conspecifics (Réale et al. 2007). Animals use aggression as a means to outcompete other individuals for resources such as food, mates, territory or social rank (Spence et al. 2008). Anxiety on the other hand, is anticipatory fear when uncertainty of a potential threat is high (Maximino et al. 2012). In the presence of uncertainty of an unknown threat, animals behave in a manner that maximises their self preservation, but the strength or duration of these behaviours will tend to differ between individuals and across contexts, such as a novel environment (Thomson 2017).

A first step to understand the functional outcomes of atrazine exposure on behaviour is to investigate the associations exposure has on molecular endpoints. Recent transcriptomic studies from adult zebrafish embryonically exposed to atrazine have highlighted the serotonergic system as a potential target of developmental atrazine exposures (Weber et al. 2013; Wirbisky et al. 2015; 2016). Moreover, Wirbisky et al. (2015) demonstrated that atrazine exposure altered serotonin (5-HT) turnover in adult females brains, indicating increased serotonergic activity. 5-HT is one of the primary neurotransmitters present in the brain and has been implicated in the facilitation of anxiety and aggression behaviours (among a myriad of other endpoints; see review Backström et al. 2017). In addition, developmental atrazine exposure has been previously shown to alter anxiety-like behaviours in rodents (Lin et al. 2013; Walters et al. 2015) and aggression in fish (Shenoy 2014). The aims of this study were two-fold: firstly, to investigate if atrazine influenced the level of transcript number in the offspring of exposed males; and secondly, if changes in transcript number occurred, are these related to changes observed in personality, particularly aggression and anxiety behavioural phenotypes?

Understanding the way in which 5-HT regulates anxiety or aggression appears complex (Clotfelter et al. 2007; Maximino et al. 2012; Maximino et al. 2013; Herculano et al. 2014), even in different strains of zebrafish (Barbosa et al. 2018a). The role 5-HT plays is not straight forward, but generally, 5-HT activity tends to repress aggression (Popova 2008). In mice, the knock-out of the 5-HT transporter results in reduced aggression (Holmes et al. 2002; Heiming et al. 2013). For measures of anxiety, the understanding is less clear. For example, two distinct behavioural tests that are both considered paradigms for testing anxiety in zebrafish (novel tank compared to the light/dark test) result in altered 5-HT levels, but in opposite directions to one another (Maximino et al. 2012). The behavioural and functional endpoints of

5-HT neurotransmitter system appears to be strictly conserved across vertebrate systems (and apparently in invertebrate systems; Fossat et al. 2014) with differences occurring in the organisation in the brain (e.g. in teleosts compared to mammals; Lillesaar 2011). In comparison to other vertebrates, a duplication event in the teleost evolutionary history lead to duplications in 5-$HT_{1A}$ receptor and the 5-HT transporters (Norton et al. 2008), leading to possible innovations and increased complexity in how the serotonergic signalling system may operate within the teleost brain.

As candidate genes, this study selected three 5-HT receptor genes *htr1Aa*, *htr1B*, and *htr2B*, as well as the two 5-HT transporter genes *slc6a4a* and *slc6a4b* for their involvement in the regulation of the serotonergic system and in anxiety and aggression behaviours. The three 5-HT receptors are G-protein-coupled receptors that function by propagating neurotransmission in the post-synaptic membrane by relaying 5-HT activity and are involved in controlling 5-HT release in the pre-synaptic membrane (Piñeyro et al. 1999; Aghajanian et al. 1972; Norton et al. 2008). The two 5-HT transporters (Wang et al. 2006) function by mediating the removal of 5-HT from the synaptic cleft and transporting it back into the presynaptic neurons, thereby regulating the strength and duration of neurotransmission (Norton et al. 2008). Drugs that target the functional proteins of these genes and expression levels of these genes are implicated in anxiety-like and dominance (via aggression) behaviours (Filby et al. 2010b; Maximino et al. 2013). Moreover single nucleotide polymorphism (SNPs) of 5-HT transporters have been associated with personality differences in other taxa (Craig et al. 2009; Miller-Butterworth et al. 2007; Müller et al. 2013; Holtmann et al. 2016). But it is not well understood to what extent these genes together influence consistent differences between individuals.

I predicted that aggressive personality would correlate negatively with mRNA transcript number of all candidate genes based on previous experiments in dominant and subordinate relationships in zebrafish and their mRNA transcripts (e.g. Filby et al. 2010b; Theodoridi et al. 2017). I predicted that an anxious personality would correlate positively with mRNA levels (in particular with *htr1Aa*; Maximino et al. 2013). I also predicted that offspring from atrazine treated males would have higher transcript number of the candidate genes compared to controls (Wirbisky et al. 2015), indicative of their higher anxiety and lower aggression behavioural profiles.

## 3.2 Methods

### 3.2.1 Phenotype selection, tissue extraction, RNA extraction and purification

After behavioural phenotyping (see Chapter 2), the two zebrafish that spent the most and the least amount of time at the bottom in the novel arena (a high and low anxious phenotype) from each treatment per family

and per sex, were selected (Appendix X). Once selected, fish were then euthanised in an ice water bath for 10 min. Fish were then dissected in PBS and whole brain tissue was extracted and immediately placed in eppendorf tubes with 50-100$\mu$L of RNA Later (*in vitro*) and subsequently stored at $-30$°C. Based on the measures of anxiety (Figure A10.1), $F_1$ fish from controls and from the 0.3ppb treatment were selected for qPCR analysis due to the greater difference between these treatment groups. Additionally, as there was a lack of a strong relationship found between the anxiety phenotype and the aggression phenotype (time spent interacting with the mirror; Figure A11.1), this method of selection enabled coverage of both animal personalities.

RNA was extracted using a modified protocol from the Norgen Biotek RNA kit and using Trizol reagent (Ambion). Briefly, tissues were homogenised with 1mL of Trizol reagent to separate the RNA phase from that of the protein and DNA. The RNA phase was then transferred to the Norgen column and processed according to the Norgen protocol. RNA concentration was measured using a ND-1000 nanodrop spectrophotometer. Samples were eliminated of contaminants using Turbo DNase (Invitrogen) and then re-nanodropped to confirm 260/280 ratio was between 1.8-2. The RNA was reverse transcribed to obtain cDNA using 400ng of RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). The parameters for reverse transcription consisted of one cycle of 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The cDNA was then diluted into 10ng/$\mu$L for qPCR.

### 3.2.2 Quantitative real-time PCR (qPCR)

The mRNA expression of genes encoding for *slc6a4a*, *slc6a4b*, *htr1Aa*, *htr1B*, and *htr2B* (see Table 3.1 for primers used) was determined in brain samples of adult offspring from atrazine exposed and non-exposed fathers with quantitative polymerase chain reaction (qPCR) assays. Primers were ordered from Integrated DNA Technologies (DIT; Singapore). qPCR was done with Quant studio 5 (Thermofisher). The total reaction volume was 10$\mu$L, of which contained 5$\mu$L SYBR green (Takara), 0.5$\mu$L forward primer, 0.5$\mu$L reverse primer, 2.8$\mu$L MilliQ water, 0.2$\mu$L ROX and 1$\mu$L of cDNA. For each primer, a gradient temperature assessment was carried out from 60-64°C in order to find the optimal annealing temperature (Table 3.1). Each cycle of qPCR was 95°C for 2 min, 95°C for 5 sec, optimal annealing temperature of gene primer for 10 sec, 72°C for 5 sec, 95°C for 1 min, 55°C for 30 sec, 95°C for 30sec, for 30-40 cycles per plate. qPCR standards for each gene were made using pooled cDNA (of all 46 samples) at a ratio of 1:3 dilution, and serially diluted 6 times. Efficiency for all qPCR runs was between 95-105% (see Appendix XII). *b-actin* was validated and proved to be a suitable reference gene (Appendix XIII).

Table 3.1: Primers used for qPCR analysis, validated by Theodoridi et al. (2017).

| Gene | Forward primer | Reverse primer | Annealing temperature |
|---|---|---|---|
| *b-actin* | 5' TGTCCCTGTATGCCTCTGGT 3' | 5' AAGTCCAGACGGAGGATGG 3' | 62°C |
| *slc6a4a* | 5' GTCTCCAATGGTTATCGCAGTA 3' | 5' GATGACCGACAACAGGAAGT 3' | 60°C |
| *slc6a4b* | 5' GAATCCTCTGGGCTTGGTAATG 3' | 5' GCTGAAGTAGACAATGGTGAAGAT 3' | 60°C |
| *htr1Aa* | 5' CAGAGCAGAGCAGCACAAG 3' | 5' TGGTCTGAGAGTTCTGGTCTAATC 3' | 60°C |
| *htr1B* | 5' GTGTCGGTGCTCGTGATG 3' | 5' CAGCCAGATGTCGCAGATG 3' | 60°C |
| *htr2B* | 5' GCTGCTCATTCTTCTGGTCAT 3' | 5' GTTAGTGGCGTTCTGGAGTT 3' | 60°C |

### 3.2.3 Statistical analyses

All statistical analyses were conducted in R, version 3.5.0 (Team 2018). To test if paternal atrazine exposure altered mRNA transcript number in the brains of $F_1$ offspring, I ran several linear mixed effect models (LMMs) using the package '*lme4*' version 1.1-1.3 (Bates et al. 2015). In all models described below, family ID was used as a random effect to account for genetic differences and all candidate genes were normalised by *b-actin* expression. Treatment group (control and 0.3ppb), sex (male and female) and an interaction term were predictors and gene mRNA were response variables. To investigate associations between mRNA transcripts of candidate genes and behaviour, LMMs were conducted between behavioural measures (time spent in the bottom zone and time spent interacting with the mirror) as response variables and candidate genes, treatment and an interaction term as predictors (see Appendix XVI for additional analyses where fish were categorised as the least and most anxious/aggressive). Sex as a main effect was non-significant and thus removed from further analyses between behavioural phenotypes. For these tests, after normalising, the candidate genes were further centred using the '*scale*' function. If an interactive effect occurred, control and 0.3ppb offspring were additionally analysed separately.

Model residuals were checked for normality and models involving *slc6a4a* and *htr1Aa* were log transformed. The interaction term was removed if non-significant and re-analysed. Occasionally, when the interaction term influenced model outcomes (i.e. main predictors statistical significance was $p < 0.05$), but no significant interactive effect was present ($p > 0.05$), small-sample-size-corrected Akaike Information Criterion ($AIC_C$; Burnham et al. 2003) was run to determine the inclusion or exclusion of the interaction term using the '*dredge*' function implemented in the '*MuMIn*' package (Bartoń 2017). Parameters were model-averaged within 6 $AIC_C$ units (when $\Delta AIC_C \leq 6$; Symonds et al. 2011) using the natural average method, '*model.avg*' function (Burnham et al. 2003) also available in the '*MuMIn*' package (Bartoń 2017). $AIC_C$ tables are provided in Appendix XIV Table A14.1. For all models, results are presented with estimates and 95% confidence intervals (CI). Confidence intervals were calculated using the '*confint*' function within base R

(Team 2018). For reference, 95% CI that do not overlap with zero are statistically significant. The full model output, including parameter coefficients, parameter standard error (SE), adjusted SE (for models that underwent $AIC_C$ analyses), test statistics and p-values are reported in Appendix XV. P-values for LMMs were calculated using the '*lmerTest*' package (Kuznetsova et al. 2017).

## 3.3 Results

### 3.3.1 mRNA expression levels between offspring from treated and non-treated fathers

Overall, *htr1Aa* mRNA was significantly down-regulated in control males compared to females (Est. −0.71 [−1.39, −0.02 CI]); however, the overall pattern was significantly reversed in atrazine 0.3ppb treatment offspring with *htr1Aa* mRNA significantly up-regulated in males instead (Est. 1.05 [0.11, 2.00 CI]; Figure 3.1 C). Similarly, control females had, on average, higher *slc6a4a* mRNA than control males. The trend was reversed between the sexes of offspring from treated fathers, suggesting an interactive treatment:sex effect, but no significant differences in either treatment, sex or an interaction between treatment and sex were observed (Figure 3.1 A). On average, there was a down-regulation of *slc6a4b* mRNA in control males compared to control females, whereas the pattern between sexes was absent in the atrazine treated offspring. After model averaging, however, no significant differences were observed (Figure 3.1 B). There was no statistically significant difference in the mRNA transcripts of *htr1B* and *htr2B* between fish from either atrazine treated or non-treated fathers (Figure 3.1 D and E respectively).

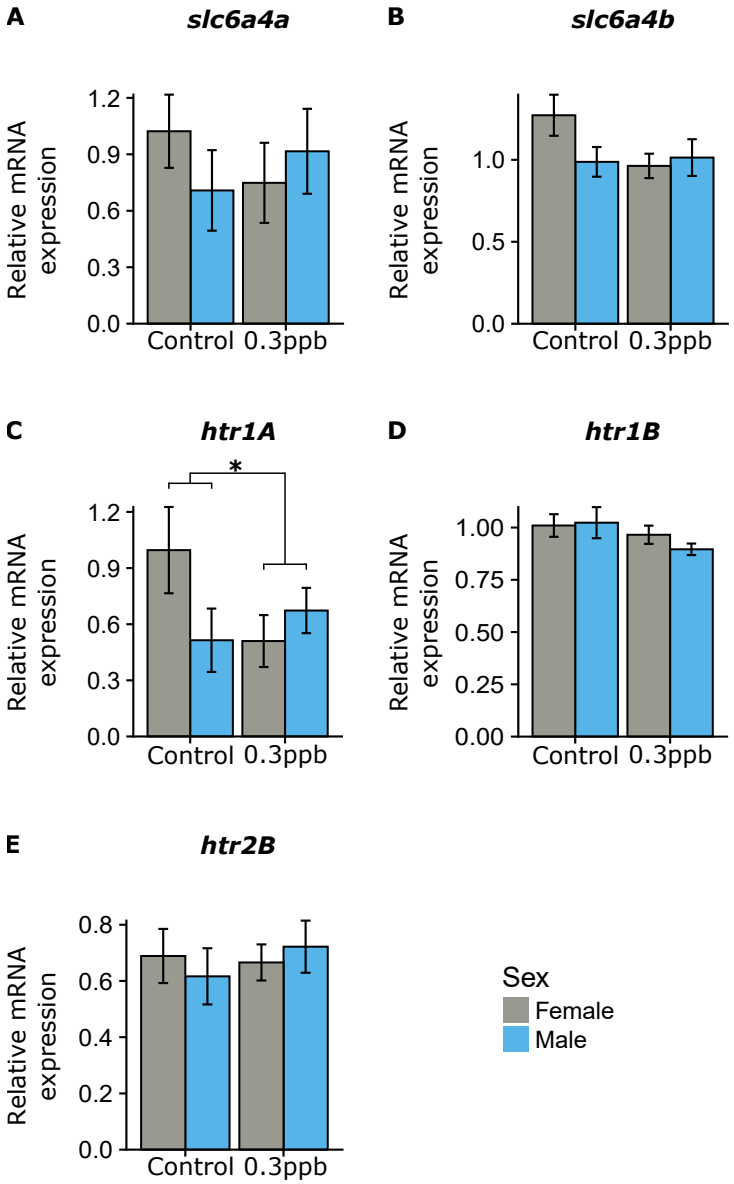


Figure 3.1: Atrazine effects on the relative mRNA levels of the candidate genes (A) *slc6a4a*; (B) *slc6a4b*; (C) *htr1Aa*; (D) *htr1B* and (E) *htr2B* of $F_1$ offspring from control and 0.3ppb atrazine treated males normalised to *b-actin* (from whole brain samples). Bars represent means, with error bars representing standard errors of the mean. Sample sizes for control offspring were n=22 (males=10; females=12; progeny per family=6-8; families n=3), and for 0.3ppb offspring were n=24 (males=12; females=12; progeny per family=8; families n=3).

### 3.3.2 Behavioural phenotype and corresponding mRNA expression levels

#### Anxiety

Time spent in the bottom zone did not significantly correlated with *slc6a4a* or *slc6a4b* mRNA transcript number; however, a marginally non-significant interactive relationship was observed between *slc6a4a* mRNA and treatment group (Est. −22.27 [−46.64, 2.09 CI]; Figure 3.2 A; B). On further inspection, no significant relationships were observed in time spent in the bottom zone and *slc6a4a* mRNA when controls and atrazine treated fish were analysed separately, owing to high variation present in both treatment groups. Paternal atrazine exposure altered the relationship between *htr1Aa* mRNA and time spent in the bottom zone (Figure 3.2 C). Overall, a significant positive relationship between time spent in the bottom zone and *htr1Aa* mRNA was observed (Est. 17.28 [2.42, 32.13 CI]) and a marginally non-significant interaction was observed between *htr1Aa* mRNA and the treatment group (Est. −21.08 [−43.98, 1.82 CI]). On further analysis, control fish drove the significant positive relationship (Control; Est. 17.28 [2.31, 32.24 cI]), whereas the relationship was absent in the 0.3ppb atrazine treatment fish (0.3ppb; Est. −3.80 [−21.12, 13.51 CI]; Figure 3.2 C). Lastly, no relationship between time spent in the bottom zone and *htr1B* and *htr2B* mRNA transcript was observed (Figure 3.2 D; E).

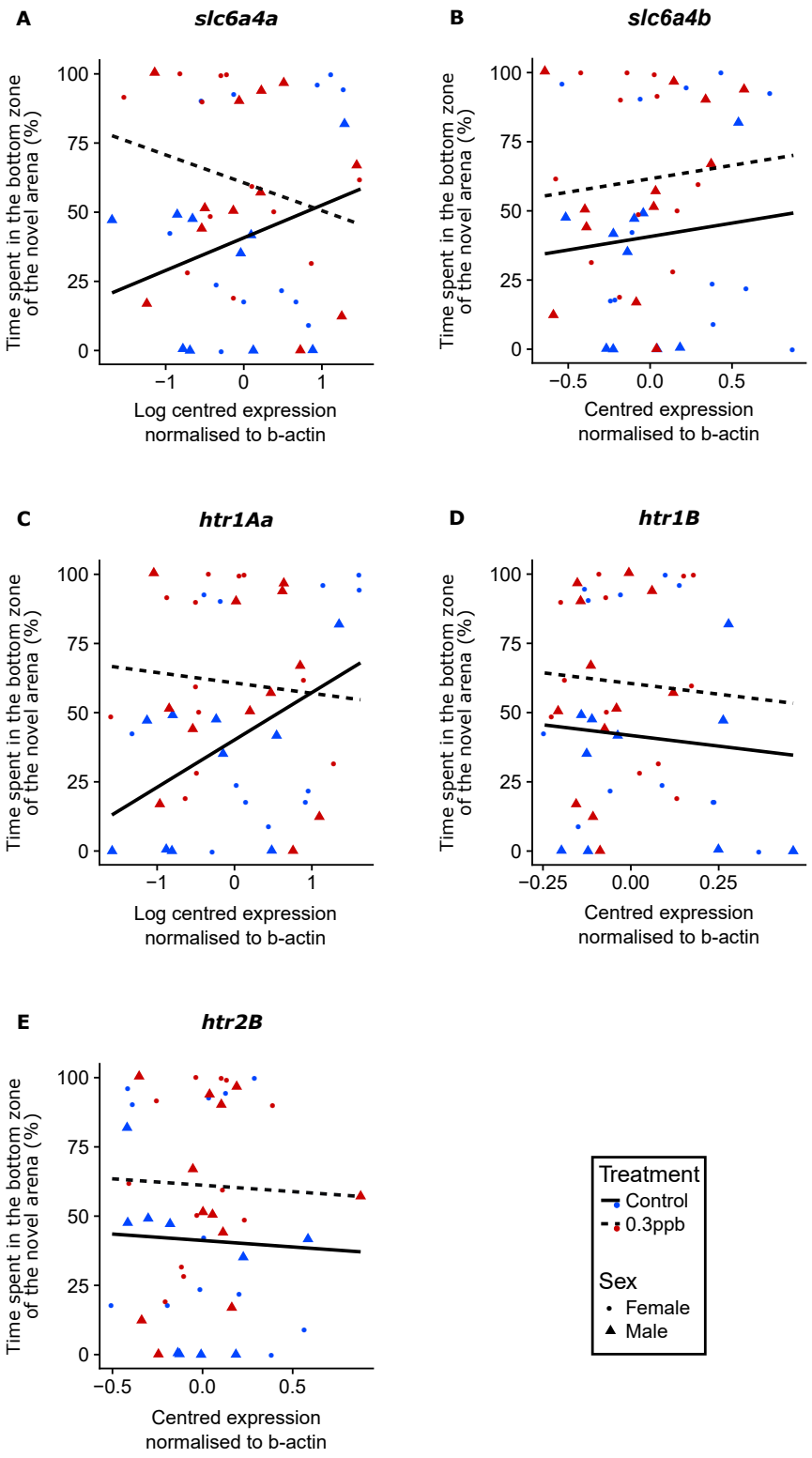


Figure 3.2: Relationship between anxiety (time spent in the bottom zone of the novel arena, sec) and relative mRNA levels of the candidate genes (A) *slc6a4a*; (B) *slc6a4b*; (C) *htr1Aa*; (D) *htr1B* and (E) *htr2B* of $F_1$ offspring from control (solid line) and 0.3ppb atrazine treated (dashed line) males noramlised to *b-actin* and then further centred (from whole brain samples). Both lines are based on model predictions. Control (blue) and 0.3ppb treated (red) offspring for both males (triangles) and females (circles) raw data is overlaid. Sample sizes for control offspring were n=22 (males=10; females=12; progeny per family=6-8; families n=3), and for 0.3ppb offspring were n=24 (males=12; females=12; progeny per family=8; families n=3).

**Aggression**

No relationship with the mRNA transcripts of *slc6a4a* or *htr1Aa* and the time spent interacting with the mirror was observed in either candidate (Figure 3.3 A and C respectively). But both *slc6a4b* and *htr1B* mRNA transcript number significantly predicted the amount of time a fish spent interacting with the mirror (*slc6a4b* Est. −69.07 [−116.11, −22.03 CI]; *htr1B* Est. −408.952 [−683.82, −134.08 CI]; Figure 3.3 B and D respectively), with higher amounts of mRNA transcripts (in both *slc6a4b* and *htr1B*) correlating with a less aggressive personality. A high amount of variation precluded a significant correlation between *htr2B* mRNA transcript number and the time a fish spent interacting with the mirror, though a suggestive negative relationship was observed (Figure 3.3 E).

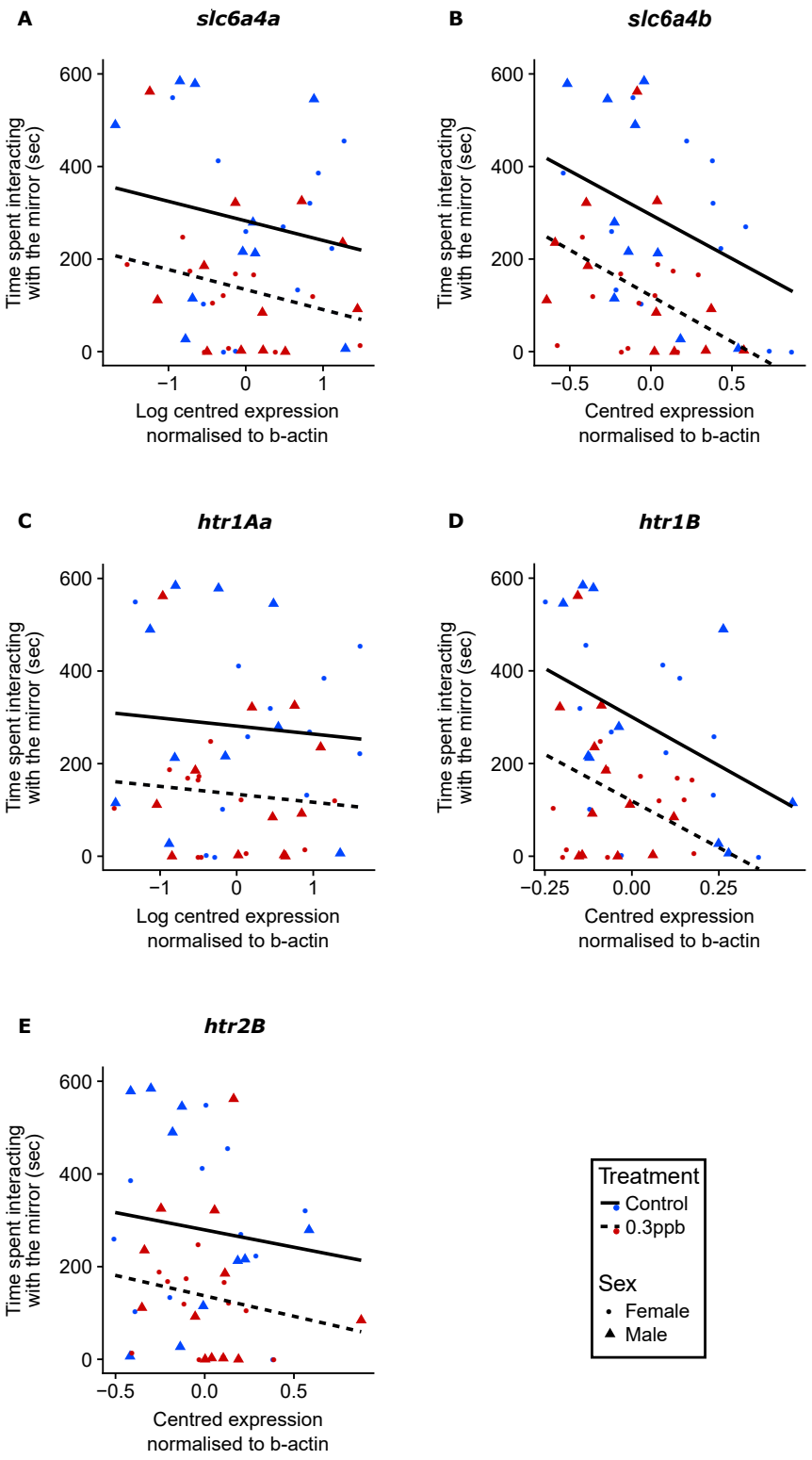


Figure 3.3: Relationship between aggression (time spent interacting with the mirror, sec) and relative mRNA levels of the candidate genes (A) *slc6a4a*; (B) *slc6a4b*; (C) *htr1Aa*; (D) *htr1B* and (E) *htr2B* of $F_1$ offspring from control (solid line) and 0.3ppb atrazine treated (dashed line) males noramlised to *b-actin* and then further centred (from whole brain samples). Both lines are based on model predictions. Control (blue) and 0.3ppb treated (red) offspring for both males (triangles) and females (circles) raw data is overlaid. Sample sizes for control offspring were n=22 (males=10; females=12; progeny per family=6-8; families n=3), and for 0.3ppb offspring were n=24 (males=12; females=12; progeny per family=8; families n=3).

## 3.4 Discussion

The aim of this study was to investigate if $F_1$ behavioural differences in anxiety and aggression (observed in Chapter 2) from atrazine treated and non-treated fathers, could be explained by differences in mRNA transcript number of genes involved in the regulation of the serotonergic system. Specifically, I investigated the two serotonin transporters genes, *slc6a4a*, *slc6a4b* and three serotonergic receptor genes, *htr1Aa*, *htr1B*, *htr2B*. Irrespective of behaviour, I found that *htr1Aa* mRNA was down-regulated in atrazine females and slightly up-regulated in atrazine males, compared to controls. There was similar relationships altered in other genes (e.g. *slc6a4a* and *htr2B*), but not significantly so. A positive relationship was found for *htr1Aa* (and non-significantly with *slc6a4a*) mRNA with increasing time spent spent in the bottom zone. However, paternal atrazine exposures appears to have deteriorated the relationship between time spent in the bottom zone and *htr1Aa* mRNA transcript number (and non-significantly with *slc6a4a* mRNA levels as well). Time spent interacting with the mirror was negatively associated with increased *slc6a4b* and *htr1B* mRNA, regardless of paternal atrazine exposure.

The results of this study support previous research associating atrazine exposure with disruption of some aspects of the serotonergic system (Lin et al. 2013; Lin et al. 2014a; Wirbisky et al. 2015). Indeed, the significant decrease in *htr1Aa* mRNA levels (females in particular) is also observed in adult zebrafish females directly exposed during embryonic development (at the same concentrations; Wirbisky et al. 2015). Wirbisky et al. (2015) also found altered 5-HT turnover in the brain (in females only), so it would be interesting to test for a similar response here. For the most part, it appears that the effects of atrazine on the serotonergic system are sex, development and dose specific with some overlap between these parameters. For example, adult mice exposed to atrazine (at 3mg/kg) during gestation and lactation, via atrazine in the mothers drinking water, had sex specific decreases in 5-HT levels in specific brain regions (both sexes exhibited 5-HT decreases in the striatum, whereas only females exhibited decreases in the perirhinal cortex; Lin et al. 2014a). Another study found disparities in the metabolite profile of exposed male mice, including tryptophan (precursor to 5-HT) and other metabolites important in normal 5-HT function (e.g. linoleic acid and $\alpha$-linolenic acid), at concentrations >5mg/kg (Lin et al. 2014b). At higher doses, (>125 mg/kg; but not at concentrations tested between 0-25 mg/kg) atrazine increased the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) levels and altered 5-HT turnover in the brain (Lin et al. 2013).

It is interesting to note that while sperm are the source of non-genetic atrazine exposure (on the assumption that both male and female offspring inherited the same environmental epigenetic marks), the EDC still appears to differentially affect males and females (at the mRNA transcript level; Figure 3.1 C, and

sometimes at the behavioural level e.g. Figure 2.7 B). This finding indicates that indirect effects of atrazine are also likely to be dependent on developmental and sex specific interactions, just as they are under direct exposure (e.g. Wirbisky et al. 2015; 2016). Other EDCs have seen similar sex-dependent intergenerational/transgenerational effects (Crews et al. 2007). For example, paternal bisphenol-A exposure decreases spatial memory retention in $F_1$ female rats (Fan et al. 2013), and $F_3$ female rats ancestrally exposed to vinclozolin discriminate against potential mates that have a history of vinclozolin exposure, an effect that was absent in males (Crews et al. 2007).

In the previous chapter (Chapter 2) I identified that $F_1$ fish from the 0.3ppb treatment spent more time, on average, in the bottom zone (indicative of a heightened anxiety phenotype). Though this pattern was not observed consistently across treatments (Figure 2.5 B), the selection criteria (for further qPCR analysis) at the extremes of this phenotype (i.e. the least and most anxious fish) suggests that paternal atrazine exposure accentuates the ranges and shifts the medians of this anxiety phenotype (Figure A10.1). When fish were ranked as either the least or most anxious behavioural phenotype from their family cohorts, high variation precluded observable differences of mRNA levels between treatment (Figure A16.1). When continuous data was considered, it revealed that *htr1Aa* mRNA was associated with anxiety (time spent in the bottom zone; Figure 3.2 C). In addition, paternal atrazine treatment appears to have significantly deteriorated the pattern between time spent in the bottom zone and *htr1Aa* mRNA transcript number (and non-significantly in *slc6a4a* mRNA levels as well).

This study provides supporting evidence that up-regulation of *htr1Aa* mRNA is associated with increased anxiety levels, as are the functional proteins of this gene (Maximino et al. 2013). Previous work has shown that bottom dwelling behaviour is positively correlated with 5-HT levels, but that drugs that interfere with *htr1Aa* protein products can alter the anxiety phenotype (Maximino et al. 2013). For example, blocking 5-HT from binding to $HT_{1A}R$ receptor on post synaptic membranes by the drugs, busporine (an agonist of the pre-synapse and antagonist on the post synapse) and WAY 100 635 (antagonist at both pre and post synapse), is associated with producing an anxiolytic effect in zebrafish (Maximino et al. 2013). $HT_{1A}R$ knockout mice exhibit increased anxiety at adulthood (Zhuang et al. 1999) and it appears that retaining proper function of $HT_{1A}R$ during development is critical for normal anxiety behavior as an adult (Gross et al. 2002). Disruption of $HT_{1A}R$ during adolescence in mice is enough to sustain increased anxiety levels through to adulthood (Garcia-Garcia et al. 2016). While this study did not test for effects over development, it is likely that *htr1Aa* mRNA disruption was present over ontogeny and possibly lead to perturbations in anxiety. Future studies should examine ontogenic features of atrazine, anxiety and their relationship to *htr1Aa* mRNA, either directly or indirectly.

One reason why paternal atrazine exposure appears to have been associated with *htr1Aa* mRNA only could be due to the methodology used here. Using a whole brain approach, rather than investigating specific regions of the brain, may not truly reflect the nuances of gene expression activity, especially in regard to behavioural changes. For instance, 5-HT activity is non-uniform across the brain, and so too are receptor and transporter gene expression (Wang et al. 2006; Norton et al. 2008; Filby et al. 2010b). The non-significant interaction between *slc6a4a* and paternal atrazine exposure is likely due to lack of sample size and increased variability, precluding observable differences, as no significant correlation was observed when controls and atrazine treated fish were tested separately (Figure 3.2; Table A15.4). Fluoxetine (a 5-HT transporter antagonist) is shown to produce an anxiolytic phenotype (Maximino et al. 2013), indicating some involvement of *slc6a4a* and *slc6a4b* in anxiety. Other drugs such as SB 224289 (an inverse agonist to the $HT_{1B}R$ receptor) also produces an anxiolytic effect in a novel arena assay (Maximino et al. 2013), further suggesting that this study failed to capture involvement of the genes tested. Another reason for lack of a trend may be that an anxiety phenotype is produced via epistatic interactions between genes (many genes of small effect culminating together) and that mRNA levels of these candidate genes (except for *htr1Aa* and *slc6a4a*) in isolation do not overall reflect the many processes that lead to an anxiety phenotype. Indeed, quantitative trait loci mapping in wild and laboratory strain zebrafish show that some measures of boldness (among other behaviours) are epistatic in nature (Wright et al. 2006b).

The relationship between aggression and *slc6a4b* expression was partially expected. Previous work by Theodoridi et al. (2017) demonstrated that whole brain *slc6a4b* expression was related to dominant and subordinate positions in male zebrafish hierarchies (subordinates express *slc6a4b* mRNA relatively more than dominant individuals). The authors further suggested that differences in expression may be due to associated coping strategies, i.e personality differences. Moreover, the lack of a significant relationship between *slc6a4a* and aggression is further supported by Theodoridi et al. (2017) who found no difference between dominant and subordinate individuals (though in that study fluoxetine treatment still altered expression levels and caused behavioural changes). Other work has found similar patterns in rainbow trout (*Oncorhynchus mykiss*) between low reactive (consistently more aggressive) and high reactive (consistently less aggressive) individuals; low reactive individuals have down-regulated *slc6a4b* expression and no difference in *slc6a4a* expression (Rosengren et al. 2017). A negative relationship between 5-HT transporter mRNA and aggression levels in zebrafish was proposed by Filby et al. (2010b). However, Filby et al. (2010b) did not investigate *slc6a4b* levels, and presided this hypothesis on *slc6a4a* mRNA levels only. Filby et al. (2010b) found evidence of altered expression of *slc6a4a* between dominant and subordinate females only, but when the authors compared day 1 expression to day 5 expression of dominant and subordinate fish, the relationship in

females with *slc6a4a* expression levels disappeared and appeared in males (higher *slc6a4a* levels in dominant individuals). This research by Filby et al. (2010b) suggests a more temporal component of *slc6a4a* mRNA, that might in function, be involved in reactive aggression, whereas *slc6a4b* might be involved in a more stable phenotype as it correlates with aggression personality (in this study). Overall, this study suggests that *slc6a4b* transcripts might be more important for personality differences than *slc6a4a* mRNA levels.

The 5-HT receptors $HT_{1B}R$ and $HT_{2B}R$ are shown to be involved in offensive and defensive aggression behaviours (Fish et al. 1999; Miczek et al. 2001; Almeida et al. 2002; Takahashi et al. 2011; Theodoridi et al. 2017). The lack of relationship between aggression levels and *htr2B* mRNA expression in this study is in contrast with what has been found previously. For instance, dominant zebrafish exhibit significantly lower transcripts of *slc6a4b* and *htr2B* in comparison to subordinate conspecifics (with suggestible evidence for lower *htr1B* expression levels in dominants; Theodoridi et al. 2017). Aggressive encounters in zebrafish involve two transient phases; first an appetitive element (overt offensive engagement, e.g. displaying, circling and biting, by both parties), followed by a resolution element (dominant or subordinate specific behaviours, e.g. chases or flights/freezing respectively; Kalueff et al. 2013; Theodoridi et al. 2017). The lack of relationship between mirror induced aggression and *htr2B* mRNA is possibly a reflection of the methodology used to capture zebrafish aggression in this study. The mirror test offers a proxy look at aggression (theoretically restricting aggression to its appetitive element) without the dynamic effect of winner or loser outcomes (Kalueff et al. 2013; Oliveira et al. 2016). The relationship between mirror induced aggression and *htr1B* mRNA, and lack thereof with *htr2B* mRNA, possibly reflects *htr1B* mRNA is important for personality differences pertaining to an individual's engagement in aggression. Whereas *htr2B* may be more involved in defensive aggression behaviours when there is an ability to process feedback information about an opponent. This remains speculation and more work is needed to clarify how offensive and defensive aggression behaviours may be related to gene expression.

Although paternal atrazine exposure altered aggression personality in this study, the lack of an effect on these genes suggests other factors are responsible for the phenotypic differences. The system that leads to aggression is complex (encompassing more than 40 genes of at least eight pathways; Filby et al. 2010b). In general, this study is limited by the whole brain approach of which there are clearly massive differences in expression across distinct regions of the brain (Filby et al. 2010b). Also, compared to Filby et al. (2010b), who used the WIK zebrafish strain, this strain is wildtype AB. Different strains of zebrafish appear to differ in their behaviour, in baseline levels of 5-HT and also react differently to chemicals that interfere with the serotonin system differently (Barbosa et al. 2018a). This study was not able to account for the dominance of individuals in their home tank, as social experience can influence future aggressive encounters

(Kloke et al. 2011). As stated before, the mirror test used here offers a limited proxy for aggression, though repeatability of this measure was observed (see Appendix IV), indicating this method likely captures personality differences between individuals. A recent study provides improved methodology to quantify the full range of aggression in zebrafish and other fish species (e.g. Li et al. 2018).

Overall, paternal atrazine exposure appears to alter offspring anxiety and aggression. These data suggest that disruption in *htr1Aa* mRNA levels (and possibly *slc6a4a* mRNA levels) contribute to an increase in time spent in the bottom zone (a shift in anxiety phenotype). In contrast, paternal atrazine exposure decreased offspring aggression, but the atrazine treatment did not appear to influence expression of any of the candidate genes and as such, shifts in behavioural phenotype appear to be under the control of different systems. Additional candidates are likely to be numerous due to the complexity of the aggressive phenotype (Filby et al. 2010b). Moreover, transcriptome and DNA methylation studies suggest a wide array of pathways are affected by early developmental exposure to atrazine (Wirbisky et al. 2015;; Hao et al. 2016; Wirbisky et al. 2016a; McBirney et al. 2017). Changes in gene expression underlying anxiety and aggression may also be potentially propagated transgenerationally. Hence, future studies combining changes in behaviour and gene expression due to atrazine exposure should frame their research with transgenerational effects in mind, as exposure to atrazine has been demonstrated to induce transgenerational effects (Hao et al. 2016; McBirney et al. 2017). Indeed, McBirney et al. (2017) consistently found that the systems associated with signalling, transcription, metabolism and receptors (among others) to be differentially methylated after transgenerational atrazine exposure (despite DNA-methylation profiles being discordant between generations, $F_1$-$F_3$). These studies suggest that mechanisms behind atrazine induced behaviours are likely to be affected transgenerationally as seen in other EDCs (e.g. Crews et al. 2007; Volkova et al. 2015a; see O'Dea et al. 2016). This study is one of a few to investigate paternal atrazine exposure on offspring phenotype, but more studies are needed to identify the extent of paternal contribution after atrazine exposure.

# Chapter 4

# General discussion

## 4.1 Thesis objectives

The main objective of this thesis was to investigate the influence of an endocrine disruptor, atrazine on non-genetic inheritance of personality traits in zebrafish. In Chapter 2, I used a series of behavioural assays (novel arena test, novel object test and a mirror test) to investigate if exposure to atrazine during juvenile development affected the five behavioural traits (aggression, anxiety, activity, boldness and exploration) of the exposed individuals and more importantly, their unexposed progeny. Chapter 3 used whole brain tissue to investigate if behavioural changes observed in the $F_1$ progeny were able to be explained by changes in mRNA transcript number in genes involved in regulating the serotonergic system (two serotonin (5-HT) transporters, *slc6a4a* and *slc6a4b*, and three 5-HT receptors: *htr1Aa*, *htr1B*, *htr2B*). I focused on two personality traits, anxiety and aggression, because I found evidence for an intergenerational effect on these two traits. Moreover, anxiety and aggression related behaviours are strongly associated with the serotonergic system, and developmental atrazine exposure is associated with the serotonergic system dis-regulation (Chiavegatto et al. 2001; Gross et al. 2002; Filby et al. 2010b; Wirbisky et al. 2015). Therefore, this study also sought to extend previously observed direct serotonergic system effects to a further generation (Wirbisky et al. 2015).

For the experiments in Chapter 2, I predicted increases in anxiety and activity levels and decreases in boldness, aggression and exploration levels in both generations. Furthermore I predicted that increases in anxiety would be more prominent in females than males. For the experiments in Chapter 3, I predicted that $F_1$ progeny aggression levels would correlate negatively with mRNA transcript number and that anxiety levels would correlate positively with all candidate genes. In particular, I predicted a stronger positive

association of *htr1Aa* mRNA with anxiety levels. I also predicted that $F_1$ progeny from atrazine treated males would have higher transcript number at the candidate genes, compared to controls, indicative of their higher anxiety and lower aggression behavioural profiles.

## 4.2 Summary of main findings

Several behavioural measures across a range of personality phenotypes were altered by either developmental exposure or paternal exposure to the herbicide, atrazine, at typical environmental concentrations (summarised in Table 4.1). These findings support the hypothesis that atrazine exposure has the potential to alter personality traits. The personality trait most affected by developmental atrazine exposure was boldness (measured by latency to enter the top zone of the novel arena) - $F_0$ female latency increased with increasing atrazine concentration, whereas males exhibited a threshold drop in latency compared to controls. $F_0$ fish exhibited increased anxiety (measured by the time spent in the bottom zone of the novel arena) and decreased exploration (measured by the standard deviation of time spent in each zone of the novel arena) in the 0.3ppb group only. There was some evidence for decreased aggression (measured by time spent interacting with the mirror) amongst $F_0$ fish, but not significantly so. No treatment differences in activity levels (measured by the total distance moved) were observed across any of assays in the $F_0$'s.

Despite never being directly exposed, several behavioural changes were observed amongst the $F_1$ progeny, supporting the hypothesis that effects of atrazine may be further transmitted down the germ line. Paternal atrazine exposure significantly increased the latency of the $F_1$ progeny, in all treatment groups compared to controls. In addition, there was a suggestive pattern of decreasing latency to enter the top zone with increasing atrazine concentration amongst the $F_1$ progeny from atrazine treated males. Significant decreases in aggression were observed amongst the treated $F_1$'s (in the 0.3ppb and 30ppb groups) that was paralleled with significant decreases in activity during the mirror test in the treatment groups, 0.3ppb and 30ppb, compared to controls. No other treatment differences in $F_1$ activity were observed during the novel arena test or in the novel object test. There was no treatment differences in $F_1$ exploration, but there was some evidence of increased anxiety across the treatment groups compared to controls, but not significantly so. These findings suggest that the offspring of exposed males have decreased aggression, decreased boldness and potentially increased anxiety, which can have consequences for how these fish interact with predators, conspecifics and their environment, such as competition for food, mates, territories or social rank (Spence et al. 2008; Paull et al. 2010).

Paternal atrazine exposure significantly down-regulated *htr1Aa* mRNA expression in females and slightly

up-regulated it in males, compared to controls. Moreover, *htr1Aa* mRNA (and *slc6a4a* - but not significantly so) was found to correlate positively with anxiety levels in controls, but this relationship was disrupted in the atrazine treatment group. While the candidate genes tested here did not explain the significant reduction in aggression observed in the offspring of atrazine treated males, both *slc6a4b* and *htr1B* mRNA correlated negatively with an aggressive personality phenotype.

Though several studies before me have previously identified a range of behavioural changes associated with atrazine exposure (e.g. male-male aggression, shoal-cohesion, anxiety-related behaviours; Belloni et al. 2011; Shenoy 2012; Lin et al. 2013; Lin et al. 2014a; Schmidel et al. 2014; Shenoy 2014), this is, to my knowledge, the first study to investigate if behavioural changes from atrazine exposure are associated with personality differences. These results also support the hypothesis that non-genetic inheritance can contribute to animal personality (Reddon 2011) and that mRNA transcript of genes (i.e., a measure of gene expression) involved in regulating the serotonergic system are associated with personality differences.

Table 4.1: Summary of significant personality differences from direct and indirect atrazine exposure for $F_0$ and $F_1$ zebrafish. Each concentration where a significant treatment effect was observed, relative to controls, is specified. Arrows indicate either a positive (up) or negative (down) direction of effect. Sex differences are presented as females (F) compared to males (M). For treatment:sex interactive differences, the first arrow indicates the direction of effect for males and second arrow indicates female direction. To remind the reader, an increased SD (standard deviation) of exploration indicates a less exploratory phenotype.

| | **$F_0$** | | | **$F_1$** | | |
|---|---|---|---|---|---|---|
| **Personality trait** | **Treatment** | **Sex** | **Interaction** | **Treatment** | **Sex** | **Interaction** |
| **Boldness** | | | | | | |
| Latency to enter the top zone | ↑(30ppb) | NS | ↓(M)↑(F)(3, 30ppb) | ↑(0.3, 3, 30ppb) | ↑(F) | NS |
| **Anxiety** | | | | | | |
| Time spent in bottom zone of novel arena | ↑(0.3ppb) | NS | NS | NS | ↑(F) | NS |
| **Exploration** | | | | | | |
| Exploration SD of the novel arena | ↑(0.3ppb) | NS | NS | NS | ↑(F) | NS |
| **Aggression** | | | | | | |
| Time spent interacting with the mirror | NS | NS | NS | ↓(0.3, 30ppb) | ↓(F) | NS |
| Total distance moved in mirror test | NS | NS | NS | ↓(0.3, 30ppb) | ↓(F) | NS |

## 4.3 Secondary findings

While not a specific aim of this thesis, I additionally assessed the early development of $F_1$ progeny, 24hpf survival and hatching at 48hpf, to examine how atrazine might influence these important early life parameters. I found that paternal atrazine exposure reduced survival at 24hpf in the 3ppb treatment group and significantly reduced the proportion of offspring that hatched by 48hpf in the 3ppb and 30ppb treatment groups, compared to controls (Appendix II). Interestingly, theses results are in contrast to those of Wirbisky et al. (2016c), who found no difference in survival at 24hpf or hatching at 48hpf of progeny from male and female zebrafish that were embryonically exposed to atrazine (at the same concentrations; 0.3ppb, 3ppb and 30ppb). This study out-crossed atrazine exposed males with unexposed females (using IVF) whereas Wirbisky et al. (2016c) employed natural matings and crossed both exposed males and females. The results found in this study may therefore be a consequence of either the difference in the timing of exposure (during juvenile development i.e. during sexual differentiation in this study versus during embryonic development in Wirbisky et al. (2016c)). Alternatively, atrazine has been shown to induce a variety of testicular and sperm quality issues (Abarikwu et al. 2010; Feyzi Dehkhargani et al. 2011; Farombi et al. 2013; Abarikwu et al. 2015); if the probability of defective sperm contacting released oocytes is lower during natural mating conditions compared to *in vitro* fertilisation conditions, then this could explain the contrasting findings between this study and Wirbisky et al. (2016c).

## 4.4 Limitations of the study

No study is without its limitations, as there were several aspects of each experiment that could have been improved or where the assumptions needed further testing. One of the key assumptions was that zebrafish exhibit long-term repeatable behaviour when given a single 'tank experience' followed by testing one week later (Thomson 2017). In our lab, this regime has been demonstrated to be able to discriminate between individual behavioural phenotypes (Thomson 2017). However, it is also known that several EDCs have the ability to disrupt individual consistency and disrupt behavioural syndromes between related traits (e.g. Dzieweczynski et al. 2013; Dzieweczynski et al. 2014; Dzieweczynski et al. 2016). So it may be probable that the behavioural data shown here is not the full extent to which atrazine is capable of altering behavioural phenotypes. Moreover, it remains to be seen whether or not behavioural consistency (i.e. the repeatability of traits) is also affected indirectly. Understanding early life exposure and intergenerational (or transgenerational) effects on behavioural consistency is a promising gap in the literature that should be explored with additional studies. If so, it is likely to have ramifications for how researchers are able

to predict long term behavioural outcomes. Nevertheless, exposure to atrazine either directly or indirectly appears to alter a variety of personality traits in a directional manner, based on treatment group averages and reasonably minimal error bars (for behavioural data).

As males were randomly selected for breeding (via IVF) and there was no markers for individual identity during behavioural testing, the ability to investigate if offspring phenotype was similar to that of the father was not possible. Zebrafish paternal personality and social status appears to non-genetically influence the activity of their offspring (at least during the larval stage; 7 and 10 dpf; Zajitschek et al. 2017). So it may be possible that this phenomena extends to other personality traits and lasts through to adulthood.

Timing and logistical reasons did not allow the possibility to test for trangenerational effects. Indeed, it is this limitation that prevents this study from discriminating between a true epigenetic explanation of inheritance from the possibility that effects were caused via exposure of the $F_0$ developing germ cells i.e., the future cells of the $F_1$ progeny (Szyf 2015; Jacobs et al. 2017). Nonetheless, these results suggest that transgenerational effects on behaviour may be possible.

There will always be important lessons to be learned with hindsight from the testing regimes researchers use to understand personality and treatment differences in a particular species. The novel object and mirror test were two such cases that could have been improved. The aim of the novel object test was to incorporate an additional measure to investigate boldness/anxiety in zebrafish (see Chapter 2; Methods); however these data suggest that the novel object itself was inappropriate for assessing effects on zebrafish behaviour, and instead possibly induced a fear response (see Appendix VI). Furthermore, the novel object test may have influenced the mirror test that followed. These data further reinforce the need for methodological validation on zebrafish (and other fish species; as mentioned by Maximino et al. 2010). Nevertheless, the reduction (near significance) in cumulative duration spent in the bottom zone by $F_1$ fish from the 0.3ppb and 3ppb treatments suggest that treatment effects that influence fear responses might be revealed with methodological improvements to this assay, or with greater sample size as other attempts (using different novel objects) have been successful (Fangmeier et al. 2018; Kirsten et al. 2018).

The mirror test was able to capture repeatable differences between individuals (Appendix IV), and though it is known to correlate with other behavioural measures of aggression (Ariyomo et al. 2013b; Way et al. 2015), it must be noted that the mirror test provides a partial evaluation of aggressive behaviour (Oliveira et al. 2011; Kalueff et al. 2013). The mirror test is also sometimes used as a measure for sociability rather than aggression (e.g. Cattelan et al. 2017, and references therein), though in the assay utilised here, aggressive behaviours were observed and were directed towards the mirror image e.g. bites and lateral displays. A

recent study provides improved methodology to quantify the full range of aggression in zebrafish and other fish species in improved predictability of winner-loser fights (e.g. Li et al. 2018).

Another limitation was the number of full-sibling families used in this study. Full-sibling families are used to separate the environmental variation attributable to the stressor of interest and the genetic signal inherited from the genetics of the parents. This study used three families per treatment; 12 different families in total, with around 13-18 fish per family. Despite this, in many cases, the genetic contribution superseded an environmental inheritance effect even when an inherited effect was suspected (Appendix IX, Table A9.1). Therefore it is recommended that researchers should configure their experimental design of their future studies to maximise the number of families utilised. Based on the limitations of this study alone, researchers should employ at least four or more families at minimum (per treatment) as a recommendation. However, a comprehensive study comparing minimal family sample size requirements would be valuable to the epigenetics field.

While the objectives of the study were aimed at getting a more general idea for atrazine's effects on the serotonergic system, as mentioned previously in Chapter 3, using a whole-brain approach to investigate mRNA differences likely confounded the nuances of how intergenerational atrazine might have affected different brain regions. The serotonergic system is peppered throughout the zebrafish brain (see Wang et al. 2006; Norton et al. 2008) and behavioural differences resulting from knock out in some areas of the brain (but not others) are not equal (Zhuang et al. 1999; Gross et al. 2002). Therefore, future studies should aim for testing similar patterns across different brain regions. In addition, the results presented in Chapter 3 clearly show a wide amount of variation present, possibly resulting from a lack of power, which is likely due to sample size.

One particular draw back of a lab based approach to understand behavioural changes is that extrapolation to individuals in the wild must be taken with caution. The environment comprises numerous and complex interactions (e.g. prey, predators, shelter, seasonal components etc.) however, employing controlled lab experiments allow the construction of base-line effects to which effects could be expected in the wild. Testing wild population for causal effects is a greater challenge, but an intermediate step would be use mesocosm experiments. Additionally, exposure to a single contaminant in the wild is not often the norm; instead, organisms are much more likely to face exposure from a multitude of xenobiotics (Vos et al. 2000; Swanson et al. 2018).

The outcome of exposure to multiple EDCs may not be entirely predictable as EDCs in combination may result in a variety of effects, such as augmentation of one effect, suppression of another, EDCs may synergise together or possibly lead to new effects than when observed singularly (Vos et al. 2000; Hayes et al.

2006; Pérez et al. 2013). Indeed, atrazine has been known to synergise with chlorpyrifos (an organophosphate; acetylacholinesterase inhibitor), increasing the prevalence of disturbed locomotor behaviour in larval zebrafish (Pérez et al. 2013). Future studies should make use of testing later life effects (and intergenerational/transgenerational) of atrazine in combination with other likely to encounter pollutants (Swanson et al. 2018).

## 4.5 Future directions

In the present study, changes in personality traits and mRNA differences observed in Chapter 2 and Chapter 3 should be verified under differing environmental contexts to give additional weight to the hypothesis that atrazine alters personality traits. For instance, future studies should aim to provide more ecological relevance to these findings by investigating the same or related behavioural measures under differing ecological pressures, such as increased predation risk, social density, food resources or environmental complexity (e.g. Luca et al. 2012b; Luca et al. 2012a). Additionally, future studies should aim to extend personality testing under atrazine exposure to other taxa, in order to identify how broadly atrazine may affect personality traits. In Chapter 2, I proposed two different scenarios that atrazine may be affecting boldness (i.e. risk-taking) behaviour in zebrafish; 1) atrazine is altering the propensity to take risks or 2) atrazine is altering the ability to process risk. Researchers could utilise zebrafish's inclination for shoaling to disentangle these two hypotheses by measuring group dynamics of a focal (developmentally exposed to atrazine) individual within a group of unexposed individuals (Spence et al. 2008). Zebrafish use shoaling as a means of predator defence (Olst et al. 1970; Landeau et al. 1986; Wright et al. 2006c) and will alter their shoal cohesion depending on ecological pressures such as predation or food availability (Pitcher et al. 1988; Bass et al. 2008; Miller et al. 2007). If atrazine is altering the propensity to take risks then I predict that focal individuals will try to maintain shorter nearest neighbour distances and actively try to maintain a centred position in the safest part of a shoal. I also predict that the focal individual will exhibit riskier behaviour (e.g. leaving the shoal) when risk decreases. On the other hand, if atrazine is altering the ability to process risk then I predict the focal individual will show less structuring in the shoal as risk is manipulated. When exposed to high doses of atrazine (1000ppb) as adults, group cohesion decreases, so it might be more probable for an altered ability to process risk (Schmidel et al. 2014).

Only a handful of studies have investigated atrazine's role in altering male (but not female) aggression (Shenoy 2012; Shenoy 2014). Further studies should investigate how atrazine exposure alters aggression in a social or dominance context. It remains to be seen what the specific consequences of reduced aggression

in females would be; however, I posit that atrazine exposed (and indirectly exposed) females (and males) will be less likely to achieve a higher social ranking, and be less likely to maintain a higher social rank for longer if able to outcompete other individuals. Since body size is a predictor of social rank (Paull et al. 2010), I further posit that smaller unexposed females would be able to outrank larger directly or indirectly exposed females.

Several intergenerational effects on behaviour and mRNA transcript number were observed throughout this study, suggesting future research into transgenerational effects would be valuable. Though transgenerational effects have been observed previously in mice after atrazine exposure (e.g. Hao et al. 2016; McBirney et al. 2017), the studies were not conducted at environmentally relevant concentrations as in this study design. Additionally, atrazine differentially affects male and female behaviour and the mRNA transcript level after indirect exposure, suggesting that if epigenetic markers are being inherited equally (between the sexes), that atrazine is acting upon developmental and sex specific factors, such as hormones. It would be valuable for future studies to address this interesting gap and attempt to isolate what molecular components are interacting together.

The findings here exemplify the importance and sensitivity of development during early life exposure to environmental stressors, and the potential for effects to be carried through to, or manifest, at maturity (Jonsson et al. 2014). The majority of work in investigating the relationship between atrazine and the early life environment in fish have usually limited their exposure regimes to the embryonic period (e.g. Wirbisky et al. 2015; Wirbisky et al. 2016a). Therefore, this work is important because it extends the sensitivity window for later life consequences of atrazine exposure. It is not unsurprising, however, as juvenile development is a period of increased brain growth and is when numerous factors, including a variety of hormones, start the process to differentiate zebrafish males from females (Jonsson et al. 2014; Lee 2015). Interference during this critical developmental period is therefore predicted to result in adverse outcomes (Burton et al. 2014; Jonsson et al. 2014). In future, it would be valuable to investigate the difference between embryonic and juvenile development (and time points in between and after) on adult behaviours, as it might give further insight into predicting potential ecological consequences and possibly into atrazine's mechanisms of action.

Lastly, while researching, a prominent gap identified in the literature was the lack of understanding to which atrazine mechanistically exerts its effects in the nervous system and other tissues. Previous research indicates that atrazine has the potential to affect a wide variety of systems, directly or indirectly (e.g. Wirbisky et al. 2015; Wirbisky et al. 2016a; McBirney et al. 2017); therefore, this is a crucial area that needs to be addressed. Indeed, this is a common problem throughout the endocrine disrupting field (Gore

et al. 2015). This gap is likely owing to several difficulties arising from the properties of EDCs in which effects are usually sex, timing, and dose specific and are typically non-monotonic in nature (Vandenberg et al. 2012), as is the case with atrazine (e.g. this study; Shenoy 2012; Brodeur et al. 2013; McCallum et al. 2013; Riffle et al. 2014; Marcus et al. 2016). As a first step, a review of potential mechanisms is sorely needed in the atrazine field in order to collate potential hypotheses and identify where specific research gaps in knowledge are. There is also increasing realisation that EDC (and other contaminant) exposure at the individual level could translate to consequences at the population level (Crews et al. 2007; Shenoy et al. 2011; Senior et al. 2014; Araújo et al. 2018).

## 4.6 Conclusions

The major findings from this study were that exposure to environmentally relevant concentrations of atrazine during early life development influences a variety of personality traits in the direct exposed individuals and in the progeny of exposed males, despite the progeny never being directly exposed to atrazine. The affected personality traits include boldness, aggression, anxiety and exploration, with sex-specific effects on boldness observed in both $F_0$ and $F_1$. In addition, I found evidence that paternal atrazine exposure appears to have disrupted aspects of the serotonergic system; specifically, *htr1Aa* mRNA levels between the sexes was reversed in the 0.3ppb treatment, relative to controls, and that the positive relationship between *htr1Aa* mRNA and a more anxious personality was perturbed. It appears that other factors not studied here are responsible for alterations in aggression, but *sl6a4b* and *htr1B* mRNA are associated with an aggressive personality. A secondary finding was that paternal atrazine exposure can reduce early life survival and hatching of progeny. This study contributes to the growing understanding that EDCs have the ability to affect behavioural traits and shows the sensitivity of the early life environment. Moreover this research shows that effects of an EDC in one generation may be further propagated down the germ line.

# References


Abarikwu, S. O., A. C. Adesiyan, T. O. Oyeloja, M. O. Oyeyemi, and E. O. Farombi (2010). "Changes in sperm characteristics and induction of oxidative stress in the testis and epididymis of experimental rats by a herbicide, atrazine". In: *Archives of Environmental Contamination and Toxicology* 58 (3), pp. 874–882.

Abarikwu, S. O., O. F. Akiri, M. A. Durojaiye, and A. Adenike (2015). "Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats". In: *Toxicology Mechanisms and Methods* 25 (1), pp. 70–80.

Aghajanian, G. K., H. J. Haigler, and F. E. Bloom (1972). "Lysergic acid diethylamide and serotonin: direct actions on serotonin-containing neurons in rat brain". In: *Life Sciences* 11 (13), pp. 615–622.

Almeida, R. M. de and K. A. Miczek (2002). "Aggression escalated by social instigation or by discontinuation of reinforcement ("frustration") in mice: inhibition by anpirtoline: a 5-HT1B receptor agonist". In: *Neuropsychopharmacology* 27 (2), pp. 171–181.

Alter, M. D., D. B. Rubin, K. Ramsey, R. Halpern, D. A. Stephan, L. Abbott, and R. Hen (2008). "Variation in the large-scale organization of gene expression levels in the hippocampus relates to stable epigenetic variability in behavior". In: *PloS One* 3 (10), e3344.

Anway, M. D., A. S. Cupp, M. Uzumcu, and M. K. Skinner (2005). "Epigenetic transgenerational actions of endocrine disruptors and male fertility". In: *Science* 308 (5727), pp. 1466–1469.

Araújo, C. V., D. C. Silva, L. E. Gomes, R. D. Acayaba, C. C. Montagner, M. Moreira-Santos, R. Ribeiro, and M. L. Pompêo (2018). "Habitat fragmentation caused by contaminants: atrazine as a chemical barrier isolating fish populations". In: *Chemosphere* 193, pp. 24–31.

Ariyomo, T. O., M. Carter, and P. J. Watt (2013a). "Heritability of boldness and aggressiveness in the zebrafish". In: *Behavior Genetics* 43 (2), pp. 161–167.

Ariyomo, T. O. and P. J. Watt (2012). "The effect of variation in boldness and aggressiveness on the reproductive success of zebrafish". In: *Animal Behaviour* 83 (1), pp. 41–46.

Ariyomo, T. O. and P. J. Watt (2013b). "Aggression and sex differences in lateralization in the zebrafish". In: *Animal Behaviour* 86 (3), pp. 617–622.

Arunachalam, M., M. Raja, C. Vijayakumar, P. Malaiammal, and R. L. Mayden (2013). "Natural history of zebrafish (*Danio rerio*) in India". In: *Zebrafish* 10 (1), pp. 1–14.

Backström, T. and S. Winberg (2017). "Serotonin coordinates responses to social stress — what we can learn from fish". In: *Frontiers in Neuroscience* 11.

Badyaev, A. V. and T. Uller (2009). "Parental effects in ecology and evolution: mechanisms, processes and implications". In: *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 364 (1520), pp. 1169–1177.

Barbosa, H. P., M. G. Lima, and C. Maximino (2018a). "Acute fluoxetine differently affects aggressive display in zebrafish phenotypes". In: *bioRxiv*, "*In press*".

Barbosa, M., A. E. Deacon, M. J. Janeiro, I. Ramnarine, M. B. Morrissey, and A. E. Magurran (2018b). "Individual variation in reproductive behaviour is linked to temporal heterogeneity in predation risk". In: *Proceedings of the Royal Society B* 285 (1870), p. 20171499.

Bardullas, U., M. Giordano, and V. M. Rodrıguez (2013). "Atrazine is primarily responsible for the toxicity of long-term exposure to a combination of atrazine and inorganic arsenic in the nigrostriatal system of the albino rat". In: *Neurotoxicology and Teratology* 40, pp. 59–66.

Barker, D. (2004). "The developmental origins of adult disease". In: *Journal of the American College of Nutrition* 23, 588S–595S.

Barr, D. B., P. Panuwet, J. V. Nguyen, S. Udunka, and L. L. Needham (2007). "Assessing exposure to atrazine and its metabolites using biomonitoring". In: *Environmental Health Perspectives* 115 (10), pp. 1474–1478.

Bartoń, K. (2017). *MuMIn: multi-model inference, R package version 1.40.4*. URL: `https://cran.r-project.org/web/packages/MuMIn/index.html`.

Bass, S. L. and R. Gerlai (2008). "Zebrafish (*Danio rerio*) responds differentially to stimulus fish: the effects of sympatric and allopatric predators and harmless fish". In: *Behavioural Brain Research* 186 (1), pp. 107–117.

Bates, D., M. Mächler, B. Bolker, and S. Walker (2015). "Fitting linear mixed-effects models using lme4". In: *Journal of Statistical Software* 67 (1), pp. 1–48.

Bell, A. M. (2001). "Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*". In: *Animal Behaviour* 62 (4), pp. 775–780.

Bell, A. M., S. J. Hankison, and K. L. Laskowski (2009). "The repeatability of behaviour: a meta-analysis". In: *Animal Behaviour* 77 (4), pp. 771–783.

Bell, A. M. and A. Sih (2007). "Exposure to predation generates personality in threespined sticklebacks (*Gasterosteus aculeatus*)". In: *Ecology Letters* 10 (9), pp. 828–834.

Belloni, V., F. Dessı-Fulgheri, M. Zaccaroni, E. Di Consiglio, G. De Angelis, E. Testai, M. Santochirico, E. Alleva, and D. Santucci (2011). "Early exposure to low doses of atrazine affects behavior in juvenile and adult CD1 mice". In: *Toxicology* 279 (1-3), pp. 19–26.

Belzung, C. and P. Philippot (2007). "Anxiety from a phylogenetic perspective: is there a qualitative difference between human and animal anxiety?" In: *Neural Plasticity* 2007, p. 59676.

Bierbach, D., K. L. Laskowski, and M. Wolf (2017). "Behavioural individuality in clonal fish arises despite near-identical rearing conditions". In: *Nature Communications* 8, p. 15361.

Bierbach, D., C. Sommer-Trembo, J. Hanisch, M. Wolf, and M. Plath (2015). "Personality affects mate choice: bolder males show stronger audience effects under high competition". In: *Behavioral Ecology* 26 (5), pp. 1314–1325.

Biro, P. A. and J. R. Post (2008a). "Rapid depletion of genotypes with fast growth and bold personality traits from harvested fish populations". In: *Proceedings of the National Academy of Sciences* 105 (8), pp. 2919–2922.

Biro, P. A. and J. A. Stamps (2008b). "Are animal personality traits linked to life-history productivity?" In: *Trends in Ecology and Evolution* 23 (7), pp. 361–368.

Blake, C. A., M. L. Andersson, K. Hulthén, P. A. Nilsson, and C. Brönmark (2018). "Conspecific boldness and predator species determine predation-risk consequences of prey personality". In: *Behavioral Ecology and Sociobiology* 72 (133).

Blanchard, R. J., D. C. Blanchard, and K. Hori (1989). *An ethoexperimental approach to the study of defense*. Kluwer Academic/Plenum Publishers.

Bonduriansky, R., A. J. Crean, and T. Day (2012). "The implications of nongenetic inheritance for evolution in changing environments". In: *Evolutionary Applications* 5 (2), pp. 192–201.

Brian, J. V., J. Augley, and V. Braithwaite (2006). "Endocrine disrupting effects on the nesting behaviour of male three-spined stickleback *Gasterosteus aculeatus* L." In: *Journal of Fish Biology* 68 (6), pp. 1883–1890.

Brodeur, J. C., A. Sassone, G. N. Hermida, and N. Codugnello (2013). "Environmentally-relevant concentrations of atrazine induce non-monotonic acceleration of developmental rate and increased size at

metamorphosis in *Rhinella arenarum* tadpoles". In: *Ecotoxicology and Environmental Safety* 92, pp. 10–17.

Burnham, K. P. and D. R. Anderson (2003). *Model selection and multimodel inference: a practical information-theoretic approach*. Springer Science and Business Media.

Burton, T. and N. B. Metcalfe (2014). "Can environmental conditions experienced in early life influence future generations?" In: *Proceedings of the Royal Society B: Biological Sciences* 281 (1785), p. 20140311.

Carmen Alvarez, M. del and L. A. Fuiman (2005). "Environmental levels of atrazine and its degradation products impair survival skills and growth of red drum larvae". In: *Aquatic Toxicology* 74 (3), pp. 229–241.

Casas, E. and T. Vavouri (2014). "Sperm epigenomics: challenges and opportunities". In: *Frontiers in Genetics* 5, p. 330.

Cattelan, S., T. Lucon-Xiccato, A. Pilastro, and M. Griggio (2017). "Is the mirror test a valid measure of fish sociability?" In: *Animal Behaviour* 127, pp. 109–116.

Chapman, B. B., L. J. Morrell, and J. Krause (2010). "Unpredictability in food supply during early life influences boldness in fish". In: *Behavioral Ecology* 21 (3), pp. 501–506.

Chiavegatto, S., V. L. Dawson, L. A. Mamounas, V. E. Koliatsos, T. M. Dawson, and R. J. Nelson (2001). "Brain serotonin dysfunction accounts for aggression in male mice lacking neuronal nitric oxide synthase". In: *Proceedings of the National Academy of Sciences* 98 (3), pp. 1277–1281.

Clotfelter, E. D., A. M. Bell, and K. R. Levering (2004). "The role of animal behaviour in the study of endocrine-disrupting chemicals". In: *Animal Behaviour* 68 (4), pp. 665–676.

Clotfelter, E. D., E. P. O'hare, M. M. McNitt, R. E. Carpenter, and C. H. Summers (2007). "Serotonin decreases aggression via 5-HT1A receptors in the fighting fish *Betta splendens*". In: *Pharmacology Biochemistry and Behavior* 87 (2), pp. 222–231.

Coban, A. and N. Filipov (2007). "Dopaminergic toxicity associated with oral exposure to the herbicide atrazine in juvenile male C57BL/6 mice". In: *Journal of Neurochemistry* 100 (5), pp. 1177–1187.

Colborn, T., F. S. Vom Saal, and A. M. Soto (1993). "Developmental effects of endocrine-disrupting chemicals in wildlife and humans". In: *Environmental Health Perspectives* 101 (5), pp. 378–383.

Cold-Spring-Harbor-Protocols (2008). *E3 medium (for zebrafish embryos)*. `http://cshprotocols.cshlp.org/content/2011/10/pdb.rec66449.full?sid=0879f311-f15f-4031-9cf5-221519ac81ba`.

Cote, J. and J. Clobert (2007). "Social personalities influence natal dispersal in a lizard". In: *Proceedings of the Royal Society of London B: Biological Sciences* 274 (1608), pp. 383–390.

Cote, J., A. Dreiss, and J. Clobert (2008). "Social personality trait and fitness". In: *Proceedings of the Royal Society of London B: Biological Sciences* 275 (1653), pp. 2851–2858.

Cote, J., S. Fogarty, K. Weinersmith, T. Brodin, and A. Sih (2010). "Personality traits and dispersal tendency in the invasive mosquitofish (*Gambusia affinis*)". In: *Proceedings of the Royal Society of London B: Biological Sciences* 227, pp. 1571–1579.

Craig, I. W. and K. E. Halton (2009). "Genetics of human aggressive behaviour". In: *Human Genetics* 126 (1), pp. 101–113.

Crews, D., A. C. Gore, T. S. Hsu, N. L. Dangleben, M. Spinetta, T. Schallert, M. D. Anway, and M. K. Skinner (2007). "Transgenerational epigenetic imprints on mate preference". In: *Proceedings of the National Academy of Sciences* 104 (14), pp. 5942–5946.

Curley, J. P., R. Mashoodh, and F. A. Champagne (2011). "Epigenetics and the origins of paternal effects". In: *Hormones and Behavior* 59 (3), pp. 306–314.

Dall, S. R. (2004). "Behavioural biology: fortune favours bold and shy personalities". In: *Current Biology* 14 (12), R470–R472.

Dall, S. R., A. I. Houston, and J. M. McNamara (2004). "The behavioural ecology of personality: consistent individual differences from an adaptive perspective". In: *Ecology letters* 7 (8), pp. 734–739.

Das, U. N. (2013). "Autism as a disorder of deficiency of brain-derived neurotrophic factor and altered metabolism of polyunsaturated fatty acids". In: *Nutrition* 29 (10), pp. 1175–1185.

Devos, S., K. De Bosscher, B. Staels, E. Bauer, F. Roels, W. Berghe, G. Haegeman, R. Hooghe, and E. L. Hooghe-Peters (2003). "Inhibition of cytokine production by the herbicide atrazine: search for nuclear receptor targets". In: *Biochemical Pharmacology* 65 (2), pp. 303–308.

Diamanti-Kandarakis, E., J.-P. Bourguignon, L. C. Giudice, R. Hauser, G. S. Prins, A. M. Soto, R. T. Zoeller, and A. C. Gore (2009). "Endocrine-disrupting chemicals: an Endocrine Society scientific statement". In: *Endocrine Reviews* 30 (4), pp. 293–342.

Dias, B. G. and K. J. Ressler (2014). "Parental olfactory experience influences behavior and neural structure in subsequent generations". In: *Nature Neuroscience* 17 (1), pp. 89–96.

Dinarello, C. A. (2007). "Historical insights into cytokines". In: *European Journal of Immunology* 37, S34–S45.

Dingemanse, N. J., C. Both, P. J. Drent, and J. M. Tinbergen (2004). "Fitness consequences of avian personalities in a fluctuating environment". In: *Proceedings of the Royal Society B: Biological Sciences* 271 (1541), pp. 847–852.

Dingemanse, N. J., C. Both, P. J. Drent, K. Van Oers, and A. J. Van Noordwijk (2002). "Repeatability and heritability of exploratory behaviour in great tits from the wild". In: *Animal Behaviour* 64 (6), pp. 929–938.

Drent, P. J., K. van Oers, and A. J. van Noordwijk (2003). "Realized heritability of personalities in the great tit (*Parus major*)". In: *Proceedings of the Royal Society of London B: Biological Sciences* 270 (1510), pp. 45–51.

Dzieweczynski, T. L. (2011). "Short-term exposure to an endocrine disruptor affects behavioural consistency in male threespine stickleback". In: *Aquatic Toxicology* 105 (3–4), pp. 681–687.

Dzieweczynski, T. L., B. A. Campbell, J. M. Marks, and B. Logan (2014). "Acute exposure to 17$\alpha$-ethinylestradiol alters boldness behavioral syndrome in female Siamese fighting fish". In: *Hormones and Behavior* 66 (4), pp. 577–584.

Dzieweczynski, T. L. and O. L. Hebert (2013). "The effects of short-term exposure to an endocrine disrupter on behavioral consistency in male juvenile and adult Siamese fighting fish". In: *Archives of Environmental Contamination and Toxicology* 64 (2), pp. 316–326.

Dzieweczynski, T. L., J. L. Kane, B. A. Campbell, and L. E. Lavin (2016). "Fluoxetine exposure impacts boldness in female Siamese fighting fish, *Betta splendens*". In: *Ecotoxicology* 25 (1), pp. 69–79.

Egan, R. J., C. L. Bergner, P. C. Hart, J. M. Cachat, P. R. Canavello, M. F. Elegante, S. I. Elkhayat, B. K. Bartels, A. K. Tien, D. H. Tien, et al. (2009). "Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish". In: *Behavioural Brain Research* 205 (1), pp. 38–44.

Ennaceur, A. and J. Delacour (1988). "A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data". In: *Behavioural Brain Research* 31 (1), pp. 47–59.

Fa, S., K. Pogrmic-Majkic, D. Samardzija, B. Glisic, S. Kaisarevic, R. Kovacevic, and N. Andric (2013). "Involvement of ERK1/2 signaling pathway in atrazine action on FSH-stimulated LHR and CYP19A1 expression in rat granulosa cells". In: *Toxicology and Applied Pharmacology* 270 (1), pp. 1–8.

Fan, W., T. Yanase, H. Morinaga, S. Gondo, T. Okabe, M. Nomura, T. B. Hayes, R. Takayanagi, and H. Nawata (2007). "Herbicide atrazine activates SF-1 by direct affinity and concomitant co-activators recruitments to induce aromatase expression via promoter II". In: *Biochemical and Biophysical Research Communications* 355 (4), pp. 1012–1018.

Fan, Y., S. Ding, X. Ye, A. Manyande, D. He, N. Zhao, H. Yang, X. Jin, J. Liu, C. Tian, et al. (2013). "Does preconception paternal exposure to a physiologically relevant level of bisphenol A alter spatial memory in an adult rat?" In: *Hormones and Behavior* 64 (4), pp. 598–604.

Fangmeier, M. L., D. W. Noble, R. E. O'Dea, T. Usui, M. Lagisz, D. Hesselson, and S. Nakagawa (2018). "Computer animation technology in behavioral sciences: a sequential, automatic, and high-throughput approach to quantifying personality in zebrafish (*Danio rerio*)". In: *Zebrafish* 15 (2), pp. 206–210.

Farombi, E., S. Abarikwu, A. Adesiyan, and T. Oyejola (2013). "Quercetin exacerbates the effects of sub-acute treatment of atrazine on reproductive tissue antioxidant defence system, lipid peroxidation and sperm quality in rats". In: *Andrologia* 45 (4), pp. 256–265.

Feyzi Dehkhargani, S., H. Malekinejad, R. Shahrooz, and R. A. Sarkhanloo (2011). "Detrimental effect of atrazine on testicular tissue and sperm quality: implication for oxidative stress and hormonal alterations". In: *Iranian Journal of Toxicology* 5 (12), pp. 426–435.

Figueira, F. H., N. de Quadros Oliveira, L. M. de Aguiar, A. L. Escarrone, E. G. Primel, D. M. Barros, and C. E. da Rosa (2017). "Exposure to atrazine alters behaviour and disrupts the dopaminergic system in *Drosophila melanogaster*". In: *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 202, pp. 94–102.

Filby, A. L., G. C. Paull, E. J. Bartlett, K. J. Van Look, and C. R. Tyler (2010a). "Physiological and health consequences of social status in zebrafish (*Danio rerio*)". In: *Physiology and Behavior* 101 (5), pp. 576–587.

Filby, A. L., G. C. Paull, T. F. Hickmore, and C. R. Tyler (2010b). "Unravelling the neurophysiological basis of aggression in a fish model". In: *BMC Genomics* 11 (1), p. 498.

Filiano, A. J., Y. Xu, N. J. Tustison, R. L. Marsh, W. Baker, I. Smirnov, C. C. Overall, S. P. Gadani, S. D. Turner, Z. Weng, et al. (2016). "Unexpected role of interferon-$\gamma$ in regulating neuronal connectivity and social behaviour". In: *Nature* 535 (7612), pp. 425–429.

Fish, E. W., S. Faccidomo, and K. A. Miczek (1999). "Aggression heightened by alcohol or social instigation in mice: reduction by the 5-HT 1B receptor agonist CP-94,253". In: *Psychopharmacology* 146 (4), pp. 391–399.

Fleming, I. A., A. Lamberg, and B. Jonsson (1997). "Effects of early experience on the reproductive performance of Atlantic salmon". In: *Behavioral Ecology* 8 (5), pp. 470–480.

Fossat, P., J. Bacqué-Cazenave, P. De Deurwaerdère, J.-P. Delbecque, and D. Cattaert (2014). "Anxiety-like behavior in crayfish is controlled by serotonin". In: *Science* 344 (6189), pp. 1293–1297.

Fraites, M. J., R. L. Cooper, A. Buckalew, S. Jayaraman, L. Mills, and S. C. Laws (2009). "Characterization of the hypothalamic-pituitary-adrenal axis response to atrazine and metabolites in the female rat". In: *Toxicological Sciences* 112 (1), pp. 88–99.

Franklin, T. B., H. Russig, I. C. Weiss, J. Gräff, N. Linder, A. Michalon, S. Vizi, and I. M. Mansuy (2010). "Epigenetic transmission of the impact of early stress across generations". In: *Biological Psychiatry* 68 (5), pp. 408–415.

Frost, A. J., A. Winrow-Giffen, P. J. Ashley, and L. U. Sneddon (2007). "Plasticity in animal personality traits: does prior experience alter the degree of boldness?" In: *Proceedings of the Royal Society B-Biological Sciences* 274 (1608), pp. 333–339.

Garcia-Garcia, A. L., Q. Meng, J. Richardson-Jones, A. Dranovsky, and E. D. Leonardo (2016). "Disruption of 5-HT1A function in adolescence but not early adulthood leads to sustained increases of anxiety". In: *Neuroscience* 321, pp. 210–221.

Gely-Pernot, A., C. Hao, E. Becker, I. Stuparevic, C. Kervarrec, F. Chalmel, M. Primig, B. Jégou, and F. Smagulova (2015). "The epigenetic processes of meiosis in male mice are broadly affected by the widely used herbicide atrazine". In: *BMC Genomics* 16 (1), p. 885.

Gerlach, G. (2006). "Pheromonal regulation of reproductive success in female zebrafish: female suppression and male enhancement". In: *Animal Behaviour* 72 (5), pp. 1119–1124.

Gioiosa, L., S. Parmigiani, F. S. Vom Saal, and P. Palanza (2013). "The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice". In: *Hormones and Behavior* 63 (4), pp. 598–605.

Godfrey, S. S., J. K. Bradley, A. Sih, and C. M. Bull (2012). "Lovers and fighters in sleepy lizard land: where do aggressive males fit in a social network?" In: *Animal Behaviour* 83 (1), pp. 209–215.

Gore, A. C., V. Chappell, S. Fenton, J. A. Flaws, A. Nadal, G. S. Prins, J. Toppari, and R. Zoeller (2015). "EDC-2: the Endocrine Society's second scientific statement on endocrine-disrupting chemicals". In: *Endocrine Reviews* 36 (6), E1–E150.

Grant, J. W. and D. L. Kramer (1992). "Temporal clumping of food arrival reduces its monopolization and defence by zebrafish, *Brachydanio rerio*". In: *Animal Behaviour* 44 (1), pp. 101–110.

Graymore, M., F. Stagnitti, and G. Allinson (2001). "Impacts of atrazine in aquatic ecosystems". In: *Environment International* 26 (7-8), pp. 483–495.

Gross, C., X. Zhuang, K. Stark, S. Ramboz, R. Oosting, L. Kirby, L. Santarelli, S. Beck, and R. Hen (2002). "Serotonin 1A receptor acts during development to establish normal anxiety-like behaviour in the adult". In: *Nature* 416 (6879), pp. 396–400.

Grunst, A. S., M. L. Grunst, B. Thys, T. Raap, N. Daem, R. Pinxten, and M. Eens (2018). "Variation in personality traits across a metal pollution gradient in a free-living songbird". In: *Science of The Total Environment* 630, pp. 668–678.

Hackett, J. A. and M. A. Surani (2013). "Beyond DNA: programming and inheritance of parental methylomes". In: *Cell* 153 (4), pp. 737–739.

Hao, C., A. Gely-Pernot, C. Kervarrec, M. Boudjema, E. Becker, P. Khil, S. Tevosian, B. Jégou, and F. Smagulova (2016). "Exposure to the widely used herbicide atrazine results in deregulation of global tissue-specific RNA transcription in the third generation and is associated with a global decrease of histone trimethylation in mice". In: *Nucleic Acids Research* 44 (20), pp. 9784–9802.

Harris, S., I. W. Ramnarine, H. G. Smith, and L. B. Pettersson (2010). "Picking personalities apart: estimating the influence of predation, sex and body size on boldness in the guppy *Poecilia reticulata*". In: *Oikos* 119 (11), pp. 1711–1718.

Harrison, X. A. (2014). "Using observation-level random effects to model overdispersion in count data in ecology and evolution". In: *PeerJ* 2, e616.

Hayes, T. B., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. P. Mai, Y. Marjuoa, J. Parker, et al. (2006). "Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact?" In: *Environmental Health Perspectives* 114, pp. 40–50.

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk (2002). "Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses". In: *Proceedings of the National Academy of Sciences* 99 (8), pp. 5476–5480.

Hayes, T. B., V. Khoury, A. Narayan, M. Nazir, A. Park, T. Brown, L. Adame, E. Chan, D. Buchholz, T. Stueve, et al. (2010). "Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*)". In: *Proceedings of the National Academy of Sciences* 107 (10), pp. 4612–4617.

Hebert, O. L., L. E. Lavin, J. M. Marks, and T. L. Dzieweczynski (2014). "The effects of 17$\alpha$-ethinyloestradiol on boldness and its relationship to decision making in male Siamese fighting fish". In: *Animal Behaviour* 87, pp. 203–212.

Hecker, M., J.-W. Park, M. B. Murphy, P. D. Jones, K. R. Solomon, G. Van Der Kraak, J. A. Carr, E. E. Smith, L. du Preez, R. J. Kendall, et al. (2005). "Effects of atrazine on CYP19 gene expression and aromatase activity in testes and on plasma sex steroid concentrations of male African clawed frogs (*Xenopus laevis*)". In: *Toxicological Sciences* 86 (2), pp. 273–280.

Heiming, R. S., A. Mönning, F. Jansen, V. Kloke, K.-P. Lesch, and N. Sachser (2013). "To attack, or not to attack? The role of serotonin transporter genotype in the display of maternal aggression". In: *Behavioural Brain Research* 242, pp. 135–141.

Heisler, L. K., N. Pronchuk, K. Nonogaki, L. Zhou, J. Raber, L. Tung, G. S. Yeo, S. O'Rahilly, W. F. Colmers, J. K. Elmquist, et al. (2007). "Serotonin activates the hypothalamic–pituitary–adrenal axis via serotonin 2C receptor stimulation". In: *Journal of Neuroscience* 27 (26), pp. 6956–6964.

Herculano, A. M. and C. Maximino (2014). "Serotonergic modulation of zebrafish behavior: towards a paradox". In: *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 55, pp. 50–66.

Hisaoka, K. and C. Firlit (1962). "Ovarian cycle and egg production in the zebrafish, *Brachydanio rerio*". In: *Copeia*, pp. 788–792.

Holmes, A., D. L. Murphy, and J. N. Crawley (2002). "Reduced aggression in mice lacking the serotonin transporter". In: *Psychopharmacology* 161 (2), pp. 160–167.

Holtmann, B., S. Grosser, M. Lagisz, S. Johnson, E. Santos, C. Lara, B. Robertson, and S. Nakagawa (2016). "Population differentiation and behavioural association of the two 'personality' genes DRD4 and SERT in dunnocks (*Prunella modularis*)". In: *Molecular Ecology* 25 (3), pp. 706–722.

Hotchkiss, A. K., C. V. Rider, C. R. Blystone, V. S. Wilson, P. C. Hartig, G. T. Ankley, P. M. Foster, C. L. Gray, and L. E. Gray (2008). "Fifteen years after "Wingspread" - environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go". In: *Toxicological Sciences* 105 (2), pp. 235–259.

Howe, K., M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K. McLaren, L. Matthews, et al. (2013). "The zebrafish reference genome sequence and its relationship to the human genome". In: *Nature* 496 (7446), pp. 498–503.

Immler, S. (2018). "The sperm factor: paternal impact beyond genes." In: *Heredity* 121, pp. 239–247.

Jablonka, E. and G. Raz (2009). "Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution". In: *The Quarterly Review of Biology* 84 (2), pp. 131–176.

Jacobs, M. N., E. L. Marczylo, C. Guerrero-Bosagna, and J. Rüegg (2017). "Marked for Life: epigenetic effects of endocrine disrupting chemicals". In: *Annual Review of Environment and Resources* 42, pp. 105–160.

Jandt, J. M., S. Bengston, N. Pinter-Wollman, J. N. Pruitt, N. E. Raine, A. Dornhaus, and A. Sih (2014). "Behavioural syndromes and social insects: personality at multiple levels". In: *Biological Reviews* 89 (1), pp. 48–67.

Jenssen, B. M. (2006). "Endocrine-disrupting chemicals and climate change: a worst-case combination for arctic marine mammals and seabirds?" In: *Environmental Health Perspectives* 114, pp. 76–80.

Jiang, L., J. Zhang, J.-J. Wang, L. Wang, L. Zhang, G. Li, X. Yang, X. Ma, X. Sun, J. Cai, et al. (2013). "Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos". In: *Cell* 153 (4), pp. 773–784.

Johnson, S. L., S. Zellhuber-McMillan, J. Gillum, J. Dunleavy, J. P. Evans, S. Nakagawa, and N. J. Gemmell (2018). "Evidence that fertility trades off with early offspring fitness as males age". In: *Proceedings of the Royal Society B* 285 (1871), p. 20172174.

Jöngren, M., J. Westander, D. Nätt, and P. Jensen (2010). "Brain gene expression in relation to fearfulness in female red junglefowl (*Gallus gallus*)". In: *Genes, Brain and Behavior* 9 (7), pp. 751–758.

Jonsson, B. and N. Jonsson (2014). "Early environment influences later performance in fishes". In: *Journal of Fish Biology* 85 (2), pp. 151–188.

Kalueff, A. V., M. Gebhardt, A. M. Stewart, J. M. Cachat, M. Brimmer, J. S. Chawla, C. Craddock, E. J. Kyzar, A. Roth, S. Landsman, et al. (2013). "Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond". In: *Zebrafish* 10 (1), pp. 70–86.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling (1995). "Stages of embryonic development of the zebrafish". In: *Developmental Dynamics* 203 (3), pp. 253–310.

Kinch, C. D., K. Ibhazehiebo, J.-H. Jeong, H. R. Habibi, and D. M. Kurrasch (2015). "Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish". In: *Proceedings of the National Academy of Sciences* 112 (5), pp. 1475–1480.

Kirsten, K. S., R. Canova, L. de Figueiredo Soveral, M. T. Friedrich, R. Frandoloso, and L. C. Kreutz (2017). "Reduced expression of selective immune-related genes in silver catfish (*Rhamdia quelen*) monocytes exposed to atrazine". In: *Fish and Shellfish Immunology* 64, pp. 78–83.

Kirsten, K., D. Fior, L. C. Kreutz, and L. J. G. Barcellos (2018). "First description of behavior and immune system relationship in fish". In: *Scientific Reports* 8, p. 846.

Kloke, V., F. Jansen, R. S. Heiming, R. Palme, K.-P. Lesch, and N. Sachser (2011). "The winner and loser effect, serotonin transporter genotype, and the display of offensive aggression". In: *Physiology and Behavior* 103 (5), pp. 565–574.

Koolhaas, J., S. Korte, S. De Boer, B. Van Der Vegt, C. Van Reenen, H. Hopster, I. De Jong, M. Ruis, and H. Blokhuis (1999). "Coping styles in animals: current status in behavior and stress-physiology". In: *Neuroscience and Biobehavioral Reviews* 23 (7), pp. 925–935.

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen (2017). "lmerTest Package: Tests in Linear Mixed Effects Models". In: *Journal of Statistical Software* 82 (13), pp. 1–26.

Landeau, L. and J. Terborgh (1986). "Oddity and the 'confusion effect' in predation". In: *Animal Behaviour* 34 (5), pp. 1372–1380.

Le Fol, V., S. Aıt-Aıssa, M. Sonavane, J.-M. Porcher, P. Balaguer, J.-P. Cravedi, D. Zalko, and F. Brion (2017). "In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays". In: *Ecotoxicology and Environmental Safety* 142, pp. 150–156.

Lee, S. L. (2015). "A transcriptomic examination of sexual differentiation in zebrafish, *Danio rerio*". PhD thesis. New Zealand: University of Otago.

Li, C.-Y., C. Curtis, and R. L. Earley (2018). "Nonreversing mirrors elicit behaviour that more accurately predicts performance against live opponents". In: *Animal Behaviour* 137, pp. 95–105.

Lillesaar, C. (2011). "The serotonergic system in fish". In: *Journal of Chemical Neuroanatomy* 41 (4), pp. 294–308.

Lin, Z., C. A. Dodd, and N. M. Filipov (2013). "Short-term atrazine exposure causes behavioral deficits and disrupts monoaminergic systems in male C57BL/6 mice". In: *Neurotoxicology and Teratology* 39, pp. 26–35.

Lin, Z., C. A. Dodd, S. Xiao, S. Krishna, X. Ye, and N. M. Filipov (2014a). "Gestational and lactational exposure to atrazine via the drinking water causes specific behavioral deficits and selectively alters monoaminergic systems in C57BL/6 mouse dams, juvenile and adult offspring". In: *Toxicological Sciences* 141 (1), pp. 90–102.

Lin, Z., J. R. Roede, C. He, D. P. Jones, and N. M. Filipov (2014b). "Short-term oral atrazine exposure alters the plasma metabolome of male C57BL/6 mice and disrupts $\alpha$-linolenate, tryptophan, tyrosine and other major metabolic pathways". In: *Toxicology* 326, pp. 130–141.

Luca, R. M. and R. Gerlai (2012a). "Animated bird silhouette above the tank: acute alcohol diminishes fear responses in zebrafish". In: *Behavioural Brain Research* 229 (1), pp. 194–201.

Luca, R. M. and R. Gerlai (2012b). "In search of optimal fear inducing stimuli: differential behavioral responses to computer animated images in zebrafish". In: *Behavioural Brain Research* 226 (1), pp. 66–76.

MacPherson, B., M. Mashayekhi, R. Gras, and R. Scott (2017). "Exploring the connection between emergent animal personality and fitness using a novel individual-based model and decision tree approach". In: *Ecological Informatics* 40, pp. 81–92.

Madsen, T. and R. Shine (2000). "Silver spoons and snake body sizes: prey availability early in life influences long-term growth rates of free-ranging pythons". In: *Journal of Animal Ecology* 69 (6), pp. 952–958.

Mainwaring, M. C. and I. R. Hartley (2013). "Hatching asynchrony and offspring sex influence the subsequent exploratory behaviour of zebra finches". In: *Animal Behaviour* 85 (1), pp. 77–81.

Marcus, S. R. and A. C. Fiumera (2016). "Atrazine exposure affects longevity, development time and body size in *Drosophila melanogaster*". In: *Journal of Insect Physiology* 91, pp. 18–25.

Marshall, J. D. and T. Uller (2007). "When is a maternal effect adaptive?" In: *Oikos* 116 (12), pp. 1957–1963.

Martins, E. P. and A. Bhat (2014). "Population-level personalities in zebrafish: aggression-boldness across but not within populations". In: *Behavioral Ecology* 25 (2), pp. 368–373.

Maximino, C., R. Benzecry, K. R. M. Oliveira, E. d. J. O. Batista, A. M. Herculano, D. B. Rosemberg, D. L. de Oliveira, and R. Blaser (2012). "A comparison of the light/dark and novel tank tests in zebrafish". In: *Behaviour* 149 (10-12), pp. 1099–1123.

Maximino, C., T. M. de Brito, B. A. W. da Silva, A. M. Herculano, S. Morato, and J. A. Gouveia (2010). "Measuring anxiety in zebrafish: a critical review". In: *Behavioural Brain Research* 214 (2), pp. 157–171.

Maximino, C., B. Puty, R. Benzecry, J. Araújo, M. G. Lima, E. d. J. O. Batista, K. R. de Matos Oliveira, M. E. Crespo-Lopez, and A. M. Herculano (2013). "Role of serotonin in zebrafish (*Danio rerio*) anxiety: relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-chlorophenylalanine (pCPA) in two behavioral models". In: *Neuropharmacology* 71, pp. 83–97.

McBirney, M., S. E. King, M. Pappalardo, E. Houser, M. Unkefer, E. Nilsson, I. Sadler-Riggleman, D. Beck, P. Winchester, and M. K. Skinner (2017). "Atrazine induced epigenetic transgenerational inheritance of disease, lean phenotype and sperm epimutation pathology biomarkers". In: *PloS One* 12 (9), e0184306.

McCallum, M. L., M. Matlock, J. Treas, B. Safi, W. Sanson, and J. L. McCallum (2013). "Endocrine disruption of sexual selection by an estrogenic herbicide in the mealworm beetle (*Tenebrio molitor*)". In: *Ecotoxicology* 22 (10), pp. 1461–1466.

McClure, M., P. McIntyre, and A. McCune (2006). "Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish *Danio rerio*". In: *Journal of Fish Biology* 69 (2), pp. 553–570.

Miczek, K. A. and R. M. de Almeida (2001). "Oral drug self-administration in the home cage of mice: alcohol-heightened aggression and inhibition by the 5-HT 1B agonist anpirtoline". In: *Psychopharmacology* 157 (4), pp. 421–429.

Miller-Butterworth, C. M., J. R. Kaplan, M. M. Barmada, S. B. Manuck, and R. E. Ferrell (2007). "The serotonin transporter: sequence variation in *Macaca fascicularis* and its relationship to dominance". In: *Behavior Genetics* 37 (5), pp. 678–696.

Miller, N. and R. Gerlai (2007). "Quantification of shoaling behaviour in zebrafish (*Danio rerio*)". In: *Behavioural Brain Research* 184 (2), pp. 157–166.

Montiglio, P.-O., D. Garant, D. Thomas, and D. Réale (2010). "Individual variation in temporal activity patterns in open-field tests". In: *Animal Behaviour* 80 (5), pp. 905–912.

Moon, M. L., J. J. Joesting, N. A. Blevins, M. A. Lawson, S. J. Gainey, A. E. Towers, L. K. McNeil, and G. G. Freund (2015). "IL-4 knock out mice display anxiety-like behavior". In: *Behavior Genetics* 45 (4), pp. 451–460.

Müller, J. C., J. Partecke, B. J. Hatchwell, K. J. Gaston, and K. L. Evans (2013). "Candidate gene polymorphisms for behavioural adaptations during urbanization in blackbirds". In: *Molecular Ecology* 22 (13), pp. 3629–3637.

Nesan, D., L. C. Sewell, and D. M. Kurrasch (2018). "Opening the black box of endocrine disruption of brain development: lessons from the characterization of Bisphenol A". In: *Hormones and Behavior* 101, pp. 50–58.

Nettle, D. and L. Penke (2010). "Personality: bridging the literatures from human psychology and behavioural ecology". In: *Philosophical Transactions of the Royal Society B: Biological Sciences* 365 (1560), pp. 4043–4050.

Nishi, M., N. Horii-Hayashi, and T. Sasagawa (2014). "Effects of early life adverse experiences on the brain: implications from maternal separation models in rodents". In: *Frontiers in Neuroscience* 8, p. 166.

Nishi, M., N. Horii-Hayashi, T. Sasagawa, and W. Matsunaga (2013). "Effects of early life stress on brain activity: implications from maternal separation model in rodents". In: *General and Comparative Endocrinology* 181, pp. 306–309.

Noldus, L. P., A. J. Spink, and R. A. Tegelenbosch (2001). "EthoVision: a versatile video tracking system for automation of behavioral experiments". In: *Behavior Research Methods, Instruments, and Computers* 33 (3), pp. 398–414.

Norton, W. H., A. Folchert, and L. Bally-Cuif (2008). "Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (*slc6a4a/b*) gene expression in the zebrafish brain". In: *Journal of Comparative Neurology* 511 (4), pp. 521–542.

Norton, W. and L. Bally-Cuif (2010). "Adult zebrafish as a model organism for behavioural genetics". In: *BMC Neuroscience* 11 (1), p. 90.

Noyes, P. D. and S. C. Lema (2015). "Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife". In: *Current Zoology* 61 (4), pp. 669–689.

O'Dea, R. E., D. W. Noble, S. L. Johnson, D. Hesselson, and S. Nakagawa (2016). "The role of non-genetic inheritance in evolutionary rescue: epigenetic buffering, heritable bet hedging and epigenetic traps". In: *Environmental Epigenetics* 2 (1), pp. 1–12.

Oers, K. van, M. Klunder, and P. J. Drent (2005). "Context dependence of personalities: risk-taking behavior in a social and a nonsocial situation". In: *Behavioral Ecology* 16 (4), pp. 716–723.

Ogwang, S. P. (2017). "Inspection of a novel object by wild and laboratory zebrafish (*Danio rerio*)". MA thesis. Norway: University of Bergen.

Oliveira, R. F., L. A. Carneiro, and A. V. Canário (2005). "Behavioural endocrinology: no hormonal response in tied fights". In: *Nature* 437 (7056), pp. 207–208.

Oliveira, R. F., J. F. Silva, and J. M. Simoes (2011). "Fighting zebrafish: characterization of aggressive behavior and winner–loser effects". In: *Zebrafish* 8 (2), pp. 73–81.

Oliveira, R. F., J. M. Simões, M. C. Teles, C. R. Oliveira, J. D. Becker, and J. S. Lopes (2016). "Assessment of fight outcome is needed to activate socially driven transcriptional changes in the zebrafish brain". In: *Proceedings of the National Academy of Sciences* 113 (5), pp. 1–8.

Olst, J. C. v. and J. R. Hunter (1970). "Some aspects of the organization of fish schools". In: *Journal of the Fisheries Board of Canada* 27 (7), pp. 1225–1238.

Palanza, P., S. C. Nagel, S. Parmigiani, and F. S. vom Saal (2016). "Perinatal exposure to endocrine disruptors: sex, timing and behavioral endpoints". In: *Current Opinion in Behavioral Sciences* 7, pp. 69–75.

Papoulias, D. M., D. E. Tillitt, M. G. Talykina, J. J. Whyte, and C. A. Richter (2014). "Atrazine reduces reproduction in Japanese medaka (*Oryzias latipes*)". In: *Aquatic Toxicology* 154, pp. 230–239.

Paull, G. C., A. L. Filby, H. G. Giddins, T. S. Coe, P. B. Hamilton, and C. R. Tyler (2010). "Dominance hierarchies in zebrafish (*Danio rerio*) and their relationship with reproductive success". In: *Zebrafish* 7 (1), pp. 109–117.

Peña-Contreras, Z., L. Miranda-Contreras, Y. Morales-Ovalles, M. Colmenares-Sulbarán, D. Dávila-Vera, A. Balza-Quintero, S. Salmen, and R. V. Mendoza-Briceño (2016). "Atrazine and mancozeb induce excitotoxicity and cytotoxicity in primary cultures of mouse cerebellar cortex". In: *Toxicological and Environmental Chemistry* 98 (8), pp. 959–976.

Pérez, J., I. Domingues, M. Monteiro, A. M. Soares, and S. Loureiro (2013). "Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish *Danio rerio*". In: *Environmental Science and Pollution Research* 20 (7), pp. 4671–4680.

Pike, T. W., M. Samanta, J. Lindström, and N. J. Royle (2008). "Behavioural phenotype affects social interactions in an animal network". In: *Proceedings of the Royal Society of London B: Biological Sciences* 275 (1650), pp. 2515–2520.

Piñeyro, G. and P. Blier (1999). "Autoregulation of serotonin neurons: role in antidepressant drug action". In: *Pharmacological Reviews* 51 (3), pp. 533–591.

Pink, M. and M. V. Abrahams (2018). "In shallow water ecosytems the abiotic environment is more important than prey abundance for foraging terns". In: *Environmental Biology of Fishes*, pp. 1–8.

Pitcher, T., S. Lang, and J. Turner (1988). "A risk-balancing trade off between foraging rewards and predation hazard in a shoaling fish". In: *Behavioral Ecology and Sociobiology* 22 (3), pp. 225–228.

Popova, N. (2008). "From gene to aggressive behavior: the role of brain serotonin". In: *Neuroscience and Behavioral Physiology* 38 (5), pp. 471–475.

Porseryd, T., K. Volkova, N. R. Caspillo, T. Kaallman, P. Dinnetz, and I. P. Hallstrom (2017a). "Persistent effects of developmental exposure to 17 alpha-ethinylestradiol on the zebrafish (*Danio rerio*) brain transcriptome and behavior". In: *Frontiers in Behavioral Neuroscience* 11, p. 69.

Porseryd, T., M. Kellner, N. R. Caspillo, K. Volkova, L. Elabbas, S. Ullah, H. Olsén, P. Dinnétz, and I. P. Hällström (2017b). "Combinatory effects of low concentrations of 17$\alpha$-etinylestradiol and citalopram on non-reproductive behavior in adult zebrafish (*Danio rerio*)". In: *Aquatic Toxicology* 193, pp. 9–17.

Potok, M. E., D. A. Nix, T. J. Parnell, and B. R. Cairns (2013). "Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern". In: *Cell* 153 (4), pp. 759–772.

Rando, O. J. (2016). "Intergenerational transfer of epigenetic information in sperm". In: *Cold Spring Harbor Perspectives in Medicine*, a022988.

Raoult, V., C. Brown, A. Zuberi, and J. E. Williamson (2012). "Blood cortisol concentrations predict boldness in juvenile mulloway (*Argyosomus japonicus*)". In: *Journal of Ethology* 30 (2), pp. 225–232.

Réale, D., N. J. Dingemanse, A. J. Kazem, and J. Wright (2010). "Evolutionary and ecological approaches to the study of personality". In: *Philosophical Transactions of the Royal Society B* 365 (1560), pp. 3937–3946.

Réale, D. and M. Festa-Bianchet (2003). "Predator-induced natural selection on temperament in bighorn ewes". In: *Animal Behaviour* 65 (3), pp. 463–470.

Réale, D., S. M. Reader, D. Sol, P. T. McDougall, and N. J. Dingemanse (2007). "Integrating animal temperament within ecology and evolution". In: *Biological Reviews* 82 (2), pp. 291–318.

Reddon, A. R. (2011). "Parental effects on animal personality". In: *Behavioral Ecology* 23 (2), pp. 242–245.

Reed, T. E., R. S. Waples, D. E. Schindler, J. J. Hard, and M. T. Kinnison (2010). "Phenotypic plasticity and population viability: the importance of environmental predictability". In: *Proceedings of the Royal Society of London B: Biological Sciences* 277 (1699), pp. 3391–3400.

Riffle, B. W., G. R. Klinefelter, R. L. Cooper, W. M. Winnik, A. Swank, S. Jayaraman, J. Suarez, D. Best, and S. C. Laws (2014). "Novel molecular events associated with altered steroidogenesis induced by exposure to atrazine in the intact and castrate male rat". In: *Reproductive Toxicology* 47, pp. 59–69.

Riyahi, S., M. Sánchez-Delgado, F. Calafell, D. Monk, and J. C. Senar (2015). "Combined epigenetic and intraspecific variation of the DRD4 and SERT genes influence novelty seeking behavior in great tit *Parus major*". In: *Epigenetics* 10 (6), pp. 516–525.

Rodriguez, V., M. Mendoza-Trejo, I. Hernandez-Plata, and M. Giordano (2017). "Behavioral effects and neuroanatomical targets of acute atrazine exposure in the male Sprague-Dawley rat". In: *Neurotoxicology* 58, pp. 161–170.

Rokka, K., M. Pihlaja, H. Siitari, and C. D. Soulsbury (2014). "Sex-specific differences in offspring personalities across the laying order in magpies *Pica pica*". In: *Behavioural Processes* 107, pp. 79–87.

Rosengren, M., P.-O. Thörnqvist, S. Winberg, and K. Sundell (2017). "The brain-gut axis of fish: rainbow trout with low and high cortisol response show innate differences in intestinal integrity and brain gene expression". In: *General and Comparative Endocrinology* 257, pp. 235–245.

Ross, M. K., T. L. Jones, and N. M. Filipov (2009). "Disposition of the herbicide 2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine (atrazine) and its major metabolites in mice: a liquid chromatography/mass spectrometry analysis of urine, plasma, and tissue levels". In: *Drug Metabolism and Disposition* 37 (4), pp. 776–786.

Rypel, A. L., C. A. Layman, and D. A. Arrington (2007). "Water depth modifies relative predation risk for a motile fish taxon in Bahamian tidal creeks". In: *Estuaries and Coasts* 30 (3), pp. 518–525.

Saaristo, M., J. A. Craft, K. K. Lehtonen, and K. Lindström (2010). "Exposure to 17$\alpha$-ethinyl estradiol impairs courtship and aggressive behaviour of male sand gobies (*Pomatoschistus minutus*)". In: *Chemosphere* 79 (5), pp. 541–546.

Sasaki, T., R. P. Mann, K. N. Warren, T. Herbert, T. Wilson, and D. Biro (2018). "Personality and the collective: bold homing pigeons occupy higher leadership ranks in flocks". In: *Philosophical Transactions of the Royal Society B* 373 (1746), p. 20170038.

Schmidel, A. J., K. L. Assmann, C. C. Werlang, K. T. Bertoncello, F. Francescon, C. L. Rambo, G. M. Beltrame, D. Calegari, C. B. Batista, R. E. Blaser, et al. (2014). "Subchronic atrazine exposure changes

defensive behaviour profile and disrupts brain acetylcholinesterase activity of zebrafish". In: *Neurotoxicology and Teratology* 44, pp. 62–69.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri (2012). "NIH Image to ImageJ: 25 years of image analysis". In: *Nature Methods* 9 (7), pp. 671–675.

Schuett, W., S. R. Dall, M. H. Kloesener, J. Baeumer, F. Beinlich, and T. Eggers (2015). "Life-history trade-offs mediate 'personality' variation in two colour morphs of the pea aphid, *Acyrthosiphon pisum*". In: *Journal of Animal Ecology* 84 (1), pp. 90–101.

Schuster, A. C., T. Carl, and K. Foerster (2017). "Repeatability and consistency of individual behaviour in juvenile and adult Eurasian harvest mice". In: *The Science of Nature* 104 (10).

Senior, A. M., S. Nakagawa, and V. Grimm (2014). "The evolutionary consequences of disrupted male mating signals: an agent-based modelling exploration of endocrine disrupting chemicals in the guppy". In: *PloS One* 9 (7), e103100.

Shenoy, K. (2012). "Environmentally realistic exposure to the herbicide atrazine alters some sexually selected traits in male guppies". In: *PLoS One* 7 (2), e30611.

Shenoy, K. (2014). "Prenatal exposure to low doses of atrazine affects mating behaviors in male guppies". In: *Hormones and Behavior* 66 (2), pp. 439–448.

Shenoy, K. and P. H. Crowley (2011). "Endocrine disruption of male mating signals: ecological and evolutionary implications". In: *Functional Ecology* 25 (3), pp. 433–448.

Sih, A., A. M. Bell, J. C. Johnson, and R. E. Ziemba (2004a). "Behavioral syndromes: an integrative overview". In: *The Quarterly Review of Biology* 79 (3), pp. 241–277.

Sih, A., A. Bell, and J. C. Johnson (2004b). "Behavioral syndromes: an ecological and evolutionary overview". In: *Trends in Ecology and Evolution* 19 (7), pp. 372–378.

Sih, A., J. Cote, M. Evans, S. Fogarty, and J. Pruitt (2012). "Ecological implications of behavioural syndromes". In: *Ecology Letters* 15 (3), pp. 278–289.

Siviter, H., D. C. Deeming, J. Rosenberger, O. H. Burman, S. A. Moszuti, and A. Wilkinson (2017). "The impact of egg incubation temperature on the personality of oviparous reptiles". In: *Animal Cognition* 20 (1), pp. 109–116.

Skinner, M. K., M. D. Anway, M. I. Savenkova, A. C. Gore, and D. Crews (2008). "Transgenerational epigenetic programming of the brain transcriptome and anxiety behavior". In: *PloS One* 3 (11), e3745.

Skinner, M. K., M. Manikkam, and C. Guerrero-Bosagna (2010). "Epigenetic transgenerational actions of environmental factors in disease etiology". In: *Trends in Endocrinology and Metabolism* 21 (4), pp. 214–222.

Skinner, M. K., M. Manikkam, and C. Guerrero-Bosagna (2011). "Epigenetic transgenerational actions of endocrine disruptors". In: *Reproductive Toxicology* 31 (3), pp. 337–343.

Smith, B. R. and D. T. Blumstein (2008). "Fitness consequences of personality: a meta-analysis". In: *Behavioral Ecology* 19 (2), pp. 448–455.

Söffker, M. and C. R. Tyler (2012). "Endocrine disrupting chemicals and sexual behaviors in fish–a critical review on effects and possible consequences". In: *Critical Reviews in Toxicology* 42 (8), pp. 653–668.

Solomon, K. R., J. A. Carr, L. H. Du Preez, J. P. Giesy, R. J. Kendall, E. E. Smith, and G. J. Van Der Kraak (2008). "Effects of atrazine on fish, amphibians, and aquatic reptiles: a critical review". In: *Critical Reviews in Toxicology* 38 (9), pp. 721–772.

Sopinka, N., P. Capelle, C. A. Semeniuk, and O. P. Love (2017). "Glucocorticoids in fish eggs: variation, interactions with the environment, and the potential to shape offspring fitness". In: *Physiological and Biochemical Zoology* 90 (1), pp. 15–33.

Spanò, L., C. R. Tyler, R. van Aerle, P. Devos, S. Mandiki, F. Silvestre, J.-P. Thomé, and P. Kestemont (2004). "Effects of atrazine on sex steroid dynamics, plasma vitellogenin concentration and gonad development in adult goldfish (*Carassius auratus*)". In: *Aquatic Toxicology* 66 (4), pp. 369–379.

Spence, R., M. Fatema, S. Ellis, Z. Ahmed, and C. Smith (2007). "Diet, growth and recruitment of wild zebrafish in Bangladesh". In: *Journal of Fish Biology* 71 (1), pp. 304–309.

Spence, R., M. Fatema, M. Reichard, K. Huq, M. Wahab, Z. Ahmed, and C. Smith (2006). "The distribution and habitat preferences of the zebrafish in Bangladesh". In: *Journal of Fish Biology* 69 (5), pp. 1435–1448.

Spence, R., G. Gerlach, C. Lawrence, and C. Smith (2008). "The behaviour and ecology of the zebrafish, *Danio rerio*". In: *Biological Reviews* 83 (1), pp. 13–34.

Steinberg, C. E., R. Lorenz, and O. H. Spieser (1995). "Effects of atrazine on swimming behavior of zebrafish, *Brachydanio rerio*". In: *Water Research* 29 (3), pp. 981–985.

Stoffel, M. A., S. Nakagawa, and H. Schielzeth (2017). "rptR: Repeatability estimation and variance decomposition by generalized linear mixed-effects models". In: *Methods in Ecology and Evolution* 8 (11), pp. 1639–1644.

Swanson, J. E., E. Muths, C. L. Pierce, S. J. Dinsmore, M. W. Vandever, M. L. Hladik, and K. L. Smalling (2018). "Exploring the amphibian exposome in an agricultural landscape using telemetry and passive sampling". In: *Scientific Reports* 8, p. 10045.

Symonds, M. R. and A. Moussalli (2011). "A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion". In: *Behavioral Ecology and Sociobiology* 65 (1), pp. 13–21.

Szyf, M. (2015). "Nongenetic inheritance and transgenerational epigenetics". In: *Trends in Molecular Medicine* 21 (2), pp. 134–144.

Takahashi, A., I. M. Quadros, R. M. de Almeida, and K. A. Miczek (2011). "Brain serotonin receptors and transporters: initiation vs. termination of escalated aggression". In: *Psychopharmacology* 213 (2-3), pp. 183–212.

Takahashi, H. (1977). "Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*". In: *Bulletin of the Faculty of Fisheries Hokkaido University* 28 (2), pp. 57–65.

Taylor, R. W., A. Boon, B. Dantzer, D. Reale, M. Humphries, S. Boutin, J. Gorrell, D. Coltman, and A. McAdam (2012). "Low heritabilities, but genetic and maternal correlations between red squirrel behaviours". In: *Journal of Evolutionary Biology* 25 (4), pp. 614–624.

Team, R. C. (2018). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. Vienna, Austria. URL: `https://www.R-project.org/`.

Teles, M. C. and R. F. Oliveira (2016). "Androgen response to social competition in a shoaling fish". In: *Hormones and Behavior* 78, pp. 8–12.

Theodoridi, A., A. Tsalafouta, and M. Pavlidis (2017). "Acute exposure to fluoxetine alters aggressive behavior of zebrafish and expression of genes involved in serotonergic system regulation". In: *Frontiers in Neuroscience* 11, p. 223.

Thomson, H. (2017). "Consistency of behaviours over time and context in zebrafish". MA thesis. New Zealand: University of Otago.

Thörnqvist, P.-O., E. Höglund, and S. Winberg (2015). "Natural selection constrain personality and brain gene expression differences in Atlantic salmon (*Salmo salar*)". In: *Journal of Experimental Biology* 218, pp. 1077–1083.

Trentacoste, S. V., A. S. Friedmann, R. T. Youker, C. B. Breckenridge, and B. R. Zirkin (2001). "Atrazine effects on testosterone levels and androgen-dependent reproductive organs in peripubertal male rats". In: *Journal of Andrology* 22 (1), pp. 142–148.

Trillmich, F., T. Müller, and C. Müller (2018). "Understanding the evolution of personality requires the study of mechanisms behind the development and life history of personality traits". In: *Biology Letters* 14 (2).

Uchida, D., M. Yamashita, T. Kitano, and T. Iguchi (2002). "Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish". In: *Journal of Experimental Biology* 205 (6), pp. 711–718.

Vandenberg, L. N. (2014). "Low-dose effects of hormones and endocrine disruptors". In: *Vitamins and Hormones* 94, pp. 129–165.

Vandenberg, L. N. et al. (2012). "Hormones and endocrine-disrupting chemicals: low-dose effects and non-monotonic dose responses". In: *Endocrine Reviews* 33 (3), pp. 378–455.

Verhulst, E. C., A. C. Mateman, M. V. Zwier, S. P. Caro, K. J. Verhoeven, and K. Oers (2016). "Evidence from pyrosequencing indicates that natural variation in animal personality is associated with DRD4 DNA methylation". In: *Molecular Ecology* 25 (8), pp. 1801–1811.

Visser, M. E. (2008). "Keeping up with a warming world; assessing the rate of adaptation to climate change". In: *Proceedings of the Royal Society of London B: Biological Sciences* 275 (1635), pp. 649–659.

Volkova, K., N. R. Caspillo, T. Porseryd, S. Hallgren, P. Dinnetz, H. Olsén, and I. P. Hällström (2015a). "Transgenerational effects of 17$\alpha$-ethinyl estradiol on anxiety behavior in the guppy, *Poecilia reticulata*". In: *General and Comparative Endocrinology* 223, pp. 66–72.

Volkova, K., N. R. Caspillo, T. Porseryd, S. Hallgren, P. Dinnétz, and I. Porsch-Hällström (2015b). "Developmental exposure of zebrafish (*Danio rerio*) to 17$\alpha$-ethinylestradiol affects non-reproductive behavior and fertility as adults, and increases anxiety in unexposed progeny". In: *Hormones and Behavior* 73, pp. 30–38.

Vos, J. G., E. Dybing, H. A. Greim, O. Ladefoged, C. Lambré, J. V. Tarazona, I. Brandt, and A. D. Vethaak (2000). "Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation". In: *Critical Reviews in Toxicology* 30 (1), pp. 71–133.

Walters, J. L., T. A. Lansdell, K. J. Lookingland, and L. E. Baker (2015). "The effects of gestational and chronic atrazine exposure on motor behaviors and striatal dopamine in male Sprague-Dawley rats". In: *Toxicology and Applied Pharmacology* 289 (2), pp. 185–192.

Wang, J., X. Wang, C. Xiong, J. Liu, B. Hu, and L. Zheng (2015). "Chronic bisphenol A exposure alters behaviors of zebrafish (*Danio rerio*)". In: *Environmental Pollution* 206, pp. 275–281.

Wang, X., H. Xing, X. Li, S. Xu, and X. Wang (2011). "Effects of atrazine and chlorpyrifos on the mRNA levels of IL-1 and IFN-$\gamma$2b in immune organs of common carp". In: *Fish and Shellfish Immunology* 31 (1), pp. 126–133.

Wang, Y., H. Liu, and Z. Sun (2017). "Lamarck rises from his grave: parental environment-induced epigenetic inheritance in model organisms and humans". In: *Biological Reviews* 92 (4), pp. 2084–2111.

Wang, Y., R. Takai, H. Yoshioka, and K. Shirabe (2006). "Characterization and expression of serotonin transporter genes in zebrafish". In: *The Tohoku Journal of Experimental Medicine* 208 (3), pp. 267–274.

Way, G. P., N. Ruhl, J. L. Snekser, A. L. Kiesel, and S. P. McRobert (2015). "A comparison of methodologies to test aggression in zebrafish". In: *Zebrafish* 12 (2), pp. 144–151.

Weber, G. J., M. S. Sepúlveda, S. M. Peterson, S. S. Lewis, and J. L. Freeman (2013). "Transcriptome alterations following developmental atrazine exposure in zebrafish are associated with disruption of neuroendocrine and reproductive system function, cell cycle, and carcinogenesis". In: *Toxicological Sciences* 132 (2), pp. 458–466.

Werner, E. E., J. F. Gilliam, D. J. Hall, and G. G. Mittelbach (1983). "An experimental test of the effects of predation risk on habitat use in fish". In: *Ecology* 64 (6), pp. 1540–1548.

White, J. W., B. J. Cole, G. N. Cherr, R. E. Connon, and S. M. Brander (2017). "Scaling up endocrine disruption effects from individuals to populations: outcomes depend on how many males a population needs". In: *Environmental Science and Technology* 51 (3), pp. 1802–1810.

White, S. J. and A. J. Wilson (2018). "Evolutionary genetics of personality in the Trinidadian guppy I: maternal and additive genetic effects across ontogeny". In: *Heredity*.

Whitfield, C. W., A.-M. Cziko, and G. E. Robinson (2003). "Gene expression profiles in the brain predict behavior in individual honey bees". In: *Science* 302 (5643), pp. 296–299.

Wiese, A.-S., E. K. Needham, C. L. Noer, T. J. S. Balsby, T. Dabelsteen, and B. Pakkenberg (2018). "The number of neurons in specific amygdala regions is associated with boldness in mink: a study in animal personality". In: *Brain Structure and Function* 223 (4), pp. 1989–1998.

Wilcox, C., N. J. Mallos, G. H. Leonard, A. Rodriguez, and B. D. Hardesty (2016). "Using expert elicitation to estimate the impacts of plastic pollution on marine wildlife". In: *Marine Policy* 65, pp. 107–114.

Wilson, D. S., A. B. Clark, K. Coleman, and T. Dearstyne (1994). "Shyness and boldness in humans and other animals". In: *Trends in Ecology and Evolution* 9 (11), pp. 442–446.

Wirbisky-Hershberger, S. E., O. F. Sanchez, K. A. Horzmann, D. Thanki, C. Yuan, and J. L. Freeman (2017). "Atrazine exposure decreases the activity of DNMTs, global DNA methylation levels, and *dnmt* expression". In: *Food and Chemical Toxicology* 109, pp. 727–734.

Wirbisky, S. E., M. S. Sepúlveda, G. J. Weber, A. S. Jannasch, K. A. Horzmann, and J. L. Freeman (2016a). "Embryonic atrazine exposure elicits alterations in genes associated with neuroendocrine function in adult male zebrafish". In: *Toxicological Sciences* 153 (1), pp. 149–164.

Wirbisky, S. E., G. J. Weber, K. E. Schlotman, M. S. Sepúlveda, and J. L. Freeman (2016b). "Embryonic atrazine exposure alters zebrafish and human miRNAs associated with angiogenesis, cancer, and neurodevelopment". In: *Food and Chemical Toxicology* 98, pp. 25–33.

Wirbisky, S. E., G. J. Weber, M. S. Sepúlveda, T.-L. Lin, A. S. Jannasch, and J. L. Freeman (2016c). "An embryonic atrazine exposure results in reproductive dysfunction in adult zebrafish and morphological alterations in their offspring". In: *Scientific Reports* 6, p. 21337.

Wirbisky, S. E., G. J. Weber, M. S. Sepúlveda, C. Xiao, J. R. Cannon, and J. L. Freeman (2015). "Developmental origins of neurotransmitter and transcriptome alterations in adult female zebrafish exposed to atrazine during embryogenesis". In: *Toxicology* 333, pp. 156–167.

Wolf, M., G. S. Van Doorn, O. Leimar, and F. J. Weissing (2007). "Life-history trade-offs favour the evolution of animal personalities". In: *Nature* 447 (7144), pp. 581–584.

Wolf, M. and F. J. Weissing (2012). "Animal personalities: consequences for ecology and evolution". In: *Trends in Ecology and Evolution* 27 (8), pp. 452–461.

Wolstenholme, J. T., M. Edwards, S. R. Shetty, J. D. Gatewood, J. A. Taylor, E. F. Rissman, and J. J. Connelly (2012). "Gestational exposure to bisphenol A produces transgenerational changes in behaviors and gene expression". In: *Endocrinology* 153 (8), pp. 3828–3838.

Wright, D., R. Nakamichi, J. Krause, and R. K. Butlin (2006a). "QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*)". In: *Behavior Genetics* 36 (2), pp. 271–284.

Wright, D., R. K. Butlin, and Ö. Carlborg (2006b). "Epistatic regulation of behavioural and morphological traits in the zebrafish (*Danio rerio*)". In: *Behavior Genetics* 36 (6), pp. 914–922.

Wright, D., L. B. Rimmer, V. L. Pritchard, R. Butlin, and J. Krause (2003). "Inter and intra-population variation in shoaling and boldness in the zebrafish (*Danio rerio*)". In: *Naturwissenschaften* 90 (8), pp. 374–377.

Wright, D., A. Ward, D. Croft, and J. Krause (2006c). "Social organization, grouping, and domestication in fish". In: *Zebrafish* 3 (2), pp. 141–155.

Xia, J., C. Niu, and X. Pei (2010). "Effects of chronic exposure to nonylphenol on locomotor activity and social behavior in zebrafish (*Danio rerio*)". In: *Journal of Environmental Sciences* 22 (9), pp. 1435–1440.

Zajitschek, S., J. E. Herbert-Read, N. M. Abbasi, F. Zajitschek, and S. Immler (2017). "Paternal personality and social status influence offspring activity in zebrafish". In: *BMC Evolutionary Biology* 17 (1), p. 157.

Zhuang, X., C. Gross, L. Santarelli, V. Compan, A.-C. Trillat, and R. Hen (1999). "Altered emotional states in knockout mice lacking 5-HT1A or 5-HT1B receptors". In: *Neuropsychopharmacology* 21, 52S–60S.

# Appendix I: IVF protocol

The following protocol describes the method of *in-vitro-fertilisation* (IVF) written by Dr Sheri Johnson. This method is also available from Johnson et al. (2018). IVF was chosen over other natural breeding methods, because the time of conception can be easily manipulated. Doing IVF over natural spawning also removes the behavioural uncertainty of successful mating between zebrafish males and females. The process describes several steps needed to ensure successful IVF.

**Squeezing**

**Day before experiments:**

1. Put one vial of Tricaine in fridge to thaw overnight. Check that have buffers for ZSI (see recipe below). Note that 1 X PBS can be used in place of ZSI, if necessary.

**Day of experiment:**

1. Prepare zfish sperm immobilizer (ZSI; see recipe below)
2. Prepare tubes with 10 uL of ZSI in each tube (close tubes so no ice or water get in tubes)
3. Set up two 250 mL beakers with anaesthetic: add 200 mL of fish water to the 8.2 mL aliquot, and then split between two beakers.
4. Anesthetise males until breathing slows, remove beaker, blot off excess anesthetic on paper towel, rinse fish in fish water and then place on paper towel and blot gonadal region with Q-tip dipped in ZSI.
5. Collect sperm in capillary tube and mouth pipette into pre-prepared tubes. Make a note on datasheet whether you collected 0.5-1 uL or more than 1 uL.
6. Once all males are squeezed, add 10 uL more of ZSI to males that gave more than 1.5-2 uL, 20 uL to males that give 2.5-3 uL and so on.

**IVF Initial Prep:**

1. Label small petri dishes (on the bottom and the top).
2. Dilute sperm collected from males. In a new tube, add 5 uL of diluted sperm to 45 uL of ZSI and mix. Return rest of sperm dilution to fridge for CASA analysis, etc. Keep IVF aliquot on ice at all times.

**IVF:**

1. Squeeze females after 5-10 min in Tricaine
2. Check for quality of eggs under microscope
3. Add 45 uL sperm (gently pipette before first replicate) on top of eggs and IMMEDIATELY add 1 mL of fish water, THEN proceed to the next replicate and switch tips between replicates
4. Wait 5 minutes and then add 5 mL of fish water
5. After 60 min, transfer to incubator @ 28.5 C
6. Assay fertilization  4 hpf and then transfer to labelled large petri dishes. The bottom should just have the male ID and replicate (e.g., AB 7A) that corresponds with that IVF sample. The lid should have this same label, plus the fish line, date and researcher initials). Enter cross information in the cross binder
7. Assess hatching rate at 3 dpf and then move to system 5dpf

**Zebrafish Reagent Recipes**

*Zebrafish Sperm Immobilizer (page 114941 in Mol. Lab book)*

- 140 mM NaCl (1M stock: 14.61 g NaCl in 250 mL MQ)
- 10 mM KCl (100 mM stock: 1.86 g of KCL in 250 mL MQ)
- 2 mM CaCl2 (100 mM stock: 2.77 g of CaCl2 in 250 mL MQ)
- 20 mM HEPES (100 mM stock: 4.78 g of HEPES in 200 mL of MQ), pH 8.5 (filtered with 0.22 uM syringe filter, kept at 4C)

*To prepare working stock morning of sampling:*

- 14 mL 1M NaCl
- 10 mL 100 mM KCl

- 2 mL 100 mM $CaCl_2$
- 20 mL 100 mM HEPES
- 54 mL of DI H20
- Keep on ice

*Tricaine:*

- 400 mg tricaine powder
- 97.9 ml UP H20 water
- ∼2.1 ml 1 M Tris (pH 9).

Tricaine (3-amino benzoic acid ethyl ester also called ethyl 3-aminobenzonate) comes in a powdered form from Sigma (Cat.# A-5040). It is also available as Finquel (Part No. C- FINQ-UE) from Argent Chemical Laboratories, Inc. Make triacaine solution for anaesthetizing fish by combing the following in a glass bottle with a screw cap:

*Adjust pH to ∼7 and aliquot:*

- 8.4 mL tricaine in 15 mL tubes
- Label tubes with instruction to dissolve in 200 mL Zfish system water
- Store aliquots in the -20 C freezer. (Buy the smallest amount possible because tricaine gets old)

# Appendix II: Early life effects of offspring from atrazine exposed fathers

## Methods

At 24hpf dead embryos were removed from petri dishes and counted. To ascertain hatching success, at 48hpf, 72hpf, 96hpf each petri dish was photographed using a Cannon EOS 700D with a cannon 50mm macro-lens and number of hatched and unhatched fry were counted in ImageJ (version 1.50i; Schneider et al. 2012). Figure A2.1 shows an example of hatched and unhatched embryos.

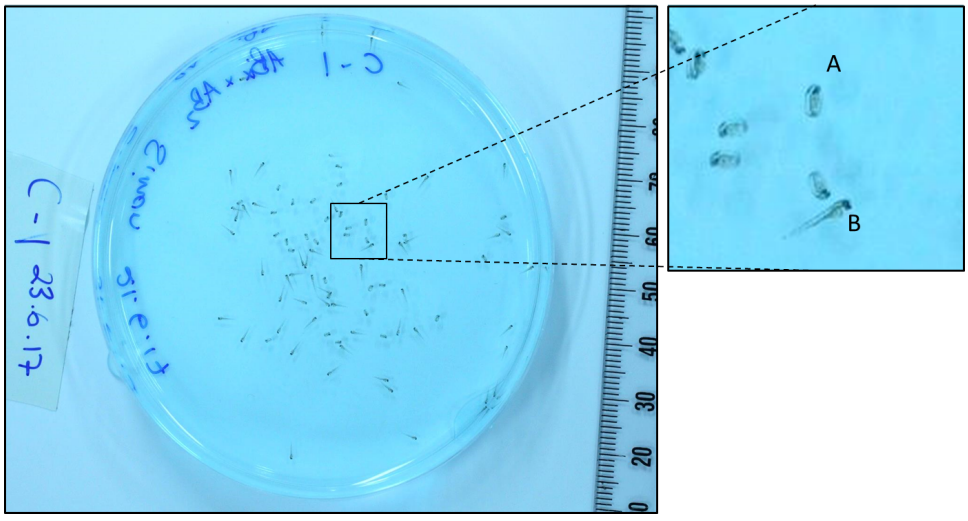


Figure A2.1: Example of zebrafish at 48hpf, photograph taken in order to count hatched and unhatched embryos. Example of A) an unhatched and B) hatched zebrafish.

## Results

Embryonic death peaked by 24hpf (see Table A2.1 for numbers) across all treatments. There was a significant reduction in the proportion of offspring that survived to 24hpf in the 3ppb treatment ($z = -6.68$, $p < 0.001$), but no other response was observed in either the 0.3ppb and 3ppb treatments (Figure A2.2 A). Interestingly, control variation was minimal relative to all other treatments, with variation greatest in the 3ppb treatment. Unexpectedly, the majority of survival in the 30ppb was above the mean of controls, whereas the majority of families from both 0.3ppb and 3ppb treatments were below. Deaths that occurred past 24hpf were minimal for each treatment (0 - 2 individuals) or did not occur whilst fish were held within the petri-dish.

The majority of hatching had occurred by 48hpf and by 72hpf $>\sim$97% had emerged (see Table A2.2 for numbers). The proportion of embryos that hatched by 48hpf was significantly less in the 3ppb ($z = -6.50$, $p < 0.001$) and 30ppb ($z = -4.57$, $p < 0.001$) treatments, and was near significance in the 0.3ppb ($p = 0.087$) treatment (Figure A2.2B). Additionally, variation in the proportion of offspring that hatched increased with increasing atrazine concentration. Note that the number of families were seven.

Table A2.1: Numbers of zebrafish offspring survived to 24hpf. Data are summarised across all families per treatment, number of families per treatment, n=7.

| **Treatment** | **n (Total)** | **n (Total survived)** | **n (Total died)** |
|---|---|---|---|
| Control | 692 | 608 | 84 |
| 0.3ppb | 768 | 650 | 118 |
| 3ppb | 720 | 529 | 191 |
| 30ppb | 614 | 553 | 61 |

Table A2.2: Numbers of zebrafish offspring that hatched at 48hpf. Data are summarised across all families per treatment, number of families per treatment n=7.

| **Treatment** | **n (Total)** | **n (Hatched)** | **n (Did not hatch)** |
|---|---|---|---|
| Control | 604 | 416 | 188 |
| 0.3ppb | 650 | 418 | 232 |
| 3ppb | 525 | 261 | 264 |
| 30ppb | 552 | 308 | 244 |

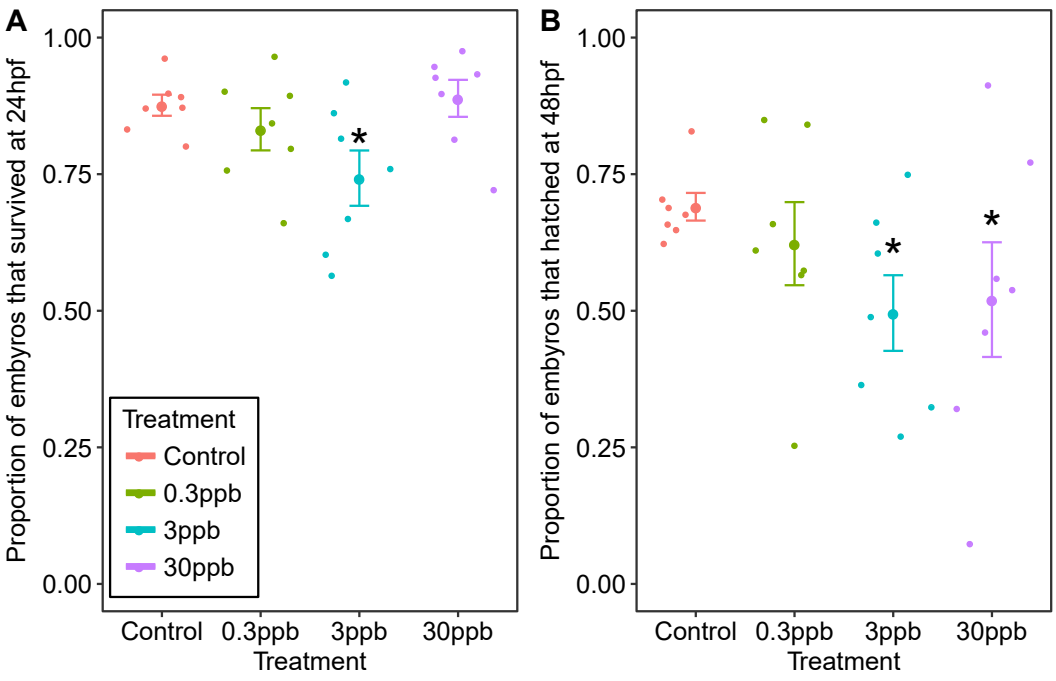


Figure A2.2: Early life parameters of zebrafish embryos from untreated and atrazine treated fathers. A) the propotion of embryos that survived to 24hpf. B) the propotion of embryos that hatched by 48hpf. A * indicates significant differences ($p < 0.05$). Large points represent means, with errors bars representing standard errors of the mean. Smaller, randomly jittered (along the x-axis) points are the raw proportions from each family, from each treatment. Total number of families per treatment were n=7.

# Appendix III: Sample sizes used in behavioural assays

Figure legends from figures depicting behavioural phenotypes (in main text, see Chapter 2) show ranges of the sample sizes used in order to improve general readability. This was due to un-evenness of samples across sex and treatments (in particular amongst the $F_1$'s), in addition, this was also due to some $F_1$ individuals (8 fish in total) being excluded from the analyses of behaviours measured in the novel object assay caused by experimental error (see Chapter 2, Methods, Faulty tracking). Therefore, below are the sample sizes used for all behavioural test (Table A3.1).

Table A3.1: Sample sizes of zebrafish used in behavioural assays across each assay.

| **Novel arena test** | **$F_0$** | | **$F_1$** | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| Control | 12 | 12 | 21 | 28 |
| 0.3ppb | 12 | 9 | 22 | 24 |
| 3ppb | 12 | 12 | 18 | 30 |
| 30ppb | 12 | 12 | 18 | 28 |
| **Novel object test** | | | | |
| Control | 12 | 12 | 19 | 27 |
| 0.3ppb | 12 | 9 | 21 | 24 |
| 3ppb | 12 | 12 | 17 | 30 |
| 30ppb | 12 | 12 | 18 | 27 |
| **Mirror test** | | | | |
| Control | 12 | 12 | 21 | 28 |
| 0.3ppb | 12 | 9 | 22 | 24 |
| 3ppb | 12 | 12 | 18 | 30 |
| 30ppb | 12 | 12 | 18 | 28 |

# Appendix IV: Repeatability of zebrafish aggression using a mirror stimulus assay

## Objectives

In order to understand the impact of adult zebrafish aggression under an atrazine exposure during juvenile development, an assessment of a mirror test was undertaken to discern consistent differences between individuals (animal personality). I assessed the repeatability of two behavioural measures thought to be representative of aggression; the time spent interacting with the mirror and the frequency entering the mirror zone in non-exposed adults wildtype (AB) strain over the course of three sampling dates.

## Methods

### Experimental overview

The zebrafish used in the experiment were part of a long term repeatability project that ran five behavioural sampling periods over the course of 27 weeks, from 13/10/16 to 14/04/17 (Thomson 2017). This study took place in between sampling point four and just after sampling point 5 (week 14 to just after week 27) of that experiment. In total, 41 zebrafish (date of birth; 22/03/2016) were used (comprising 20 females and 21 males). Each fish was uniquely tagged by injecting visible implant elastomers at the start of the long term repeatability experiments, one on either side of the fish's body resulting in a unique and identifiable colour combination (colours used were; yellow, green, red or orange). Zebrafish were fed twice daily with dry food; ZM400 and once a day with live Artemia (*Artemia salina*). On the day of testing, zebrafish were

fed after the assay. Temperature during housing was maintained at 25°C with a 14:12 hour light:dark, dawn-dusk lighting regime. Three different time points were chosen to assess repeatability; 0 days, 7 days and 28 days, initial behavioural tests were performed on the 18th and 19th of March 2017, after which the seven day repeatability tests were conducted on the 25th and 26st of March, the 28 day repeatability tests were conducted on the 15th and 16th of April 2017.

## Experimental setup

The experimental setup follows the design described in methods of Chapter 2 (see Chapter 2, Methods, Behavioural phenotyping; Experimental protocols; adapted from Ariyomo et al. (2012); Way et al. (2015)), with slight differences in filming equipment and volume of water used. In brief, three identical glass tanks (length=30cm, width=15.5cm and height=27cm) were used side by side as test arenas for the assay. A mirror (length=19cm, width=15cm) was fitted externally to the side of the tank; two tanks with mirrors fixed on the right and one mirror placed on the left. White plastic film was fitted to the inside of tanks to block external stimulus between tanks and to minimise unwanted reflections during filming, the side facing the camera was not covered. A removable opaque plastic partition was used to cover up mirrors. Tanks were filled with a combination of 3L system water and 3L aerated adjusted water and left to age overnight. Trials took place over two days, testing half of the fish on one day, and half the next. In preparation for repeatability tests the following day, 3L of tested water was replaced in each tank and left to age overnight. Two-three days before assessment, males and females were separated within their holding tanks (length=18cm, width=11cm and height=8.5cm) to allow easy individual identification after each assay. Before entering the test arena, fish were netted from their holding tanks to an intermediary tank for 2-4min to homogenise entering time. Three fish were filmed simultaneously per assay with one individual per test tank at a time. The aggression behavioural assay started with an initial 10min acclimation period (i.e. a novel arena test), after which, the opaque plastic partition was removed, revealing the mirror stimulus. To minimise the interference of unsettled water in tracking ability, filming commenced 1min after the last mirror was revealed. Filming during the mirror assay lasted for 10min. Fish were netted out of the test arena into their intermediary holding tanks and their unique colour combinations were identified with a UV torch. Trials were filmed using GoPro Hero3+ cameras at a resolution of 1080p at 60 frames per second. Cameras were placed at distance of ($\sim$ 15cm away from the tank) along the mirrors edges, capturing roughly 1/2 ($\sim$15cm) of the entire tank during filming (see Figure A4.1). Each camera was placed on a make-shift LEGO® platform to achieve required height relative to the distance to maximise vision in the assay. Platforms were covered in white plastic film to block colour variation from the LEGO® blocks. To control for variation in circadian

rhythms, each tank was tested within 30 minutes of the last time it was tested; i.e. tank one was always tested between 09:30 and 12:00, while tank two was always tested between 12:50 and 15:00. After filming, measures of aggression were recorded using EthoVision (Noldus et al. 2001). A mirror zone ~5cm from the mirror was drawn and the time spent in the mirror zone as well as the frequency of times entering the mirror zone (sec) was recorded (Figure A4.1).

Figure A4.1: EthoVision setup for investigating repeatability during the mirror test. The light green region depicts the mirror zone area, while the orange zone depicts the rest of the testing arena.

### Statistical analysis

Repeatability of each behavioural measure was analysed using the '*rptR*' package (Stoffel et al. 2017) in R, version 3.5.0 (Team 2018), with permutations and bootstrap set to 1000. The cumulative duration spent in the mirror zone was normally distributed and analysed under the 'guassian datatype'. The frequency of times entering the mirror zone was analysed using the 'poisson datatype'.

## Results

The time spent within the mirror zone was significantly repeatable, for both sexes and across all time points, with the exception between week and a month for males (Table A4.1). Overall, females were more repeatable than males, 0.52 compared to 0.35. Figure A4.2 depicts the raw values for all individuals.

The frequency of entering the mirror zone was significantly repeatable between the initial and one week tests, but was not repeatable overall (Table A4.2). After the week test, the majority of males declined sharply in

their frequency, whereas females showed a combination of increases and decreases in their frequency (Figure A4.3).

Table A4.1: Repeatability estimates ($R$) of the time spent within the mirror zone, including lower and upper confidence intervals (CI), standard error (SE) and $P$ values. Repeatability estimates are separated by time points and sex. Males, $n_{[individuals]}$=21, $n_{[total\ observations]}$=63; females, $n_{[individuals]}$=20, $n_{[total\ observations]}$=60.

| **Time Points** | **Group** | ***R*** | **Lower 95% CI** | **Upper 95% CI** | **SE** | ***P value*** |
|---|---|---|---|---|---|---|
| Initial - Week | Combined | 0.67 | 0.44 | 0.80 | 0.09 | **<0.001** |
| | Male | 0.57 | 0.22 | 0.81 | 0.15 | **0.001** |
| | Female | 0.72 | 0.42 | 0.88 | 0.12 | **<0.001** |
| Week - Month | Combined | 0.406 | 0.131 | 0.631 | 0.127 | **0.003** |
| | Male | 0.287 | 0.000 | 0.647 | 0.173 | 0.057 |
| | Female | 0.504 | 0.097 | 0.762 | 0.167 | **0.008** |
| Initial - Month (Overall) | Combined | 0.439 | 0.242 | 0.616 | 0.093 | **<0.001** |
| | Male | 0.351 | 0.102 | 0.609 | 0.132 | **<0.001** |
| | Female | 0.520 | 0.245 | 0.725 | 0.129 | **<0.001** |

Table A4.2: Repeatability estimates ($R$) of the frequency entering the mirror zone, including upper and lower confidence intervals (CI) standard error (SE) and $P$ values. Repeatability estimates are separated by time points and sex. Males, $n_{[individuals]}$=21, $n_{[total\ observations]}$=63; females, $n_{[individuals]}$=20, $n_{[total\ observations]}$=60.

| **Time Points** | **Group** | ***R*** | **Lower 95% CI** | **Upper 95% CI** | **SE** | ***P value*** |
|---|---|---|---|---|---|---|
| Initial - Week | Combined | 0.61 | 0.38 | 0.76 | 0.1 | **<0.001** |
| | Male | 0.70 | 0.39 | 0.85 | 0.12 | **<0.001** |
| | Female | 0.53 | 0.08 | 0.78 | 0.17 | **0.006** |
| Week - Month | Combined | 0 | 0 | 0.28 | 0.08 | 0.5 |
| | Male | 0 | 0 | 0.33 | 0.10 | 1 |
| | Female | 0 | 0 | 0.41 | 0.13 | 0.5 |
| Initial - Month (Overall) | Combined | 0.08 | 0 | 0.25 | 0.075 | 0.176 |
| | Male | 0.14 | 0 | 0.37 | 0.11 | 0.081 |
| | Female | 0.06 | 0 | 0.33 | 0.10 | 0.334 |

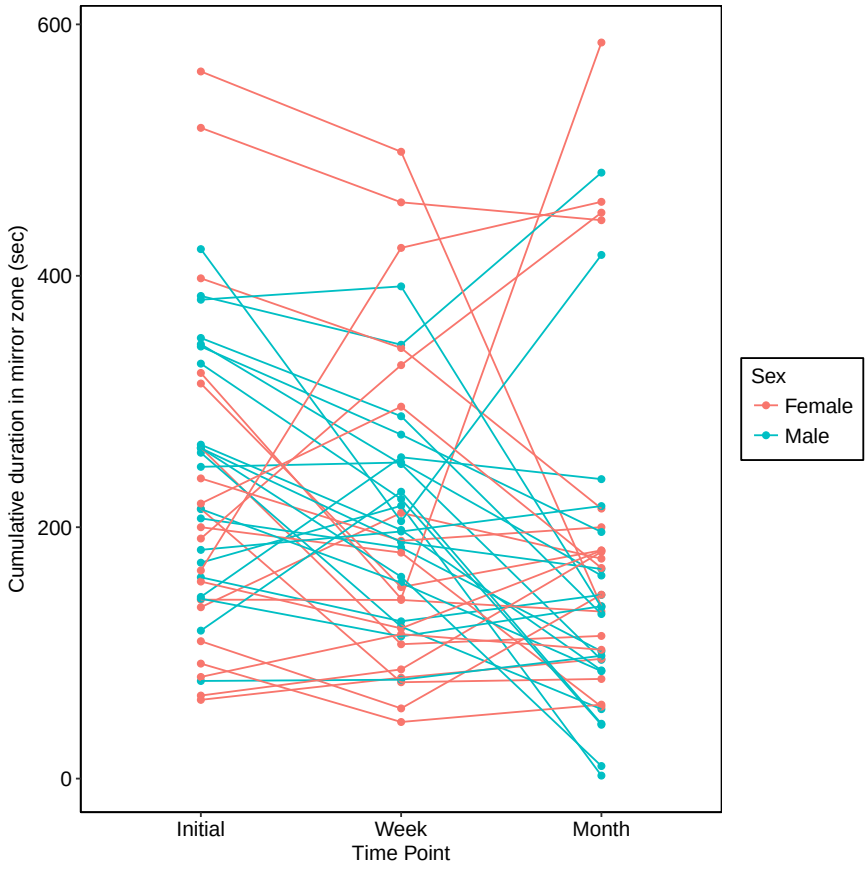


Figure A4.2: Raw values (points) of the cumulative duration spent in the mirror zone of each individual, connected across all three time points (lines); Initial, week and month. Males are coloured in blue, $n_{[individuals]}$=21, $n_{[observations]}$=63; females in pink, $n_{[individuals]}$=20, $n_{[observations]}$=60.

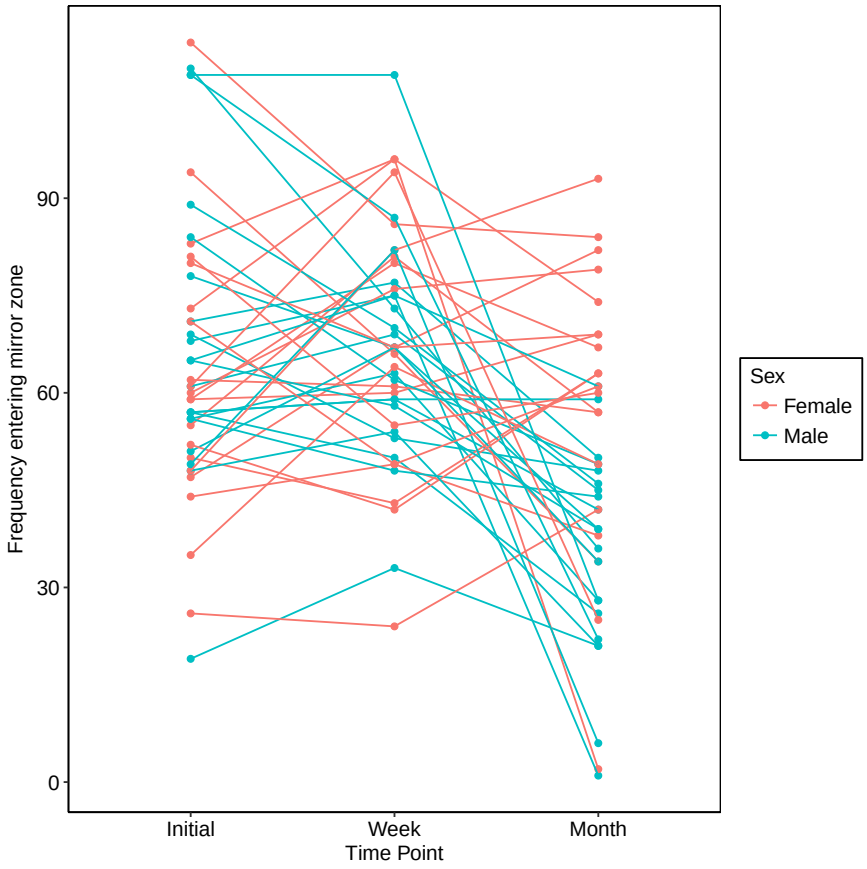


Figure A4.3: Raw values (points) of the frequency entering the mirror zone of each individual, connected across all three time points (lines); Initial, week and month. Males are coloured in blue, $n_{[individuals]}$=21, $n_{[observations]}$=63; females in pink, $n_{[individuals]}$=20, $n_{[observations]}$=60.

# Appendix V: Statistical analyses for behavioural measures

All statistical analyses were conducted in R, version 3.5.0 (Team 2018). All analyses described below contained the predictors; treatment (i.e. controls, 0.3ppb, 3ppb, 30ppb), and sex (male and female) with an interaction term. The interaction term was removed if there was no influence and was re-analysed without it. All $F_0$ behavioural measures were analysed with linear models (LMs) or generalised linear models (GLMs). All $F_1$ behavioural measures were analysed with linear mixed effects models (LMMs) or with generalised linear mixed effects models (GLMMs) using the package '*lme4*' version 1.1-1.3 (Bates et al. 2015) with family id included as a random effect to account for genetic influences (with some exceptions, see Appendix V). Specific details on each behaviour analysed can be seen in Appendix V.

Exploration and measures of anxiety, the time spent in the bottom zone of the novel arena test and the time spent in the bottom zone of the novel object test were analysed using a LM.

Analyses for measures of boldness are described hereafter. The time spent in the top zone of the novel arena test was analysed using a LM. The latency to enter the top zone was right skewed and therefore analysed with a (GLM) using a gamma error structure (log-link function). The assumptions of a GLM with a gamma error structure are that zeros are unable to be present as they skew the results, therefore if a fish was in the top zone at the start of the trial; it was given a latency of 0.1 sec, if the fish never entered the top zone; it was given a latency of 600 sec (the total duration of the assay). For the $F_0$'s, the frequency entering the top zone of the novel arena was analysed using a GLM with a quasi-poisson error structure (log-link function) to account for over-dispersion. For the $F_1$'s however, frequency was analysed with a GLMM with a poisson error structure (log-link function), with two random effects; family, to account for genetic influence and fish id, to account for over-dispersion (a quasi-poisson error structure is not possible with random effects). Interacting with the novel object was analysed using a GLM with a binomial error structure (logit-link

function) where the binary response was 'interacted with the novel object' (1), or 'did not interact with the novel object' (0).

For measures of aggression, the time spent interacting with the mirror was analysed with a LM. For the frequency entering the mirror zone, the $F_0$'s were analysed using a GLM with a quasi-poisson error structure (log-link function) to account for over-dispersion. The $F_1$'s however, frequency entering the mirror zone was analysed with a GLMM with a poisson error structure (log-link function), with two random effects; family, to account for genetic influence and fish id, to account for over-dispersion.

Analyses for measures of activity are described hereafter. The time spent not moving in the novel arena, total distance moved and the mean velocity in each of the three behavioural tests were analysed with LMs. I also investigated how the influence of the different assays employed might have affected activity levels. I ran a LMM with assay type (i.e. novel arena, novel object and mirror test), treatment and with sex as predictors and the total distance moved as the response (mean velocity reported in Appendix VI). For the $F_0$'s, fish id was included as a random effect to account for pseudo-replication. For the $F_1$'s, fish id nested within family was included as a random effect (Harrison 2014)

I investigated how the influence of the different assays employed might have affected activity levels. Assays were compared against one another using a LMM with assay type (i.e. novel arena, novel object and mirror test), treatment and sex as predictors, and the total distance moved as the response (mean velocity reported in Appendix VIII). For the $F_0$'s, fish ID was included as a random effect to account for repeated measures on the same individuals. For the $F_1$'s, fish ID nested within family was included as a random effect (results can be found in Appendix VI).

In some instances of $F_1$ analyses, family level variation was suspected of influencing the model outputs. This would indicate a genetic component of behaviour, i.e. siblings regardless of sex would exhibit a similar phenotype to each other than to individuals from another family, even if from the same treatment, and even if the behavioural measure usually exhibited sex differences. Therefore models were additionally tested against itself with and without the random effect included using a likelihood ratio test (LRT). Differences between models are reported below when they occurred with the level of variance and the standard deviation (SD) from the LMM or GLMM model output (all measures are summarised in Appendix VIII, Table A9.1). Family variation was also visually compared across treatments to investigate if atrazine altered the amount of variation present at the family level (Appendix VIII).

# Appendix VI: Additional measures of personality

## Results

### Anxiety

Overall, regardless of treatment $F_0$ males spent an average of 352.12 ± 18.78 sec (range 76.83−580.92 sec) and $F_0$ females spent an average 382.87 ± 22.45 sec (range 23.04−600 sec; the entirety of the assay) in the bottom zone during the novel arena test.

During the novel object assay the mean time spent in the bottom zone was 363.3 ± 30.92 sec for $F_0$ males (range 0−600 sec) and 419.67 ± 27.44 sec (range 0−600 sec) for $F_0$ females. For $F_1$ males mean time was 355.45 ± 24.96 sec (range 0−600 sec) and by $F_1$ females was 437.47 ± 21.80 (range 0−600 sec). There was no difference between treatments or sexes in the amount of time spent within the bottom half for the $F_0$'s (Figure A6.1). In the $F_1$'s, fish from the 0.3ppb treatment spent more in the bottom zone than controls (Est. 180.19 [13.18, 347.20 CI]; Figure A6.1) though the difference was only marginal ($p = 0.06$). Fish from the 3ppb treatment also spent more time in the bottom zone, but the difference was marginally non-significant (Est. 163.90 [−2.92, 330.71 CI]). There was no difference observed in the higher 30ppb treatment. $F_1$ males overall, spent less time in the bottom zone of the novel object test compared to females (Est. −78.44 [−137.31, −19.57 CI]). The family level variance was 8301 sec with a SD of 91.11 sec and explained ($\chi^2 = 7.2$, df = 1, $p < 0.007$) a significant portion of variation present in the LMM model (Appendix VIII). Like-wise to the novel arena test, there appears to be a suggestive trend of more time spent in the bottom zone of the novel object assay with fish from treated fathers than from fish from untreated fathers (Figure A6.1).

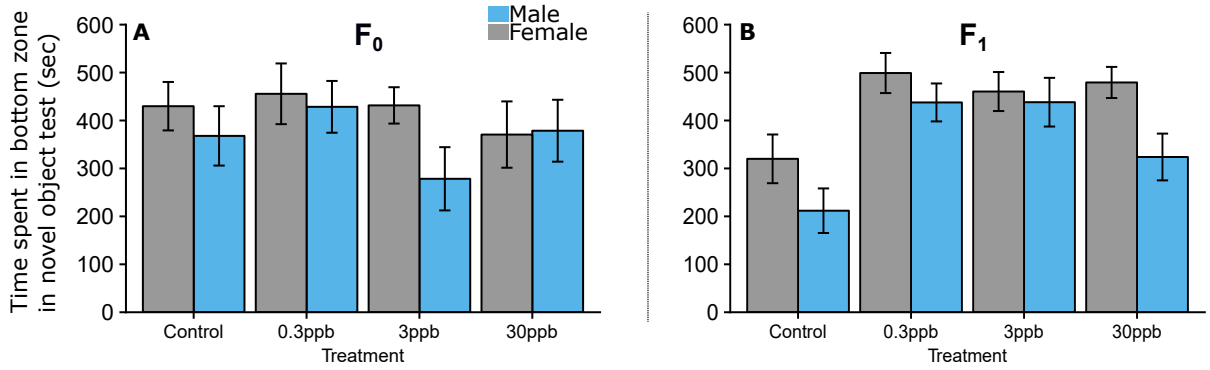


Figure A6.1: Additional measure of anxiety on atrazine exposed $F_0$ fish (left side) and $F_1$ offspring from exposed fathers (right side). (A) and (B) the time spent in the bottom zone during the novel object test. Bars represent means, with error bars representing standard errors of the mean. For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=17-30. Total sample size for $F_0$'s were n=93; $F_1$'s n=190, with n=3 families per treatment.

## Exploration

Average exploration regardless of treatment by $F_0$ males was 46.53 ± 2.52 (range 19.20−89.37) and was 53.71 ± 3.66 sec (range 23.83−158.57) by $F_0$ females. $F_1$ males averaged 48.75 ± 2.67 (range 15.38−109.69) and females averaged was 60.60 ± 3.04 (range 19.90−176.93).

## Boldness

Overall, mean latency for $F_0$ males was 111.94 ± 19.26 sec and for $F_0$ females was 170.82 ± 28.54 sec. In total, 8 fish (8.6%) of fish were in the top zone at beginning of recording and 6 fish 6.45% never entered. Excluding these individuals, latency amongst the $F_0$'s ranged from 3.50−502.60 sec for males and 2.52−518.92 sec for females. Mean latency for $F_1$ males was 84.10 ± 15.56 sec and for $F_1$ females was 170.74 ± 18.32 sec. In total, 47 fish (24.7%) of fish were in the top zone at beginning of recording and 12 fish (6.3%) never entered. Excluding these individuals, latency amongst the $F_1$'s ranged from 0.26−533.63 sec for males and 0.60−549.20 sec for females.

There was no differences in treatment in either the $F_0$ or $F_1$ generation in the frequency to enter the top zone once over-dispersion was accounted for (A6.2). $F_0$ males overall exhibited a higher frequency of entering the top zone than females (Est. 1.50 [1.08, 2.10 CI]) and so too did $F_1$ males (Est. 2.04 [1.51, 2.75 CI]). Mean frequency of $F_0$ males was 23.58 ± 2.64 (range 0−94), and $F_0$ females was 16.84 ± 1.872 (range 0−49). The mean frequency for $F_1$ males was 38.0 ± 2.74 (range 0−119) and for $F_1$ females was 20.90 ± 1.7 (range 0−82).

There was no significant difference between treatments across both generations in the time spent in the

top zone during the novel arena. However, the time spent by $F_0$ fish from the 0.3ppb treatment was non-significantly less (Est. −62.22 [−132.79, 8.36 CI] A6.2). There was no sex difference observed amongst the $F_0$'s. However, amongst the $F_1$'s, males spent significantly longer than females in the top zone (Est. 84.21 [43.09, 125.32 CI]). Family level variation was 11693 sec with a SD of 108.1 and explained a significant portion of the variance in this model ($\chi^2 = 35.53$, df = 1, p <0.001; Appendix III). Overall, $F_0$ male averaged 164.30 ± 17.025 sec (range 0−451.97 sec), and female averaged 131.71 ± 18.36 sec (range 0−512.86 sec) of time spent in the top zone. Male $F_1$'s averaged 255.87 ± 19.44 (range 0−599.10) and female $F_1$'s averaged 167.81 ± 16.06 (range 0−597.0).

In both generations there was no treatment effect observed in whether or not an individual approached the novel object (A6.2). In the $F_0$'s there was no sex effect observed, whereas in the $F_1$ generation, males approached the novel object significantly more so than females did (Est. 0.87 [1.17, 2.66 CI]). In total, 48 (52%) $F_0$ fish and 59 (32%) $F_1$ fish, regardless of treatment or sex approached the novel object over the course of the novel object assay.

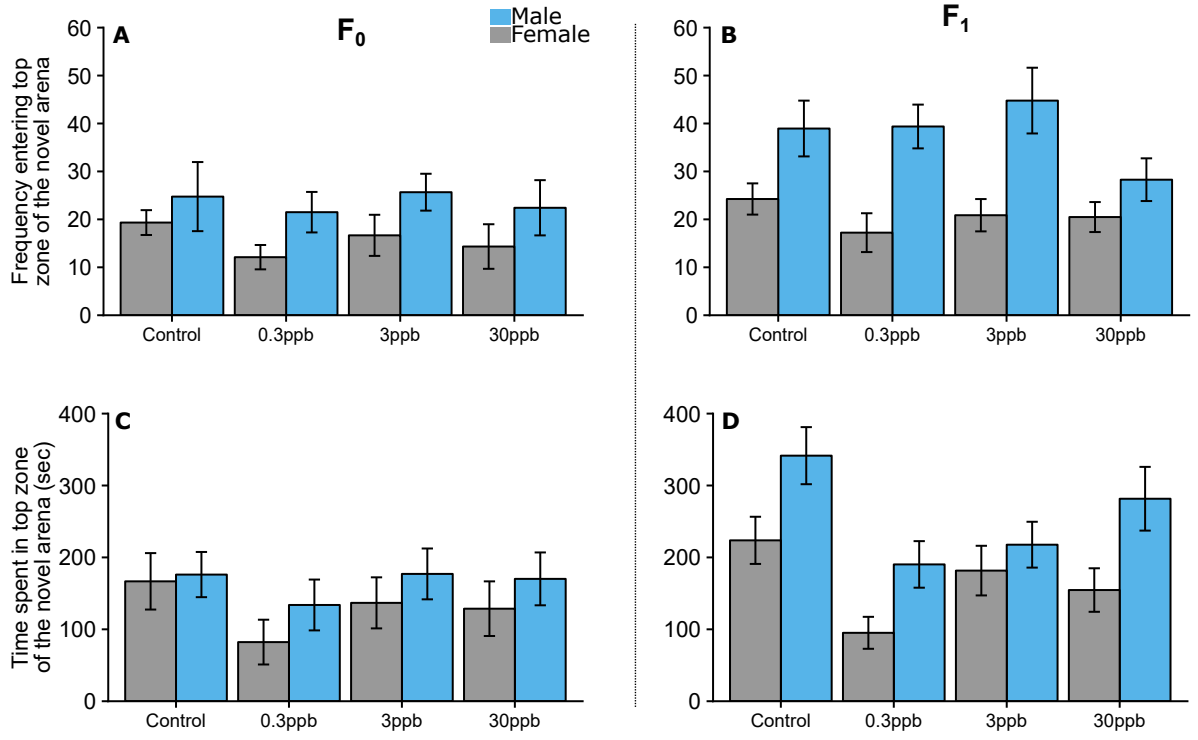


Figure A6.2: Additional measures of boldness on atrazine exposed $F_0$ fish (left side) and $F_1$ offspring from exposed fathers (right side). (A) and (B) the frequency entering the top zone of the novel arena; (C) and (D) the time spent in the top zone of the novel arena (sec). Bars represent means, with error bars representing standard errors of the mean. For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=17-30. Total sample size for $F_0$'s were n=93; $F_1$'s n=182-190, with n=3 families per treatment.

## Aggression

Mean time interacting with the mirror by $F_0$ males was 225.99 ± 189.6 (range 0−577.96) and was 189.60 ± 21.19 sec (range 0−473.0) by $F_0$ females.

There was no difference amongst treatments in the frequency a fish entered the mirror zone amongst the $F_0$'s once over-dispersion was taken into account (Figure A6.3). Overall, $F_0$ males exhibited a higher frequency of entering the mirror zone than females (Est. 1.33 [1.03, 1.73]). Male $F_0$ average frequency was 49.46 ± 3.59 (range 0−99) and female $F_0$ average frequency was 37.04 ± 4.26 (range 0−112). In the following generation, the frequency entering the mirror zone by $F_1$ females tended to decrease as atrazine concentration increased, whereas males tended to increase (Figure A6.3). However, an interactive difference between sex and treatment was only observe in the 3ppb treatment (Est. 3.75 [1.45, 9.68 CI]) compared to controls, with no other treatment effects observed. $F_1$ males were slightly more likely to enter the mirror zone at a higher frequency than females but not significantly so (Est. −1.76, [−3.42, 1.11]). $F_1$ mean male frequency was 36.77 ± 2.7 (range 0−87) and mean $F_1$ female frequency was 36.93 ± 2.44 (range 0−100). Family level variation was 0.03 with a SD of 1.16 and significantly explained a portion of the GLMM ($\chi^2$ = 127.84, df = 1, p <0.001; Appendix VIII).

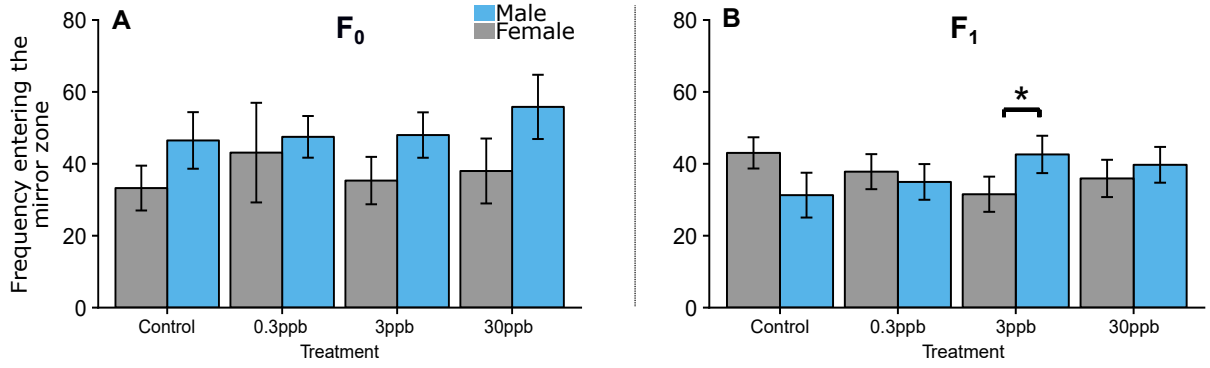


Figure A6.3: Additional measure of aggression on atrazine exposed $F_0$ fish (left side) and $F_1$ offspring from exposed fathers (right side). (A) and (B) the frequency entering the mirror zone. Bars represent means, with error bars representing standard errors of the mean. For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=17-30. Total sample size for $F_0$'s were n=93; $F_1$'s n=190, with n=3 families per treatment.

## Activity

There was a significant sex difference observed in both generation with males spending less time immobile ($F_0$ males Est. $-0.34$ [$-0.57$, $-0.11$ CI]; $F_1$ males Est. $-0.55$ [$-0.79$, $-0.31$ CI]). However no treatment differences were observed in either generation in the total distance moved nor in the time spent immobile (A6.4), though $F_1$ fish from the 30ppb treatment were marginally less immobile than controls ($p = 0.083$). In addition, despite the measures total distance moved and mean velocity being highly correlated in the novel arena assay (correlation coefficient = 0.87, $R^2 = 0.76$; Appendix VI), $F_1$ fish from the 30ppb treatment had a significantly lower mean velocity than controls (Est. $-0.83$ [$-1.62$, $-0.03$]). During the novel arena, the mean total distance for $F_0$ males was 2974.48 ± 131.36 cm (range 1131.24−5596.57 cm) and for $F_0$ females was 2445.70 ± 102.20 cm (range 902.92−4643.76 cm). Mean total distance for $F_1$ males was 4044.20 ± 170.34 cm (range 887.60−8536.37 cm) and for $F_1$ females was 2780.29 ± 96.88 cm (range 11.66−5518.67 cm). The mean time spent immobile for $F_0$ males was 98.11 ± 9.4 sec (range 15.16−340.59 sec) and for females was 134.58 ± 13.30 sec (range 50.08−452.85 sec). $F_1$ males was 90.31 ± 11.30 sec (range 10.40−480.93 sec) and for $F_1$ females was 141.58 ± 11.72 sec (4.03−600 sec).

Mean total distance moved in the novel object assay for $F_0$ males was 1806.16 ± 138.92 cm (range 145.78−4867.44 cm) and for $F_0$ females was 1173.47 ± 102.66 (range 85.09−2836.30 cm). Mean total distance for $F_1$ males was 2659.45 ± 166.99 cm (range 0−8341.81 cm) and for $F_1$ females was 1380.96 ± 82.01 cm (range 0−3820.19 cm).

Mean total distance for $F_1$ males was 2569.01 ± 114.80 cm (range 0−5580.16 cm) and for $F_1$ females was 1880.54 ± 98.76 cm (range 0−3646.06 cm).

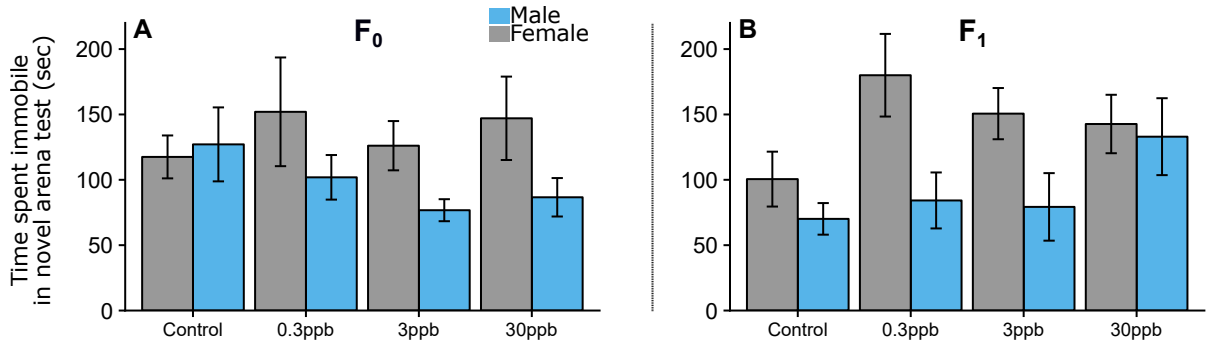


Figure A6.4: Addiontal measures of activity on atrazine exposed $F_0$ fish (left side) and $F_1$ offspring from exposed fathers (right side). (A) and (B) the time spent immobile during the novel arena test (sec); (C) and (D) the total distance moved during the novel object test. Bars represent means, with error bars representing standard errors of the mean. For each sex and treatment, sample sizes for $F_0$'s ranged from n=9-12; $F_1$'s ranged from n=17-30. Total sample size for $F_0$'s were n=93; $F_1$'s n=180-190, with n=3 families per treatment.

## Differences between assays in activity

After the conclusion of the novel arena and the start of the novel object assay, the addition of the novel object into the arena caused a strong drop in the total distance moved ($F_0$ Est. −1218.6 [−1383.43, −1053.77 CI]; $F_1$ Est. −1408.50 [−1584.37, −1232.64 CI]). On average $F_0$ males decreased by 39.3% and females decreased by 52.3%. $F_1$ males similarly decreased by 34.2% and $F_1$ females decreased by 48.2%. Activity levels increased across most of the treatments during the mirror test (there some instances of a decrease e.g. in $F_0$ 0.3ppb females, and in $F_1$ 0.3ppb and 30ppb males) but were still significantly lower than during the novel arena assay ($F_0$, Est. −994.23 [−1159.06, −829.40 CI]; $F_1$ Est. −1145.07 [−1315.96, −974.19 CI]). On average this increase in the mirror assay was relatively, much smaller than the initial decrease between the novel arena and novel object. On average $F_0$ males increased by 14.4% and females increased by 15.09%. $F_1$ males however decreased on average by 2.65% whereas females increased by 36.1%. The sexes differed overall in activity ($F_0$ male Est. 621.40 [357.44, 885.37 CI]; $F_1$ male Est. 1072.51 [806.94, 1338.07 CI]) as reported earlier (see section; Activity), but both sexes exhibited a similar reaction to the novel object. Lastly, there were no treatment differences observed in activity when compared across all three assays, though treatment effects were found previously when assays were analysed independently of each other (e.g. in the $F_1$ mirror test). Amongst the $F_0$ fish, there was high variation across individuals, with fish identification producing a variance of 310151 cm with a SD of 556.9, that significantly helps explain a portion of the variation ($\chi^2 = 56.5$, df = 1, p <0.001; Appendix VIII) in the model. The variance was similarly high in the $F_1$'s with fish id nested within family id producing a variance of 626963 cm with a SD of 791.8. The inclusion of the nested random factor helped significantly explain a portion of the model's variance ($\chi^2 = 99.2$, df = 1, p <0.001; Appendix VIII).

# Appendix VII: The correlation between the total distance moved and mean velocity

Across all assays, the total distance moved was highly correlated with mean velocity. I provide the correlation coefficient between the total distance moved and mean velocity for each assay and for each generation. I additionally provide the $R^2$ from the linear models between these two variables as an another congruent measure, and also to account for pseudoreplication at the individual level and at the family level for the $F_1$ generation. For the $F_1$ generation, the $R^2$ was calculated using the *'MuMin'* package (reference).

Table A7.1: Correlation coefficients (cor. coef), $R^2$ and *P-values* of the relationship between the total distance moved and the mean velocity during the novel arena, novel object and mirror test assay across both $F_0$ and $F_1$ generations.

| **Novel Arena** | **$F_0$** | | | **$F_1$** | |
|---|---|---|---|---|---|
| **Cor. coef** | ***$R^2$*** | ***P-value*** | **Cor. coef** | ***$R^2$*** | ***P-value*** |
| 0.99 | 0.98 | <0.001 | 0.87 | 0.76 | <0.001 |

| **Novel object** | **$F_0$** | | | **$F_1$** | |
|---|---|---|---|---|---|
| **Cor. coef** | ***$R^2$*** | ***P-value*** | **Cor. coef** | ***R2*** | ***P-value*** |
| 0.99 | 0.98 | <0.001 | 0.87 | 0.76 | <0.001 |

| **Mirror test** | **$F_0$** | | | **$F_1$** | |
|---|---|---|---|---|---|
| **Cor. coef** | ***$R^2$*** | ***P-value*** | **Cor. coef** | ***$R^2$*** | ***P-value*** |
| 0.82 | 0.66 | <0.001 | 0.64 | 0.41 | <0.001 |

# Appendix VIII: Model parameters of behavioural analyses

Table A8.1: Model coefficients of $F_0$ behavioural measures.

| Parameter | Estimate | Variance | Lwr95%CI | Upr95% | *Z/t* | *P* |
|---|---|---|---|---|---|---|
| **Anxiety measures** | | | | | | |
| Time in bottom zone NA, $F_0$,n=93 | | | | | | |
| Intercept | 349.13 | 31.97 | 286.46 | 411.79 | 10.92 | <0.001 |
| 0.3ppb | 83.03 | 41.76 | 1.18 | 164.87 | 1.99 | **0.0499** |
| 3ppb | 27.18 | 40.29 | −51.80 | 106.15 | 0.67 | 0.5018 |
| 30ppb | 37.09 | 40.29 | −41.88 | 116.06 | 0.92 | 0.3598 |
| Sex(male) | −33.83 | 29.01 | −90.70 | 23.03 | −1.17 | 0.2467 |
| Time in bottom zone NO, $F_0$,n=93 | | | | | | |
| Intercept | 428.73 | 46.00 | 338.58 | 518.88 | 9.32 | <0.001 |
| 0.3ppb | 45.56 | 60.08 | −72.19 | 163.30 | 0.76 | 0.4500 |
| 3ppb | −43.92 | 57.97 | −157.53 | 69.69 | −0.76 | 0.4510 |
| 30ppb | −24.21 | 57.97 | −137.82 | 89.41 | −0.42 | 0.6770 |
| Sex(male) | −59.72 | 41.74 | −141.53 | 22.09 | −1.43 | 0.1560 |
| **Boldness measures** | | | | | | |
| Latency to enter top zone $F_0$,n=93 | | | | | | |
| Intercept | 81.45 | 1.38 | 46.53 | 162.39 | 13.96 | <0.001 |
| 0.3ppb | 2.16 | 1.62 | −1.19 | 5.75 | 1.60 | 0.1135 |

*Continued on next page*

Table A8.1 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| 3ppb | 2.36 | 1.57 | −1.03 | 5.75 | 1.93 | 0.0574 |
| 30ppb | 2.86 | 1.57 | 1.17 | 6.96 | 2.36 | **0.0205** |
| Sex(male) | 1.99 | 1.57 | −1.22 | 4.85 | 1.55 | 0.1253 |
| 0.3ppb:Sex(male) | −3.54 | 1.93 | −13.20 | 1.02 | −1.93 | 0.0572 |
| 3ppb:Sex(male) | −3.88 | 1.88 | −13.46 | −1.12 | −2.15 | **0.0345** |
| 30ppb:Sex(male) | −5.39 | 1.88 | −18.73 | −1.55 | −2.67 | **0.0091** |
| Frequency entering top zone $F_0$,n=93 | | | | | | |
| Intercept | 17.62 | 1.20 | 12.18 | 24.78 | 15.73 | <0.001 |
| 0.3ppb | −1.30 | 1.27 | −2.10 | 1.22 | −1.09 | 0.2771 |
| 3ppb | −1.04 | 1.24 | −1.60 | 1.48 | −0.19 | 0.8526 |
| 30ppb | −1.20 | 1.25 | −1.88 | 1.30 | −0.81 | 0.4223 |
| Sex(male) | 1.50 | 1.18 | 1.08 | 2.10 | 2.43 | **0.0173** |
| Time in top zone $F_0$,n=93 | | | | | | |
| Intercept | 153.89 | 27.57 | 99.85 | 207.92 | 5.58 | <0.001 |
| 0.3ppb | −62.22 | 36.01 | −132.79 | 8.36 | −1.73 | 0.0875 |
| 3ppb | −14.51 | 34.74 | −82.60 | 53.59 | −0.42 | 0.6773 |
| 30ppb | −22.01 | 34.74 | −90.10 | 46.09 | −0.63 | 0.5281 |
| Sex(male) | 35.09 | 25.02 | −13.94 | 84.13 | 1.40 | 0.1642 |
| Approach NO $F_0$,n=93 | | | | | | |
| Intercept | 0.45 | 0.61 | −0.25 | 0.67 | −0.43 | 0.6700 |
| 0.3ppb | 0.52 | 0.65 | −0.25 | 0.78 | 0.11 | 0.9100 |
| 3ppb | 0.63 | 0.64 | −0.35 | 0.85 | 0.87 | 0.3820 |
| 30ppb | 0.42 | 0.64 | −0.18 | 0.69 | −0.58 | 0.5610 |
| Sex(male) | 0.60 | 0.60 | −0.39 | 0.77 | 0.93 | 0.3520 |
| Time in middle zone $F_0$,n=93 | | | | | | |
| Intercept | 96.50 | 10.01 | 76.89 | 116.11 | 9.65 | <0.001 |
| 0.3ppb | −21.00 | 13.07 | −46.61 | 4.61 | −1.61 | 0.1120 |
| 3ppb | −12.81 | 12.61 | −37.53 | 11.90 | −1.02 | 0.3120 |
| 30ppb | −14.89 | 12.61 | −39.60 | 9.82 | −1.18 | 0.2410 |

*Continued on next page*

Table A8.1 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Sex(male) | −0.96 | 9.08 | −18.75 | 16.84 | −0.11 | 0.9160 |
| **Exploration measures** | | | | | | |
| Exploration $F_0$,n=93 | | | | | | |
| Intercept | 47.06 | 4.76 | 37.74 | 56.39 | 9.89 | <0.001 |
| 0.3ppb | 15.12 | 6.21 | 2.94 | 27.30 | 2.43 | **0.0170** |
| 3ppb | 4.11 | 6.00 | −7.65 | 15.86 | 0.69 | 0.4953 |
| 30ppb | 9.48 | 6.00 | −2.27 | 21.23 | 1.58 | 0.1174 |
| Sex(male) | −7.71 | 4.32 | −16.17 | 0.76 | −1.78 | 0.0778 |
| **Activity measures** | | | | | | |
| Total distance moved in NA $F_0$,n=93 | | | | | | |
| Intercept | 2355.00 | 187.69 | 1987.14 | 2722.85 | 12.55 | <0.001 |
| 0.3ppb | 36.19 | 245.13 | −444.27 | 516.64 | 0.15 | 0.8830 |
| 3ppb | 192.33 | 236.53 | −271.26 | 655.92 | 0.81 | 0.4183 |
| 30ppb | 120.66 | 236.53 | −342.93 | 584.25 | 0.51 | 0.6112 |
| Sex(male) | 532.19 | 170.32 | 198.37 | 866.02 | 3.13 | **0.0024** |
| Mean velocity in NA $F_0$,n=93 | | | | | | |
| Intercept | 4.01 | 0.30 | 3.42 | 4.61 | 13.22 | <0.001 |
| 0.3ppb | −0.02 | 0.40 | −0.80 | 0.76 | −0.05 | 0.9581 |
| 3ppb | 0.30 | 0.38 | −0.45 | 1.05 | 0.78 | 0.4359 |
| 30ppb | 0.11 | 0.38 | −0.64 | 0.86 | 0.30 | 0.7667 |
| Sex(male) | 0.89 | 0.28 | 0.35 | 1.43 | 3.23 | **0.0018** |
| Time spent not moving in NA $F_0$,n=93 | | | | | | |
| Intercept | 4.80 | 0.13 | 4.55 | 5.05 | 37.49 | <0.001 |
| 0.3ppb | 0.02 | 0.17 | −0.30 | 0.35 | 0.14 | 0.8874 |
| 3ppb | −0.12 | 0.16 | −0.43 | 0.20 | −0.71 | 0.4775 |
| 30ppb | −0.06 | 0.16 | −0.38 | 0.25 | −0.39 | 0.6985 |
| Sex(male) | −0.34 | 0.12 | −0.57 | −0.11 | −2.92 | **0.0045** |
| Total distance moved in NO $F_0$,n=93 | | | | | | |
| Intercept | 1126.68 | 195.18 | 744.14 | 1509.22 | 5.77 | <0.001 |

*Continued on next page*

Table A8.1 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.3ppb | 66.36 | 254.92 | −433.27 | 566.00 | 0.26 | 0.7952 |
| 3ppb | 159.16 | 245.97 | −322.94 | 641.26 | 0.65 | 0.5193 |
| 30ppb | −33.49 | 245.97 | −515.58 | 448.61 | −0.14 | 0.8920 |
| Sex(male) | 631.47 | 177.12 | 284.32 | 978.62 | 3.57 | **0.0006** |
| Mean velocity in NO (F0),n=93 | | | | | | |
| Intercept | 2.02 | 0.32 | 1.40 | 2.64 | 6.41 | <0.001 |
| 0.3ppb | 0.03 | 0.41 | −0.78 | 0.83 | 0.06 | 0.9509 |
| 3ppb | 0.25 | 0.40 | −0.53 | 1.03 | 0.62 | 0.5349 |
| 30ppb | −0.10 | 0.40 | −0.88 | 0.68 | −0.24 | 0.8091 |
| Sex(male) | 1.02 | 0.29 | 0.46 | 1.58 | 3.55 | **0.0006** |
| Total Distance Moved in MIS (F0),n=93 | | | | | | |
| Intercept | 1344.41 | 168.68 | 1013.80 | 1675.01 | 7.97 | <0.001 |
| 0.3ppb | −41.13 | 220.31 | −472.93 | 390.67 | −0.19 | 0.8520 |
| 3ppb | 141.26 | 212.58 | −275.39 | 557.90 | 0.66 | 0.5080 |
| 30ppb | −33.92 | 212.58 | −450.57 | 382.72 | −0.16 | 0.8740 |
| Sex(male) | 700.55 | 153.07 | 400.53 | 1000.57 | 4.58 | **<0.001** |
| Mean velocity in MIS (F0),n=93 | | | | | | |
| Intercept | 2.45 | 0.25 | 1.96 | 2.93 | 9.81 | <0.001 |
| 0.3ppb | 0.04 | 0.33 | −0.59 | 0.68 | 0.13 | 0.8936 |
| 3ppb | 0.63 | 0.31 | 0.02 | 1.25 | 2.01 | **0.0477** |
| 30ppb | 0.43 | 0.31 | −0.18 | 1.05 | 1.37 | 0.1730 |
| Sex(male) | 0.97 | 0.23 | 0.53 | 1.41 | 4.29 | **<0.001** |
| **Aggression measures** | | | | | | |
| Time spent interacting with mirror (F0),n=93 | | | | | | |
| Intercept | 216.92 | 34.03 | 150.22 | 283.62 | 6.37 | <0.001 |
| 0.3ppb | 2.24 | 44.45 | −84.88 | 89.36 | 0.05 | 0.9600 |
| 3ppb | −46.76 | 42.89 | −130.82 | 37.30 | −1.09 | 0.2790 |
| 30ppb | −57.37 | 42.89 | −141.43 | 26.70 | −1.34 | 0.1850 |
| Sex(male) | 34.54 | 30.88 | −26.00 | 95.07 | 1.12 | 0.2660 |

*Continued on next page*

Table A8.1 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Fq interacting with mirror (F0),n=93 | | | | | | |
| Intercept | 34.20 | 1.17 | 25.03 | 45.60 | 22.85 | <0.001 |
| 0.3ppb | 1.12 | 1.21 | −1.30 | 1.63 | 0.60 | 0.5490 |
| 3ppb | 1.04 | 1.21 | −1.39 | 1.51 | 0.23 | 0.8160 |
| 30ppb | 1.18 | 1.20 | −1.22 | 1.68 | 0.89 | 0.3770 |
| Sex(male) | 1.33 | 1.14 | 1.03 | 1.73 | 2.15 | **0.0340** |
| **Comparison between assays** | | | | | | |
| Total Distance Moved between assays | | | | | | |
| Intercept | 2346.31 | 156.15 | 2040.26 | 2652.35 | 15.03 | <0.001 |
| Assay(Nobject) | −1218.60 | 84.10 | −1383.43 | −1053.77 | −14.49 | **<0.001** |
| Assay(Mirror) | −994.23 | 84.10 | −1159.06 | −829.40 | −11.82 | **<0.001** |
| Sex(Male) | 621.40 | 134.68 | 357.44 | 885.37 | 4.61 | **<0.001** |
| 0.3ppb | 20.47 | 193.84 | −359.44 | 400.38 | 0.11 | 0.916 |
| 3ppb | 164.25 | 187.03 | −202.33 | 530.83 | 0.88 | 0.382 |
| 30ppb | 17.75 | 187.03 | −348.83 | 384.33 | 0.10 | 0.925 |
| Random effects | | | | | | |
| Fish ID | | 310151 | | | | |
| Mean velocity between assays | | | | | | |
| Intercept | 3.94 | 0.25 | 3.45 | 4.42 | 15.93 | <0.001 |
| Assay(Nobject) | −1.98 | 0.13 | −2.24 | −1.72 | −14.89 | **<0.001** |
| Assay(Mirror) | −1.34 | 0.13 | −1.61 | −1.08 | −10.10 | **<0.001** |
| Sex(Male) | 0.96 | 0.21 | 0.54 | 1.38 | 4.50 | **<0.001** |
| 0.3ppb | 0.02 | 0.31 | −0.59 | 0.62 | 0.05 | 0.958 |
| 3ppb | 0.39 | 0.30 | −0.19 | 0.97 | 1.33 | 0.188 |
| 30ppb | 0.15 | 0.30 | −0.43 | 0.73 | 0.51 | 0.614 |
| Random effects | | | | | | |
| Fish ID | | 0.78 | | | | |

Table A8.2: Model coefficients of $F_1$ behavioural measures.

| Parameter | Estimate | Variance | Lwr95%CI | Upr95% | *Z/t* | *P* |
|---|---|---|---|---|---|---|
| **Anxiety measures** | | | | | | |
| Time in bottom zone NA $F_1$,n=190 | | | | | | |
| Intercept | 274.1 | 65.59 | 145.54 | 402.65 | 4.18 | 0.003 |
| 0.3ppb | 134.84 | 92.05 | −45.57 | 315.25 | 1.47 | 0.181 |
| 3ppb | 65.14 | 91.92 | −115.01 | 245.30 | 0.71 | 0.499 |
| 30ppb | 69.96 | 91.98 | −110.33 | 250.24 | 0.76 | 0.469 |
| Sex(male) | −86.78 | 21.19 | −128.30 | −45.25 | −4.10 | **<0.001** |
| Random effects | | | | | | |
| Family | | 11425 | | | | |
| Time in bottom zone NO $F_1$,n=182 | | | | | | |
| Intercept | 319.97 | 61.36 | 199.71 | 440.23 | 5.22 | <0.001 |
| 0.3ppb | 180.19 | 85.21 | 13.18 | 347.20 | 2.12 | 0.068 |
| 3ppb | 163.90 | 85.11 | −2.92 | 330.71 | 1.93 | 0.092 |
| 30ppb | 129.86 | 85.04 | −36.82 | 296.54 | 1.53 | 0.167 |
| Sex(male) | −78.44 | 30.04 | −137.31 | −19.57 | −2.61 | **0.010** |
| Random effects | | | | | | |
| Family | | 8297 | | | | |
| **Boldness measures** | | | | | | |
| Latency to enter top zone $F_1$,n=190 | | | | | | |
| Intercept | 40.80 | 1.00 | 40.45 | 41.26 | 769.60 | <0.001 |
| 0.3ppb | 6.16 | 1.76 | 2.01 | 18.73 | 3.20 | **0.001** |
| 3ppb | 4.07 | 1.76 | 1.34 | 12.30 | 2.50 | **0.013** |
| 30ppb | 3.71 | 1.76 | 1.22 | 11.25 | 2.30 | **0.021** |
| Sex(male) | −2.30 | 1.00 | −2.32 | −2.27 | −172.80 | **<0.001** |
| Random effects | | | | | | |
| Family | | 1.85 | | | | |
| Frequency entering top zone $F_1$,n=190 | | | | | | |

*Continued on next page*

Table A8.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Intercept | 15.85 | 1.19 | 11.25 | 22.42 | 15.62 | <0.001 |
| 0.3ppb | −1.35 | 1.27 | −2.14 | 1.17 | −1.27 | 0.203 |
| 3ppb | −1.08 | 1.26 | −1.70 | 1.46 | −0.31 | 0.755 |
| 30ppb | −1.25 | 1.26 | −1.97 | 1.26 | −0.96 | 0.337 |
| Sex(male) | 2.04 | 1.16 | 1.51 | 2.75 | 4.66 | **<0.001** |
| Random effects | | | | | | |
| Family | | 0.02 | | | | |
| Fish ID | | 0.98 | | | | |
| Time in top zone $F_1$,n=190 | | | | | | |
| Intercept | 231.82 | 66.20 | 102.07 | 361.56 | 3.50 | 0.008 |
| 0.3ppb | −126.89 | 92.92 | −309.02 | 55.24 | −1.37 | 0.209 |
| 3ppb | −64.65 | 92.80 | −246.53 | 117.23 | −0.70 | 0.506 |
| 30ppb | −55.04 | 92.86 | −237.05 | 126.97 | −0.59 | 0.570 |
| Sex(male) | 84.21 | 20.98 | 43.09 | 125.32 | 4.01 | **<0.001** |
| Random effects | | | | | | |
| Family | | 11693 | | | | |
| Approach NO $F_1$,n=182 | | | | | | |
| Intercept | 0.13 | 0.62 | −0.05 | −0.28 | −3.87 | <0.001 |
| 0.3ppb | 0.63 | 0.65 | −0.34 | 0.85 | 0.87 | 0.386 |
| 3ppb | 0.59 | 0.65 | −0.30 | 0.83 | 0.58 | 0.565 |
| 30ppb | −0.48 | 0.65 | −0.22 | 0.76 | −0.11 | 0.913 |
| Sex(male) | 0.87 | 0.59 | 0.76 | 0.93 | 5.03 | **<0.001** |
| Random effects | | | | | | |
| Family | | 0.1831 | | | | |
| Time in middle zone $F_1$,n=190 | | | | | | |
| Intercept | 322.58 | 55.40 | 214.01 | 431.15 | 5.82 | <0.001 |
| 0.3ppb | 104.52 | 77.75 | −47.87 | 256.90 | 1.34 | 0.216 |
| 3ppb | 54.21 | 77.64 | −97.96 | 206.38 | 0.70 | 0.505 |
| 30ppb | 43.40 | 77.69 | −108.87 | 195.68 | 0.56 | 0.592 |
| Sex(male) | −69.97 | 17.75 | −104.75 | −35.19 | −3.94 | **<0.001** |

*Continued on next page*

Table A8.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Random effects | | | | | | |
| Family | | 8166 | | | | |
| **Measures of exploration** | | | | | | |
| Exploration $F_1$,n=190 | | | | | | |
| Intercept | 59.55 | 4.50 | 50.73 | 68.38 | 13.23 | <0. 001 |
| 0.3ppb | 5.80 | 5.93 | −5.82 | 17.42 | 0.98 | 0.329 |
| 3ppb | −1.27 | 5.86 | −12.75 | 10.21 | −0.22 | 0.828 |
| 30ppb | 0.87 | 5.89 | −10.66 | 12.41 | 0.15 | 0.882 |
| Sex(male) | −12.43 | 4.26 | −20.77 | −4.09 | −2.92 | **0.004** |
| Random effects | | | | | | |
| Family | | 0.0 | | | | |
| **Measures of activity** | | | | | | |
| Total Distance Moved in NA $F_1$,n=190 | | | | | | |
| Intercept | 3005.79 | 213.30 | 2587.73 | 3423.85 | 14.09 | <0.001 |
| 0.3ppb | −268.97 | 284.21 | −826.02 | 288.08 | −0.95 | 0.373 |
| 3ppb | −143.93 | 281.27 | −695.22 | 407.35 | −0.51 | 0.624 |
| 30ppb | −481.99 | 282.56 | −1035.79 | 71.82 | −1.71 | 0.129 |
| Sex(male) | 1255.64 | 185.46 | 892.14 | 1619.14 | 6.77 | **<0.001** |
| Random effects | | | | | | |
| Family | | 21379 | | | | |
| Mean velocity in NA $F_1$,n=190 | | | | | | |
| Intercept | 5.46 | 0.31 | 4.85 | 6.06 | 17.64 | <0.001 |
| 0.3ppb | −0.04 | 0.41 | −0.84 | 0.76 | −0.10 | 0.918 |
| 3ppb | −0.15 | 0.40 | −0.94 | 0.63 | −0.38 | 0.704 |
| 30ppb | −0.83 | 0.40 | −1.62 | −0.03 | −2.05 | 0.042 |
| Sex(male) | 1.62 | 0.29 | 1.05 | 2.19 | 5.54 | **<0.001** |
| Random effects | | | | | | |
| Family | | 0.0 | | | | |
| Time spent not moving in NA $F_1$,n=190 | | | | | | |

*Continued on next page*

Table A8.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Intercept | 4.36 | 0.17 | 4.04 | 4.69 | 25.96 | <0.001 |
| 0.3ppb | 0.356 | 0.23 | −0.09 | 0.80 | 1.56 | 0.158 |
| 3ppb | 0.29 | 0.23 | −0.15 | 0.74 | 1.29 | 0.236 |
| 30ppb | 0.45 | 0.23 | 0.01 | 0.90 | 1.99 | 0.083 |
| Sex(male) | −0.55 | 0.12 | −0.79 | −0.31 | −4.48 | **<0.001** |
| Random effects | | | | | | |
| Family | | 0.04 | | | | |
| Total Distance Moved in NO $F_1$,n=182 | | | | | | |
| Intercept | 1204.45 | 195.45 | 821.37 | 1587.53 | 6.16 | <0.001 |
| 0.3ppb | 367.21 | 259.14 | −140.71 | 875.12 | 1.42 | 0.194 |
| 3ppb | 177.76 | 258.55 | −328.99 | 684.52 | 0.69 | 0.511 |
| 30ppb | 195.99 | 257.38 | −308.46 | 700.45 | 0.76 | 0.469 |
| Sex(male) | 1257.18 | 173.18 | 917.76 | 1596.60 | 7.26 | **<0.001** |
| Random effects | | | | | | |
| Family | | 13634 | | | | |
| Mean velocity in NO $F_1$,n=182 | | | | | | |
| Intercept | 2.21 | 0.36 | 1.50 | 2.91 | 6.12 | <0.001 |
| 0.3ppb | 0.70 | 0.48 | −0.24 | 1.64 | 1.46 | 0.183 |
| 3ppb | 0.73 | 0.48 | −0.21 | 1.67 | 1.52 | 0.167 |
| 30ppb | 0.48 | 0.48 | −0.45 | 1.42 | 1.01 | 0.341 |
| Sex(male) | 1.86 | 0.31 | 1.25 | 2.47 | 5.96 | **<0.001** |
| Random effects | | | | | | |
| Family | | 0.064 | | | | |
| Total Distance Moved in MIS $F_1$,n=190 | | | | | | |
| Intercept | 2205.50 | 159.50 | 1892.89 | 2518.20 | 13.83 | <0.001 |
| 0.3ppb | −448.20 | 210.10 | −859.95 | −36.53 | −2.13 | **0.034** |
| 3ppb | −368.70 | 207.50 | −775.41 | 37.91 | −1.78 | 0.077 |
| 30ppb | −500.10 | 208.60 | −908.81 | −91.30 | −2.40 | **0.018** |
| Sex(male) | 689.30 | 150.80 | 393.78 | 984.89 | 4.571 | **<0.001** |
| Random effects | | | | | | |

*Continued on next page*

Table A8.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Family | | ∼0 | | | | |
| Mean velocity in MIS $F_1$,n=190 | | | | | | |
| Intercept | 4.20 | 0.29 | 3.63 | 4.76 | 14.51 | <0.001 |
| 0.3ppb | −0.14 | 0.39 | −0.90 | 0.62 | −0.36 | 0.728 |
| 3ppb | −0.20 | 0.39 | −0.95 | 0.56 | −0.51 | 0.624 |
| 30ppb | −0.64 | 0.39 | −1.39 | 0.12 | −1.64 | 0.142 |
| Sex(male) | 0.79 | 0.23 | 0.33 | 1.24 | 3.39 | **<0.001** |
| Random effects | | | | | | |
| Family | | 0.07 | | | | |
| **Measures of aggression** | | | | | | |
| Time interacting with mirror $F_1$,n=190 | | | | | | |
| Intercept | 256.68 | 25.08 | 207.53 | 305.83 | 10.24 | <0.001 |
| 0.3ppb | −110.63 | 33.02 | −175.35 | −45.91 | −3.35 | **<0.001** |
| 3ppb | −56.32 | 32.62 | −120.25 | 7.61 | −1.73 | 0.086 |
| 30ppb | −95.98 | 32.79 | −160.24 | −31.72 | −2.93 | **0.004** |
| Sex(male) | 74.42 | 23.71 | 27.96 | 120.89 | 3.14 | **0.002** |
| Random effects | | | | | | |
| Family | | 0.00 | | | | |
| Fq interacting with mirror $F_1$,n=190 | | | | | | |
| Intercept | 31.62 | 1.29 | 19.11 | 51.94 | 13.51 | <0.001 |
| 0.3ppb | −1.05 | 1.42 | −2.12 | 1.90 | −0.15 | 0.882 |
| 3ppb | −1.90 | 1.43 | −3.86 | 1.06 | −1.78 | 0.075 |
| 30ppb | 1.05 | 1.40 | −1.92 | 2.12 | 0.15 | 0.884 |
| Sex(male) | −1.76 | 1.40 | −3.42 | 1.11 | −1.66 | 0.097 |
| 0.3ppb:Sex(Male) | −1.29 | 1.63 | −2.94 | 1.79 | −0.59 | 0.555 |
| 3ppb:Sex(Male) | 3.75 | 1.63 | 1.45 | 9.68 | 2.72 | **0.007** |
| 30ppb:Sex(Male) | 1.71 | 1.64 | −1.54 | 4.48 | 1.09 | 0.278 |
| Random effects | | | | | | |
| Family | | 0.052 | | | | |
| Fish ID | | 1.29 | | | | |

*Continued on next page*

Table A8.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| **Comparison between assays** | | | | | | |
| Total Distance Moved between assays | | | | | | |
| Intercept | 2997.98 | 153.94 | 2696.26 | 3299.71 | 19.48 | <0.001 |
| Assay(Nobject) | −1408.50 | 89.73 | −1584.37 | −1232.64 | −15.70 | **<0.001** |
| Assay(Mirror) | −1145.07 | 87.19 | −1315.96 | −974.19 | −13.13 | **<0.001** |
| Sex(Male) | 1072.51 | 135.49 | 806.94 | 1338.07 | 7.92 | **<0.001** |
| 0.3ppb | −123.83 | 185.84 | −488.08 | 240.42 | −0.67 | 0.506 |
| 3ppb | −122.20 | 190.01 | −494.61 | 250.22 | −0.64 | 0.521 |
| 30ppb | −278.11 | 190.75 | −651.97 | 95.75 | −1.46 | 0.147 |
| Random effects | | | | | | |
| Fish ID: Family ID | | 626963 | | | | |
| Family ID | | 0 | | | | |
| Mean velocity between assays | | | | | | |
| Intercept | 5.32 | 0.23 | 4.86 | 5.78 | 22.47 | <0.001 |
| Assay(Nobject) | −2.43 | 0.17 | −2.77 | −2.10 | −14.31 | **<0.001** |
| Assay(Mirror) | −1.60 | 0.17 | −1.92 | −1.27 | −9.39 | **<0.001** |
| Sex(Male) | 1.42 | 0.20 | 1.02 | 1.81 | 7.06 | **<0.001** |
| 0.3ppb | 0.14 | 0.28 | −0.40 | 0.68 | 0.51 | 0.608 |
| 3ppb | 0.09 | 0.28 | −0.45 | 0.64 | 0.34 | 0.738 |
| 30ppb | −0.36 | 0.28 | −0.91 | 0.19 | −1.28 | 0.202 |
| Random effects | | | | | | |
| Fish ID: Family ID | | 0.99 | | | | |
| Family ID | | ∼0 | | | | |

# Appendix IX: Behavioural family variation comparisons across treatments

Across a variety of behavioural measures (and across behavioural assays), family variation appeared to significantly influence the $F_1$ model outputs indicating a genetic component contributed to behavioural phenotypes (Table A9.1). However, it is unclear whether family variation occurred across all treatment groups, or if the atrazine treatment influenced the amount of variation present.

## Statistical analysis

To determine if family variance of a particular behavioural measure influenced model outputs, an analysis of variance (ANOVA) was run between a model with the random effect against the same model without the random effect, and a *p-value* was calculated. The amount of family variation is taken from the model outputs with the random factor. In addition, the repeatability at the family level was calculated to provide another measure of genetic influence. Repeatability of each behavioural measure (identified from Table A9.1) was analysed using the '*rptR*' package (Stoffel et al. 2017), with permutations and bootstrap set to 1000. The time spent in the bottom zone of the novel object test, time spent in the bottom zone, top zone and the latency to enter the top zone of the novel arena was analysed under the 'guassian datatype'.

To determine the level of within group variation, a test of variance was run. The variance test compares the proportion of two groups and in return gives a variance estimate ratio. The variance estimate ratio ($F$) is calculated by dividing the variance of one group by another. A proportion where $F$ <1 indicates the numerator group has less variation and inversely the denominator has more. Whereas a $F$ >1 indicates the

opposite; the numerator group has more variation and the denominator group less. As the variance tests are limited to tests along the $F$ distribution, only normally distributed behavioural measures were tested. I compared the proportion of variation across $F_1$ controls to each of the three treatment groups, and in turn all treatment groups to each other

## Results and discussion

There were several behavioural measures that were significantly explained by family level variation. From the novel arena these include; latency to enter the top zone, frequency entering the top zone, time spent in the bottom zone, time spent in the middle zone and the log+1(time spent in the top zone). From the novel object assay, only the time spent in the bottom half was influenced. And from the mirror test, the frequency entering the mirror zone (summarised in Table A9.1). There was also high variation in the total distance moved in both the novel arena and the novel object assay (but not in the mirror test), but this did not significantly influence model outcomes.

The significant repeatability estimates (Table A9.2) of the behavioural measures identified in Table A9.1, further indicate a genetic component for these behaviours. In particular, the repeatability estimates of the two behavioural measures; time spent in the bottom zone of the novel arena and novel object test are relatively higher compared to the other two measures (latency to enter the top zone and time spent in the top zone of the novel arena test), suggesting a stronger genetic component for these behaviours.
Of the behavioural measures that were normally distributed, there were several instances found in the total distance moved and mean velocity (in all three assays), the standard deviation of exploration and time spent in the middle zone of the novel arena, where the variance ratios were significantly different from one another (see Table A9.3, Table A9.4 and Table A9.5 for raw data).

Table A9.6, Table A9.7 and Table A9.8, shows ranks of which treatment contributed the least, to the most variation for all normally distributed behavioural measures. These tables also summarises the raw data from Table A9.3, Table A9.4 and Table A9.5 to showcase the minimum and maximum ranges of ratios by controls vs treatments (CvT) or treatments vs treatments (TvT).

Overall, the minimum and maximum ranges of the variance ratios show the majority of CvT variation was lower than TvT variation, suggesting at a broad scale, controls exhibited slightly lower variation for some behavioural measures, however in many cases maximum CvT variation was >1, indicating that lower family variation in the controls is not a consistent pattern. Controls exhibited the lowest variation in the total distance moved (in novel object assay and mirror test) and in the mean velocity (in all three assays), which

is also reinforced by their range of $F$ ratios <1. Controls exhibited the most variation in the time spent in the bottom half of the novel object assay and time spent interacting with the mirror in the mirror test.

As the time spent in the bottom half of the novel object assay is the only measure that was influenced by family variation, it is possible that less variation in the treatments could be attributed to the atrazine treatments. However, due to the lack of a pattern amongst all other behavioural measures, the lack of a pattern amongst the other treatment levels for this particular behaviour (i.e. the treatment ranks are not ordered from lowest to highest concentration, or vice versa) and the lack of a treatment effect observed (see Chapter 2), suggest that the variation is likely, more attributable to genetic differences. Lastly, I might not have been able to capture differences in the variation produced by families, if they occurred due to the low number of families (three families per treatment) used in this study. More studies with greater numbers of families would be more likely to be able to discern difference if they occur.

In summary, based on this data and for all the behavioural measures, there doesn't appear to be a discernible pattern that would suggest an atrazine treatment effect could influence family level variation, and that difference in at the family level are more likely a result of genetic differences.

Table A9.1: Summary of behavioural models where family level variation influenced model outputs across the novel arena, novel object and mirror test assays. Given are the all models, family variation (variance) and the standard deviation (SD) produced by the inclusion of the random factor (family id), whether or not the model was influenced by family level variation (Model infl.), and the $\chi^2$ test statistic, the degrees of freedom (d.f.) and its p-value.

| **Novel arena** | | | | | | |
|---|---|---|---|---|---|---|
| **Models** | **Variance** | **SD** | **Model infl.** | $\chi^2$ | **d.f.** | ***P-value*** |
| Total distance moved ~ Treatment + Sex | 21379 | 146.2 | No | 0.0 | 1 | 1 |
| Mean velocity ~ Treatment + Sex | 0.0 | 0.0 | No | 0.0 | 1 | 1 |
| Exploration ~ Treatment + Sex | 0.0 | 0.0 | No | 0.0 | 1 | 1 |
| Time spent in bottom zone ~ Treatment + Sex | 11425 | 106.9 | Yes | 33.6 | 1 | <0.001 |
| Latency to enter the top zone ~ Treatment + Sex | 1.85 | 1.36 | Yes | 157.4 | 1 | <0.001 |
| Frequency entering the top zone ~ Treatment × Sex | 0.98 | 0.99 | No | 0.27 | 1 | 0.60 |
| Time spent in the top zone ~ Treatment + Sex | 11693 | 108.1 | Yes | 35.53 | 1 | <0.001 |
| Time in the middle zone ~ Treatment + Sex | 0.0 | 0.0 | No | 0 | 1 | 1 |
| Time spent not moving ~ Treatment + Sex | 0.036 | 0.19 | No | 0.19 | 1 | 0.66 |

| **Novel object** | | | | | | |
|---|---|---|---|---|---|---|
| **Models** | **Variance** | **SD** | **Model infl.** | $\chi^2$ | **d.f.** | ***P-value*** |
| Total distance moved ~ Treatment + Sex | 13634 | 116.8 | No | 0.0 | 1 | 1 |
| Mean velocity ~ Treatment + Sex | 0.066 | 0.25 | No | 0.0 | 1 | 1 |
| Approached the novel object ~ Treatment + Sex | 0.18 | 0.43 | No | 0.9 | 1 | 0.34 |
| Time spent in the bottom half ~ Treatment + Sex | 8301 | 91.11 | Yes | 7.2 | 1 | 0.007 |

| **Mirror test** | | | | | | |
|---|---|---|---|---|---|---|
| **Models** | **Variance** | **SD** | **Model infl.** | $\chi^2$ | **d.f.** | ***P-value*** |
| Total distance moved ~ Treatment + Sex | ~0 | ~0 | No | 0 | 1 | 1 |
| Mean velocity ~ Treatment + Sex | 0.07 | 0.27 | No | 0 | 1 | 1 |
| Time spent interacting with the Mirror ~ Treatment + Sex | ~0 | ~0 | No | 0 | 1 | 1 |
| Frequency entering mirror zone ~ Treatment × Sex | 1.29 | 1.14 | No | 2.46 | 1 | 0.12 |

Table A9.2: Family level repeatability estimates ($R$) for behavioural traits identified as having a genetic influence in Table A9.1; i.e. time spent in the bottom zone, latency to enter the top zone and time spent in the top zone of the novel arena, as well as the time spent in the bottom zone of the novel object test. Lower and upper confidence intervals (CI), standard error (SE) and $P$ values are reported

| **Behaviour** | ***R*** | **Lower 95% CI** | **Upper 95% CI** | **SE** | ***P-value*** |
|---|---|---|---|---|---|
| Time spent in the bottom zone of the novel arena | 0.33 | 0.11 | 0.52 | 0.11 | 7.68E-12 |
| Latency to enter the top zone of the novel arena | 0.12 | 0.00 | 0.26 | 0.07 | 1.74E-03 |
| Time spent in the top zone of the novel arena | 0.34 | 0.12 | 0.54 | 0.11 | 5.59E-12 |
| Time spent in the bottom zone of the novel object test | 0.22 | 0.05 | 0.40 | 0.09 | 3.58E-07 |

Table A9.3: Relative family variation during the novel arena test.

**Total distance moved**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.67 | 0.37 | 1.20 | 0.17 | 0.96 | 0.54 | 1.71 | 0.89 | 1.21 | 0.68 | 2.16 | 0.52 |
| 0.3ppb | | | | | 1.44 | 0.80 | 2.58 | 0.22 | 1.81 | 1.0 | 3.26 | 0.05 |
| 3ppb | | | | | | | | | 1.26 | 0.70 | 2.25 | 0.43 |
| 30ppb | | | | | | | | | | | | |

**Mean velocity**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.59 | 0.33 | 1.06 | 0.08 | 0.88 | 0.50 | 1.57 | 0.67 | 0.9 | 0.51 | 1.61 | 0.73 |
| 0.3ppb | | | | | 1.49 | 0.83 | 2.67 | 0.18 | 1.52 | 0.85 | 2.75 | 0.16 |
| 3ppb | | | | | | | | | 1.03 | 0.57 | 1.83 | 0.93 |
| 30ppb | | | | | | | | | | | | |

**Exploration**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.83 | 0.46 | 1.48 | 0.53 | 1.80 | 1.01 | 3.20 | 0.05 | 2.08 | 1.16 | 3.70 | 0.001 |
| 0.3ppb | | | | | 2.17 | 1.21 | 3.91 | 0.01 | 2.50 | 1.39 | 4.51 | 0.00 |
| 3ppb | | | | | | | | | 1.15 | 0.64 | 2.06 | 0.63 |
| 30ppb | | | | | | | | | | | | |

*Continued on next page*

Table A9.3 – *Continued from previous page*

**Time spent in the bottom zone**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 1.16 | 0.65 | 2.07 | 0.62 | 0.96 | 0.54 | 1.71 | 0.90 | 1.04 | 0.58 | 1.85 | 0.90 |
| 0.3ppb | | | | | 0.83 | 0.46 | 1.50 | 0.53 | 0.90 | 0.50 | 1.62 | 0.71 |
| 3ppb | | | | | | | | | 1.08 | 0.60 | 1.93 | 0.80 |
| 30ppb | | | | | | | | | | | | |

**Time spent in the middle zone**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 1.37 | 0.76 | 2.44 | 0.29 | 1.42 | 0.80 | 2.52 | 0.23 | 2.15 | 1.20 | 3.83 | 0.01 |
| 0.3ppb | | | | | 1.04 | 0.58 | 1.86 | 0.90 | 1.57 | 0.87 | 2.83 | 0.13 |
| 3ppb | | | | | | | | | 1.51 | 0.84 | 2.71 | 0.16 |
| 30ppb | | | | | | | | | | | | |

**log+1(Time spent in the top zone)**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.80 | 0.44 | 1.42 | 0.44 | 1.05 | 0.59 | 1.87 | 0.86 | 1.08 | 0.60 | 1.92 | 0.79 |
| 0.3ppb | | | | | 1.33 | 0.74 | 2.38 | 0.34 | 1.36 | 0.75 | 2.45 | 0.30 |
| 3ppb | | | | | | | | | 1.02 | 0.57 | 1.83 | 0.94 |
| 30ppb | | | | | | | | | | | | |

*Continued on next page*

Table A9.3 – *Continued from previous page*

**log+1(Time spent not moving)**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.83 | 0.46 | 1.49 | 0.54 | 0.96 | 0.54 | 1.71 | 0.90 | 1.06 | 0.59 | 1.89 | 0.84 |
| 0.3ppb | | | | | 1.16 | 0.64 | 2.08 | 0.62 | 1.27 | 0.71 | 2.29 | 0.42 |
| 3ppb | | | | | | | | | 1.10 | 0.61 | 1.97 | 0.75 |
| 30ppb | | | | | | | | | | | | |

Table A9.4: Relative family variation during the novel object test.

**Total distance moved**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.41 | 0.23 | 0.74 | 0.00 | 0.60 | 0.33 | 1.09 | 0.09 | 0.70 | 0.39 | 1.27 | 0.24 |
| 0.3ppb | | | | | 1.47 | 0.81 | 2.67 | 0.21 | 1.71 | 0.94 | 3.10 | 0.08 |
| 3ppb | | | | | | | | | 1.16 | 0.64 | 2.11 | 0.62 |
| 30ppb | | | | | | | | | | | | |

**Mean velocity**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.39 | 0.22 | 0.71 | 0.00 | 0.40 | 0.22 | 0.72 | 0.00 | 0.66 | 0.36 | 1.19 | 0.16 |
| 0.3ppb | | | | | 1.01 | 0.56 | 1.84 | 0.97 | 1.68 | 0.93 | 3.04 | 0.09 |
| 3ppb | | | | | | | | | 1.66 | 0.92 | 3.01 | 0.09 |
| 30ppb | | | | | | | | | | | | |

**Time spent in the bottom half**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 1.60 | 0.88 | 2.89 | 0.12 | 1.34 | 0.74 | 2.43 | 0.33 | 1.50 | 0.83 | 2.71 | 0.18 |
| 0.3ppb | | | | | 0.84 | 0.46 | 1.53 | 0.56 | 0.94 | 0.52 | 1.70 | 0.84 |
| 3ppb | | | | | | | | | 1.12 | 0.62 | 2.03 | 0.71 |
| 30ppb | | | | | | | | | | | | |

Table A9.5: Relative family variation during the mirror test.

**Total distance moved**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.58 | 0.32 | 1.04 | 0.07 | 0.66 | 0.37 | 1.17 | 0.15 | 0.82 | 0.46 | 1.46 | 0.50 |
| 0.3ppb | | | | | 1.14 | 0.63 | 2.05 | 0.66 | 1.4 | 0.79 | 2.55 | 0.25 |
| 3ppb | | | | | | | | | 1.24 | 0.69 | 2.22 | 0.46 |
| 30ppb | | | | | | | | | | | | |

**Mean velocity**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 0.33 | 0.18 | 0.58 | 0.00 | 0.80 | 0.45 | 1.43 | 0.45 | 0.64 | 0.36 | 1.14 | 0.13 |
| 0.3ppb | | | | | 2.46 | 1.37 | 4.43 | 0.003 | 1.96 | 1.09 | 3.53 | 0.025 |
| 3ppb | | | | | | | | | 0.79 | 0.44 | 1.42 | 0.43 |
| 30ppb | | | | | | | | | | | | |

**Time spent interacting with the mirror**

| | 0.3ppb | | | | 3ppb | | | | 30ppb | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** | ***F*** | **L. CI** | **U. CI** | **P-value** |
| control | 1.37 | 0.77 | 2.45 | 0.28 | 1.08 | 0.61 | 1.92 | 0.80 | 1.75 | 0.98 | 3.12 | 0.059 |
| 0.3ppb | | | | | 0.79 | 0.44 | 1.41 | 0.42 | 1.27 | 0.71 | 2.30 | 0.42 |
| 3ppb | | | | | | | | | 1.62 | 0.91 | 2.90 | 0.10 |
| 30ppb | | | | | | | | | | | | |

Table A9.6: Ranked family variation produced across treatments for measures during the novel arena test. Included is the relative minimum and maximum variation when controls are compared to the three treatment levels (0.3ppb, 3ppb and 30ppb) and when the treatment levels are compared with each other.

| **Total distance moved** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.67 | 1.21 | 30ppb | Control | 3ppb | 0.3ppb |
| **Treatment vs treatment variation** | 1.26 | 1.81 | | | | |
| **Mean velocity** | **Range** | | **Ranked treatment variation** | | | |
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.59 | 0.90 | Control | 3ppb | 30ppb | 0.3ppb |
| **Treatment vs treatment variation** | 1.03 | 1.52 | | | | |
| **Standard deviation of exploration** | **Range** | | **Ranked treatment variation** | | | |
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.83 | 2.08 | 30ppb | 3ppb | Control | 0.3ppb |
| **Treatment vs treatment variation** | 1.15 | 2.50 | | | | |
| **Time spent in the bottom zone** | **Range** | | **Ranked treatment variation** | | | |
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.96 | 1.16 | 0.3ppb | 30ppb | Control | 3ppb |
| **Treatment vs treatment variation** | 0.83 | 1.08 | | | | |
| **log+1(Time spent in the top zone)** | **Range** | | **Ranked treatment variation** | | | |
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.80 | 1.08 | 30ppb | 3ppb | Control | 0.3ppb |
| **Treatment vs treatment variation** | 1.02 | 1.36 | | | | |
| **Time spent in the middle zone** | **Range** | | **Ranked treatment variation** | | | |
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 1.37 | 2.15 | 30ppb | 3ppb | Control | 0.3ppb |
| **Treatment vs treatment variation** | 1.04 | 1.57 | | | | |
| **log+1(Time spent not moving)** | **Range** | | **Ranked treatment variation** | | | |
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.83 | 1.06 | 30ppb | Control | 3ppb | 0.3ppb |
| **Treatment vs treatment variation** | 1.10 | 1.27 | | | | |

Table A9.7: Ranked family variation produced across treatments for measures during the novel object test. Included is the relative minimum and maximum variation when controls are compared to the three treatment levels (0.3ppb, 3ppb and 30ppb) and when the treatment levels are compared with each other.

| **Total distance moved** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.41 | 0.70 | Control | 30ppb | 3ppb | 0.3ppb |
| **Treatment vs treatment variation** | 1.16 | 1.71 | | | | |

| **Mean velocity** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.39 | 0.66 | Control | 30ppb | 3ppb | 0.3ppb |
| **Treatment vs treatment variation** | 1.01 | 1.68 | | | | |

| **Time spent in the bottom half** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 1.34 | 1.60 | 0.3ppb | 30ppb | 3ppb | Control |
| **Treatment vs treatment variation** | 0.84 | 1.22 | | | | |

Table A9.8: Ranked family variation produced across treatments for measures during the mirror test. Included is the relative minimum and maximum variation when controls are compared to the three treatment levels (0.3ppb, 3ppb and 30ppb) and when the treatment levels are compared with each other.

| **Total distance moved** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.58 | 0.82 | Control | 30ppb | 3ppb | 0.3ppb |
| **Treatment vs treatment variation** | 1.14 | 1.41 | | | | |

| **Mean velocity** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 0.33 | 0.80 | Control | 3ppb | 30ppb | 0.3ppb |
| **Treatment vs treatment variation** | 0.79 | 2.46 | | | | |

| **Time spent interacting with the mirror** | **Range** | | **Ranked treatment variation** | | | |
|---|---|---|---|---|---|---|
| | **Min** | **Max** | **Least** | **2nd least** | **2nd most** | **Most** |
| **Control vs treatment variation** | 1.08 | 1.75 | 30ppb | 0.3ppb | 3ppb | Control |
| **Treatment vs treatment variation** | 0.79 | 1.62 | | | | |

# Appendix X: Phenotype selection for dissections

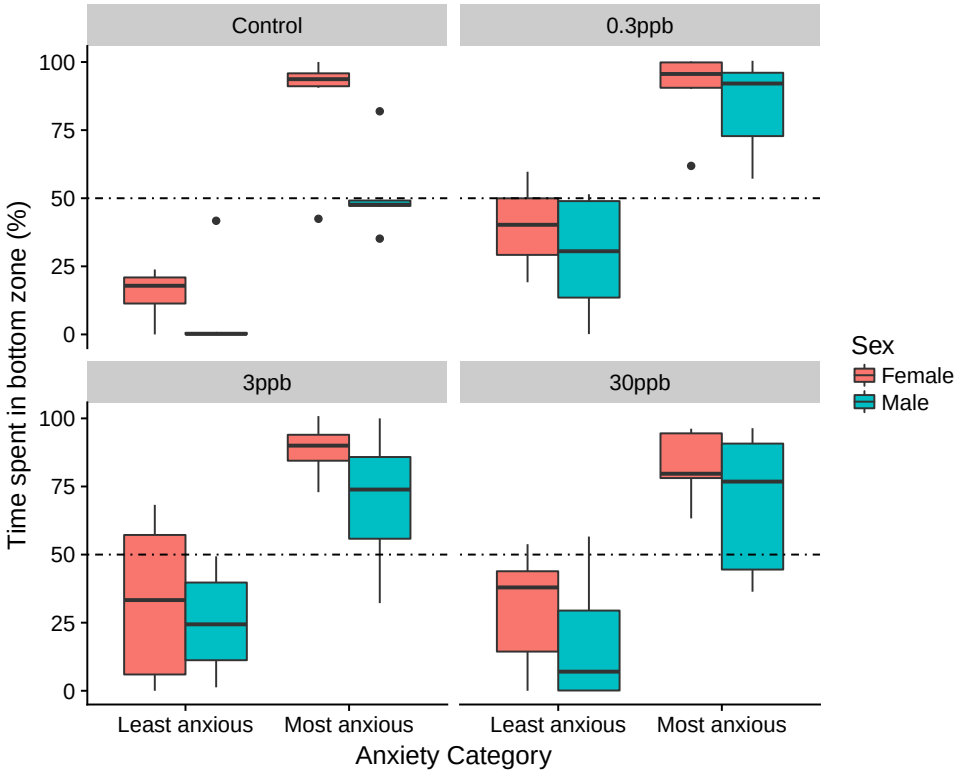


Figure A10.1: Anxiety phenotype (time spent in the bottom zone) of the least and most anxious fish across treatments and sex, for further selection for qPCR analysis. Sample sizes were: Controls n=10-12; 0.3ppb n=12; 3ppb n=10-14; 30ppb n=11 per sex.

# Appendix XI: Correlation of anxiety and aggression phenotypes

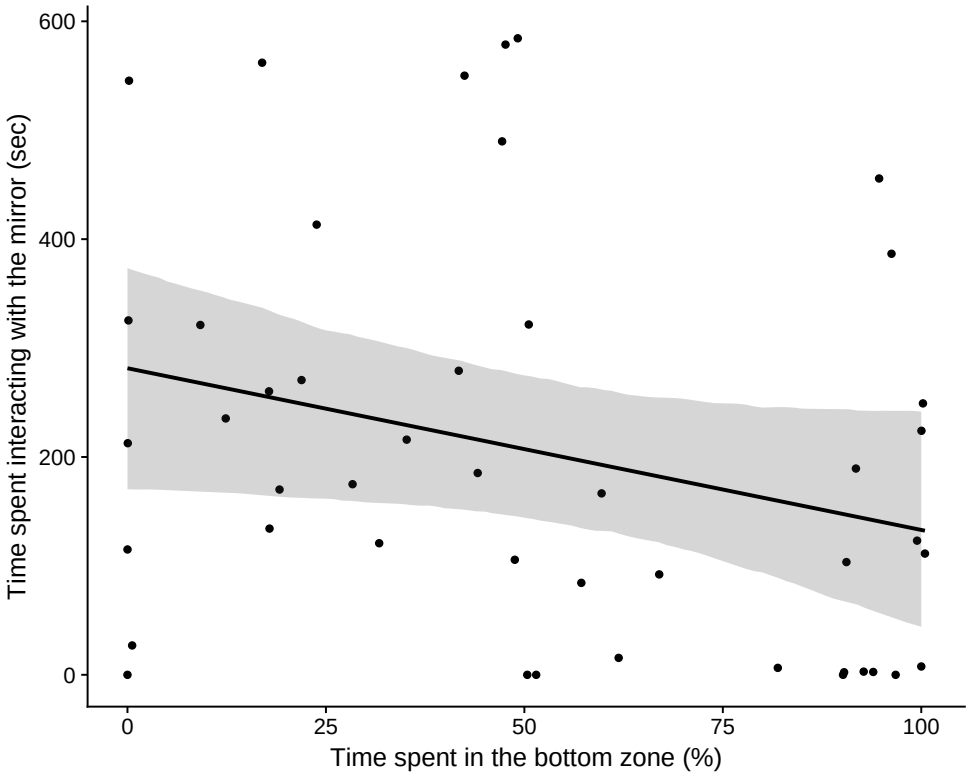


Figure A11.1: Correlation of anxiety and aggression phenotypes for qPCR, (Time spent in the bottom zone Est. -1.29 ± 0.74; −2.75, 0.17 CI; *T-value*= −1.74; $P = 0.090$).

# Appendix XII: Efficiency of qPCR runs

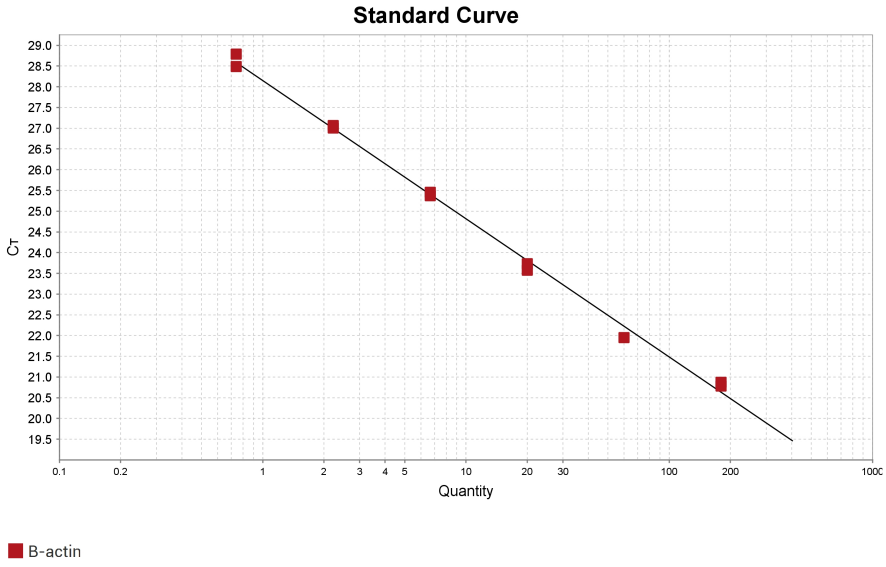


Figure A11.2: Efficiency of *b-actin*.

# Appendix XIII: Validation of *b-actin* as a reference gene

## Statistical analysis

To validated mRNA of *b-actin* as a suitable reference gene, I ran several linear mixed effects models in R (version 3.5.0; Team 2018), using the package '*lme4*' (Bates et al. 2015). The behavioural measures (anxiety phenotype [least and most anxious]; aggression phenotype [least and most aggressive]; time spent in the bottom zone and time spent interacting with the mirror (both continuous behavioural measurements were further centred), treatment (control and 0.3ppb) group and sex (male and female) were used as predictors. *b-actin* was log transformed to improve normality and was used as the response variable. Family id was used as a random effect to account for variation in genetics. Confidence intervals were calculated using the 'confint' function, p-values were calculated using the '*lmerTest*' package (Kuznetsova et al. 2017). The full model output is listed in the Table A13.1 and Table A13.2 below.

Table A13.1: Model outputs of *b-actin* mRNA transcripts for the least and most anxious/aggressive phenotypes. For all models, results are presented with estimates, lower and upper 95% confidence intervals (Lwr95%CI and Upr95%CI), the $t$ value and the $p$ value.

| Parameter | Estimate | Variance | Lwr95%CI | Upr95% | *T* | *P* |
|---|---|---|---|---|---|---|
| **Anxious phenotypes to *b-actin* mRNA** | | | | | | |
| Intercept | 4.31 | 0.37 | 3.59 | 5.04 | 11.59 | <0.001 |
| Anxious(most) | 0.14 | 0.24 | −0.32 | 0.60 | 0.60 | 0.554 |
| Treatment(0.3ppb) | 0.02 | 0.47 | −0.90 | 0.95 | 0.05 | 0.963 |
| Sex(male) | −0.16 | 0.24 | −0.62 | 0.31 | −0.67 | 0.510 |
| Random effects | | | | | | |
| Family | | 0.25 | | | | |
| **Aggressive phenotypes to *b-actin* mRNA** | | | | | | |
| Intercept | 4.52 | 0.37 | 3.79 | 5.25 | 12.17 | <0.001 |
| Aggressive(most) | −0.27 | 0.23 | −0.73 | 0.18 | −1.174 | 0.248 |
| Treatment(0.3ppb) | 0.02 | 0.47 | −0.90 | 0.95 | 0.05 | 0.963 |
| Sex(male) | −0.16 | 0.23 | −0.62 | 0.30 | −0.67 | 0.506 |

Table A13.2: Model outputs of *b-actin* mRNA transcripts for anxiety and aggression levels. For all models, results are presented with estimates, lower and upper 95% confidence intervals (Lwr95%CI and Upr95%CI), the $t$ value and the $p$ value.

| Parameter | Estimate | Variance | Lwr95%CI | Upr95% | *T* | *P* |
|---|---|---|---|---|---|---|
| **Time spent in the bottom zone** | | | | | | |
| Intercept | 4.40 | 0.35 | 3.72 | 5.08 | 12.68 | <0.001 |
| Centred bottom time | 0.00 | 0.00 | −0.00 | 0.01 | 1.102 | 0.277 |
| Treatment(0.3ppb) | −0.06 | 0.47 | −0.98 | 0.87 | −0.12 | 0.911 |
| Sex(male) | −0.10 | 0.24 | −0.58 | 0.37 | −0.43 | 0.668 |
| **Time spent interacting with mirror** | | | | | | |
| Intercept | 4.42 | 0.36 | 3.72 | 5.12 | 12.340 | <0.001 |
| Centred mirror time | −0.00 | 0.00 | −0.00 | 0.00 | −0.74 | 0.463 |
| Treatment(0.3ppb) | −0.061 | 0.49 | −1.02 | 0.90 | −0.13 | 0.906 |
| Sex(male) | −0.13 | 0.24 | −0.60 | 0.34 | −0.54 | 0.595 |

# Appendix XIV: Model selection using $\mathbf{AIC_C}$

Table A14.1: Ranked models by small-sample-size-corrected Akaike Information Criterion ($AIC_C$) for the inclusion or exclusion of interaction terms in models describing changes in mRNA of the genes: *slc6a4b*, *htr1B* or time in the bottom zone (Tbz) by treatment:sex interactions, treatment:behaviour interactions or mRNA:treatment interactions. The top five ranked models per each $AIC_C$ analysis are shown. Model number indicates the order the model was generated. A + indicates the presence of the main predictor or the interaction term: sex, treatment (Trt), sex:treatment (Sex:Trt), behavioural phenotype (Beh), behaviour phenotype:treatment (Beh:Trt) and *htr1Aa* mRNA:treatment (H1A:Trt). Presence of estimate indicates the inclusion of the continuous main predictor *htr1Aa* mRNA (H1A). Also included are the intercepts (int), degree of freedom (df), log-Likelihood (logLik), $AIC_C$, change in $AIC_C$ value ($\Delta$) and the $AIC_C$ probability for each model (weight). Models in bold indicate models with $\Delta AIC_C \leq 6$.

| **Model number** | **Int** | **Sex** | **Trt** | **Sex:Trt** | **df** | **logLik** | **$AIC_C$** | **$\Delta$** | **weight** |
|---|---|---|---|---|---|---|---|---|---|
| ***slc6a4b* mRNA∼Treatment∗Sex** | | | | | | | | | |
| **1** | **1.062** | | | | **3** | **−20.175** | **46.9** | **0** | **0.705** |
| **3** | **1.143** | | | + | **4** | **−20.445** | **49.9** | **2.94** | **0.162** |
| **2** | **1.117** | + | | | **4** | **−20.908** | **50.8** | **3.87** | **0.102** |
| 4 | 1.192 | + | + | | 5 | −21.244 | 54 | 7.07 | 0.021 |
| 8 | 1.272 | + | + | + | 6 | −20.614 | 55.4 | 8.46 | 0.01 |
| **Model number** | **Int** | **Beh** | **Trt** | **Beh:Trt** | **df** | **logLik** | **$AIC_C$** | **$\Delta$** | **weight** |
| ***htr1B* mRNA∼Anxiety*Trt** | | | | | | | | | |
| **1** | **0.9693** | | | | **3** | **22.221** | **−37.90** | **0.0** | **0.848** |
| **3** | **1.008** | | + | | **4** | **21.199** | **−33.4** | **4.45** | **0.092** |
| **2** | **0.993** | + | | | **4** | **20.674** | **−32.4** | **5.5** | **0.054** |
| 4 | 1.032 | + | + | | 5 | 19.651 | −27.8 | 10.07 | 0.006 |
| 8 | 1.063 | + | + | + | 6 | 19.274 | −24.4 | 13.48 | 0.001 |
| ***slc6a4b* mRNA∼Aggression*Trt** | | | | | | | | | |
| **1** | **1.062** | | | | **3** | **−20.175** | **46.9** | **0** | **0.624** |
| **2** | **1.146** | + | | | **4** | **−20.251** | **49.5** | **2.56** | **0.174** |
| **3** | **1.143** | | + | | **4** | **−20.445** | **49.9** | **2.94** | **0.143** |
| **4** | **1.227** | + | + | | **5** | **−20.501** | **52.5** | **5.58** | **0.038** |
| 8 | 1.315 | + | + | + | 6 | −19.799 | 53.8 | 6.83 | 0.021 |
| **Model number** | **Int** | **H1A** | **Trt** | **H1A:Trt** | **df** | **logLik** | **$AIC_C$** | **$\Delta$** | **weight** |
| **Tbz∼*htr1Aa* mRNA*Trt** | | | | | | | | | |
| **8** | **40.14** | **17.28** | + | + | **6** | **−212.15** | **438.4** | **0** | **0.972** |
| 4 | 40.79 | 8.41 | + | | 5 | −217.12 | 445.7 | 7.3 | 0.025 |
| 3 | 41.41 | | + | | 4 | −220.82 | 450.6 | 12.17 | 0.002 |
| 2 | 51.58 | 7.864 | | | 4 | −222.18 | 453.3 | 14.89 | 0.001 |
| 1 | 51.63 | | | | 3 | −225.73 | 458 | 19.58 | 0 |

# Appendix XV: Model outputs for relative mRNA level data

Table A15.1: Model outputs of $F_1$ gene expression data.

| Parameter | Estimate | Variance | Lwr95%CI | Upr95% | *Z/t* | *P* |
|---|---|---|---|---|---|---|
| ***slc6a4a* expression** | | | | | | |
| Intercept | 0.59 | 0.11 | 0.37 | 0.82 | 5.81 | 5.188 |
| Treatment(0.3ppb) | −0.03 | 0.15 | −0.32 | 0.26 | −0.20 | 0.851 |
| Sex(male) | −0.04 | 0.10 | −0.23 | 0.15 | −0.42 | 0.681 |
| Random effects | | | | | | |
| Family | 0.11 | | | | | |
| ***slc6a4b* expression** | | | | | | |
| Intercept | 1.08 | 0.07 | 0.94 | 1.22 | 15.47 | <0.001 |
| Treatment(0.3ppb) | −0.15 | 0.11 | −0.37 | 0.06 | 1.42 | 0.157 |
| Sex(male) | −0.12 | 0.11 | −0.33 | 0.10 | 1.05 | 0.294 |
| Random effects | | | | | | |
| Family | 0.0 | | | | | |
| ***htr1Aa* expression** | | | | | | |
| Intercept | −0.33 | 0.26 | −0.84 | 0.18 | −1.27 | 0.230 |
| Treatment(0.3ppb) | −0.64 | 0.37 | −1.36 | 0.07 | −1.76 | 0.109 |
| Sex(male) | −0.71 | 0.35 | −1.39 | −0.02 | −2.03 | 0.049 |
| Trt:Sex | 1.05 | 0.48 | 0.11 | 2.00 | 2.19 | 0.035 |

*Continued on next page*

Table A15.1 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Random effects | | | | | | |
| Family | 0.04 | | | | | |
| ***htr1b* expression** | | | | | | |
| Intercept | 1.03 | 0.09 | 0.86 | 1.19 | 12.02 | <0.001 |
| Treatment(0.3ppb) | −0.08 | 0.12 | −0.31 | 0.16 | −0.64 | 0.558 |
| Sex(male) | −0.04 | 0.04 | −0.11 | 0.03 | −1.04 | 0.307 |
| Random effects | | | | | | |
| Family | 0.02 | | | | | |
| ***htr2b* expression** | | | | | | |
| Intercept | 0.66 | 0.10 | 0.46 | 0.86 | 6.43 | <0.001 |
| Treatment(0.3ppb) | 0.04 | 0.13 | −0.23 | 0.30 | 0.27 | 0.800 |
| Sex(male) | 0.00 | 0.08 | −0.16 | 0.16 | −0.04 | 0.970 |
| Random effects | | | | | | |
| Family | 0.0 | | | | | |

Table A15.2: Model outputs of the least/most anxious and aggressive phenotypes and relative mRNA levels of candidate genes *slc6a4a*; *slc6a4b*; *htr1Aa*; *htr1B* and *htr2B* of $F_1$ offspring from control and 0.3ppb atrazine treated males normalised to *b-actin* (from whole brain samples). For all models, results are presented with estimates, lower and upper 95% confidence intervals (Lwr95%CI and Upr95%CI), the $t$ value and the $p$ value.

| **Parameter** | **Estimate** | **Variance** | **Lwr95%CI** | **Upr95%** | ***Z/t*** | ***P*** |
|---|---|---|---|---|---|---|
| Anxiety phenotype | | | | | | |
| log *slc6a4a* | | | | | | |
| Intercept | −0.39 | 0.24 | −0.87 | 0.08 | −1.62 | 0.15 |
| Anxious(most) | −0.07 | 0.24 | −0.53 | 0.40 | −0.29 | 0.77 |
| Treatment(0.3ppb) | −0.08 | 0.29 | −0.66 | 0.50 | −0.27 | 0.80 |
| Random effects | | | | | | |
| Family | | 0.05 | | | | |
| *slc6a4b* | | | | | | |
| Intercept | 1.14 | 0.09 | 0.96 | 1.32 | 12.12 | <0.001 |
| Anxious(most) | 0.01 | 0.11 | −0.20 | 0.22 | 0.06 | 0.955 |
| Treatment(0.3ppb) | −0.15 | 0.11 | −0.36 | 0.06 | −1.44 | 0.157 |
| Random effects | | | | | | |
| Family | | 0 | | | | |
| log *htr1Aa* Intercept | −0.76 | 0.25 | −1.25 | −0.27 | −3.048 | 0.018 |
| Anxious(most) | 0.23 | 0.25 | −0.26 | 0.71 | 0.91 | 0.37 |
| Treatment(0.3ppb) | −0.15 | 0.30 | −0.74 | 0.44 | −0.50 | 0.645 |
| Random effects | | | | | | |
| Family | | 0.044 | | | | |
| *htr1B* (Intercept) | 0.97 | 0.06 | 0.85 | 1.09 | 15.84 | <0.001 |
| Anxious(most) | −0.05 | 0.04 | 0.04 | 0.03 | 1.26 | 0.207 |
| Treatment(0.3ppb) | −0.08 | 0.12 | −0.31 | 0.16 | 0.64 | 0.521 |

*Continued on next page*

Table A15.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Random effects | | 0.02 Family | | | | |
| *htr2B* Intercept | 0.77 | 0.11 | 0.55 | 0.98 | 6.89 | <0.001 |
| Anxious(most) | −0.22 | 0.11 | −0.44 | 0.01 | −1.89 | 0.067 |
| Treatment(0.3ppb) | −0.12 | 0.16 | −0.42 | 0.19 | −0.76 | 0.474 |
| Anxious(most):Trt | 0.31 | 0.16 | 0.00 | 0.62 | 1.94 | 0.059 |
| Aggressive phenotype | | | | | | |
| log *slc6a4a* | | | | | | |
| Intercept | −0.25 | 0.24 | −0.72 | 0.23 | −1.02 | 0.339 |
| Aggressive(most) | −0.36 | 0.23 | −0.82 | 0.09 | −1.58 | 0.122 |
| Treatment(0.3ppb) | −0.08 | 0.29 | −0.66 | 0.50 | −0.27 | 0.800 |
| Random effects | | | | | | |
| Family | | 0.05 | | | | |
| *slc6a4b* | | | | | | |
| Intercept | 1.10 | 0.08 | 0.94 | 1.25 | 13.70 | <0.001 |
| Aggressive(most) | −0.15 | 0.11 | −0.37 | 0.06 | 1.42 | 0.155 |
| Treatment(0.3ppb) | −0.17 | 0.11 | −0.38 | 0.04 | 1.55 | 0.121 |
| Random effects | | | | | | |
| Family | | 0.02 | | | | |
| log *htr1Aa* | | | | | | |
| Intercept | −0.53 | 0.25 | 0.53 | 0.94 | −2.13 | 0.07 |
| Most Aggressive | −0.23 | 0.25 | −0.31 | 0.00 | −0.94 | 0.36 |
| AZT0.3 | −0.15 | 0.30 | −0.23 | 0.30 | −0.50 | 0.64 |
| Random effects | | | | | | |
| Family | | 0.04 | | | | |
| *htr1B* | | | | | | |
| Intercept | 1.01 | 0.09 | 0.84 | 1.18 | 11.84 | 0.00 |
| Aggressive(most) | 0.00 | 0.04 | −0.07 | 0.07 | −0.02 | 0.99 |
| Treatment(0.3ppb) | −0.08 | 0.12 | −0.31 | 0.15 | −0.66 | 0.54 |
| Random effects | | | | | | |
| Family | | 0.02 | | | | |

*Continued on next page*

Table A15.2 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| *htr2B* | | | | | | |
| Intercept | 0.73 | 0.10 | 0.53 | 0.94 | 7.13 | 0.00 |
| Aggressive(most) | −0.15 | 0.08 | −0.31 | 0.00 | −1.97 | 0.06 |
| Treatment(0.3ppb) | 0.04 | 0.13 | −0.23 | 0.30 | 0.27 | 0.80 |
| Random effects | | | | | | |
| Family | | 0.02 | | | | |

Table A15.3: Model outputs of anxiety (time spent in the bottom zone), aggression (time spent interacting with the mirror) and relative mRNA levels of candidate genes *slc6a4a*; *slc6a4b*; *htr1Aa*; *htr1B* and *htr2B* of $F_1$ offspring from control and 0.3ppb atrazine treated males normalised to *b-actin* (from whole brain samples). For all models, results are presented with estimates, lower and upper 95% confidence intervals (Lwr95%CI and Upr95%CI), the $t$ value and the $p$ value.

| **Parameter** | **Estimate** | **Variance** | **Lwr95%CI** | **Upr95%** | ***T*** | ***P*** |
|---|---|---|---|---|---|---|
| **Anxiety** | | | | | | |
| **Time spent in the bottom zone** | | | | | | |
| Intercept | 40.92 | 7.18 | 26.85 | 54.98 | 5.70 | <0.001 |
| c.log *slc6a4a* | 11.94 | 8.94 | −5.57 | 29.46 | 1.34 | 0.189 |
| Treatment(0.3ppb) | 19.64 | 9.93 | 0.17 | 39.11 | 1.98 | 0.055 |
| c.log *slc6a4a*:Treatment(0.3ppb) | −22.27 | 12.43 | −46.64 | 2.09 | −1.79 | 0.080 |
| Random effects | | | | | | |
| Family | | 0 | | | | |
| Intercept | 40.62 | 7.40 | 26.12 | 55.13 | 5.49 | <0.001 |
| c.*slc6a4b* | 9.77 | 14.41 | −18.48 | 38.02 | 0.68 | 0.50 |
| Treatment(0.3ppb) | 21.05 | 10.36 | 0.74 | 41.36 | 2.03 | 0.05 |
| Random effects | | | | | | |
| Family | | 0 | | | | |
| Intercept | 40.14 | 7.02 | 26.37 | 53.90 | 5.72 | <0.001 |
| c.log*htr1Aa* | 17.28 | 7.58 | 2.42 | 32.13 | 2.28 | 0.028 |
| Treatment(0.3ppb) | 20.56 | 9.73 | 1.49 | 39.62 | 2.11 | 0.041 |
| c.log*htr1Aa*:Treatment(0.3ppb) | −21.08 | 11.69 | −43.98 | 1.82 | −1.80 | 0.078 |
| Random effects | | | | | | |
| Family | 0 | | | | | |
| Intercept | 42.24 | 7.78 | 27.00 | 57.48 | 5.43 | 0.009 |

*Continued on next page*

Table A15.3 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| c.*htr1B* | −17.12 | 31.18 | −78.23 | 43.99 | −0.55 | 0.593 |
| Treatment(0.3ppb) | 18.01 | 10.94 | −3.43 | 39.45 | 1.65 | 0.191 |
| Random effects | | | | | | |
| Family | | 15.89 | | | | |
| Intercept | 41.32 | 7.35 | 26.91 | 55.72 | 5.62 | <0.001 |
| c.*htr2B* | −4.73 | 17.55 | −39.13 | 29.68 | −0.27 | 0.789 |
| Treatment(0.3ppb) | 19.72 | 10.19 | −0.25 | 39.69 | 1.94 | 0.060 |
| Random effects | | | | | | |
| Family | | 0 | | | | |
| **Aggression** | | | | | | |
| **Time spent in the mirror zone** | | | | | | |
| Intercept | 282.55 | 35.56 | 212.86 | 352.23 | 7.95 | <0.001 |
| c. log *slc6a4a* | −42.57 | 30.80 | −102.94 | 17.80 | −1.38 | 0.17 |
| Treatment(0.3ppb) | −148.91 | 49.25 | −245.44 | −52.37 | −3.02 | <0.001 |
| Random effects | | | | | | |
| Family | | 0.00 | | | | |
| Intercept | 297.64 | 37.03 | 225.07 | 370.21 | 8.04 | 0.001 |
| c. *slc6a4b* | −196.40 | 64.83 | −323.46 | −69.34 | −3.03 | <0.001 |
| Treatment(0.3ppb) | −176.89 | 51.91 | −278.63 | −75.15 | −3.41 | 0.03 |
| Random effects | | | | | | |
| Family | | 810.3 | | | | |
| Intercept | 282.03 | 36.25 | 210.99 | 353.07 | 7.78 | <0.001 |
| c.log *htr1Aa* | −16.87 | 29.81 | −75.30 | 41.56 | −0.57 | 0.574 |
| Treatment(0.3ppb) | −147.92 | 50.26 | −246.44 | −49.40 | −2.94 | 0.005 |
| Random effects | | | | | | |
| Family | | 0 | | | | |
| Intercept | 299.61 | 34.53 | 231.93 | 367.29 | 8.68 | <0.001 |
| c. *htr1B* | −420.75 | 139.49 | −694.14 | −147.35 | −3.02 | 0.01 |
| Treatment(0.3ppb) | −181.50 | 48.56 | −276.67 | −86.33 | −3.74 | 0.03 |

*Continued on next page*

Table A15.3 – *Continued from previous page*

| | | | | | | |
|---|---|---|---|---|---|---|
| Random effects | | | | | | |
| Family | | 211 | | | | |
| Intercept | 280.05 | 39.97 | 201.71 | 358.39 | 7.01 | <0.001 |
| c. *htr2B* | −95.49 | 87.04 | −266.09 | 75.11 | −1.10 | 0.28 |
| Treatment(0.3ppb) | −143.06 | 55.60 | −252.03 | −34.10 | −2.57 | 0.08 |
| Random effects | | | | | | |
| Family | | 984.1 | | | | |

Table A15.4: Cross validation model outputs of *slc6a4a* and *htr1Aa* mRNA for interactive anxiety:treatment differences. For all models, results are presented with estimates, lower and upper 95% confidence intervals (Lwr95%CI and Upr95%CI), the $t$ value and the $p$ value.

| **Parameter** | **Estimate** | **Variance** | **Lwr95%CI** | **Upr95%** | **T** | **P** |
|---|---|---|---|---|---|---|
| Controls | | | | | | |
| (Intercept) | 41.41 | 7.61 | 26.50 | 56.32 | 5.44 | <0.001 |
| c.log.*slc6a4a* | 11.94 | 9.49 | −6.65 | 30.54 | 1.26 | 0.223 |
| Treatment | | | | | | |
| (Intercept) | 60.95 | 6.45 | 48.30 | 73.60 | 9.45 | <0.001 |
| c.log.*slc6a4a* | −10.33 | 8.13 | −26.26 | 5.60 | −1.27 | 0.217 |
| Controls | | | | | | |
| (Intercept) | 41.41 | 7.05 | 27.59 | 55.23 | 5.87 | <0.001 |
| c.log.*htr1Aa* | 17.28 | 7.64 | 2.31 | 32.24 | 2.26 | **0.0349** |
| Treatment | | | | | | |
| (Intercept) | 60.95 | 6.66 | 47.90 | 74.00 | 9.16 | <0.001 |
| c.log.*htr1Aa* | −3.80 | 8.84 | −21.12 | 13.51 | −0.43 | 0.671 |

# Appendix XVI: Additional measures of relative mRNA level data

## Methods

Due to the least and most anxious fish being selected, additional analyses were carried out on the behavioural phenotype defined as a categorical variable. The least and most aggressive fish were selected based on highest and lowest time spent interacting with the mirror from their family group, per sex.

### Statistical analysis

To investigate associations between mRNA transcripts of candidate genes and behaviour, LMMs were conducted with candidate genes as response variables with treatment, behavioural phenotype (fish categorised as either the least or most anxious/ aggressive) and an interaction term as predictors. Sex as a main effect was non-significant and thus removed from further analyses between behavioural phenotypes.

## Results

### Anxiety

No significant relationship was observed for anxiety phenotype and *slc6a4a* mRNA transcript (Figure A16.1 A), nor were there any significant differences observed for *slc6a4b* and *htr1B* mRNA transcripts (after model averaging) between the least anxious and most anxious fish from either treatment (Figure A16.1 B and D respectively). On average, more anxious control fish showed an up-regulation of *htr1Aa* with the pattern reduced in atrazine treated fish. However no significant relationships were observed (Figure A16.1 C). More anxious control fish showed a marginally non-significant down-regulation of *htr2B* mRNA (Est. $-0.22$

[−0.44, 0.01 CI]). Conversely, fish from atrazine treated fathers exhibited the opposite pattern, but this interactive effect was also marginally non-significant (Est. 0.31 [0.0, 0.62 CI]; Figure A16.1 E).

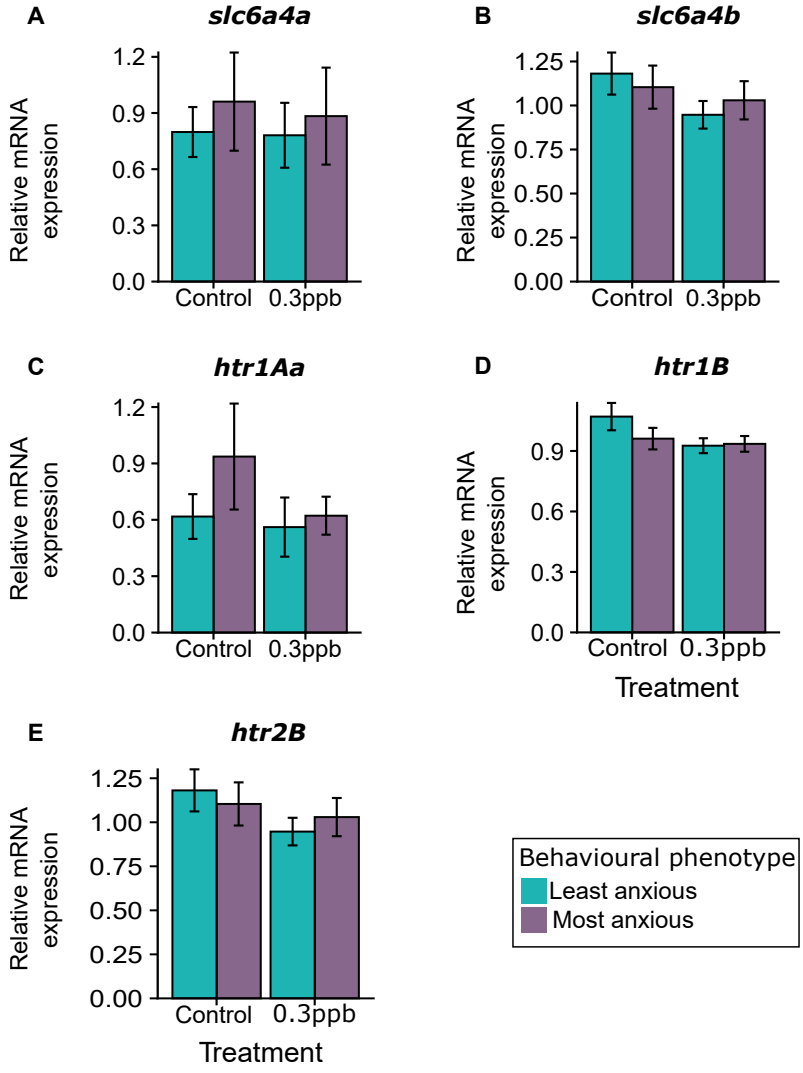


Figure A16.1: Relationship between anxiety phenotype (fish categorised as the least and most anxious) and relative mRNA levels of the candidate genes (A) *slc6a4a*; (B) *slc6a4b*; (C) *htr1Aa*; (D) *htr1B* and (E) *htr2B* of $F_1$ offspring from control and 0.3ppb atrazine treated males noramlised to *b-actin* (from whole brain samples). Sample sizes for control offspring were n=22 (males=10; females=12; progeny per family=6-8; families n=3), and for 0.3ppb offspring were n=24 (males=12; females=12; progeny per family=8; families n=3).

## Aggression

There was no statistically significant relationship in the mRNA transcripts of *slc6a4a*, *slc6a4b* (after model averaging), *htr1Aa* and *htr1B* in fish categorised as either the most or least aggressive (Figure A16.2 A; B; C; D respectively). However, more aggressive fish showed a non-significant down regulation in *htr2B* mRNA (Est. −0.15 [0.0, −0.94 CI]) regardless of treatment (Figure A16.2 E).

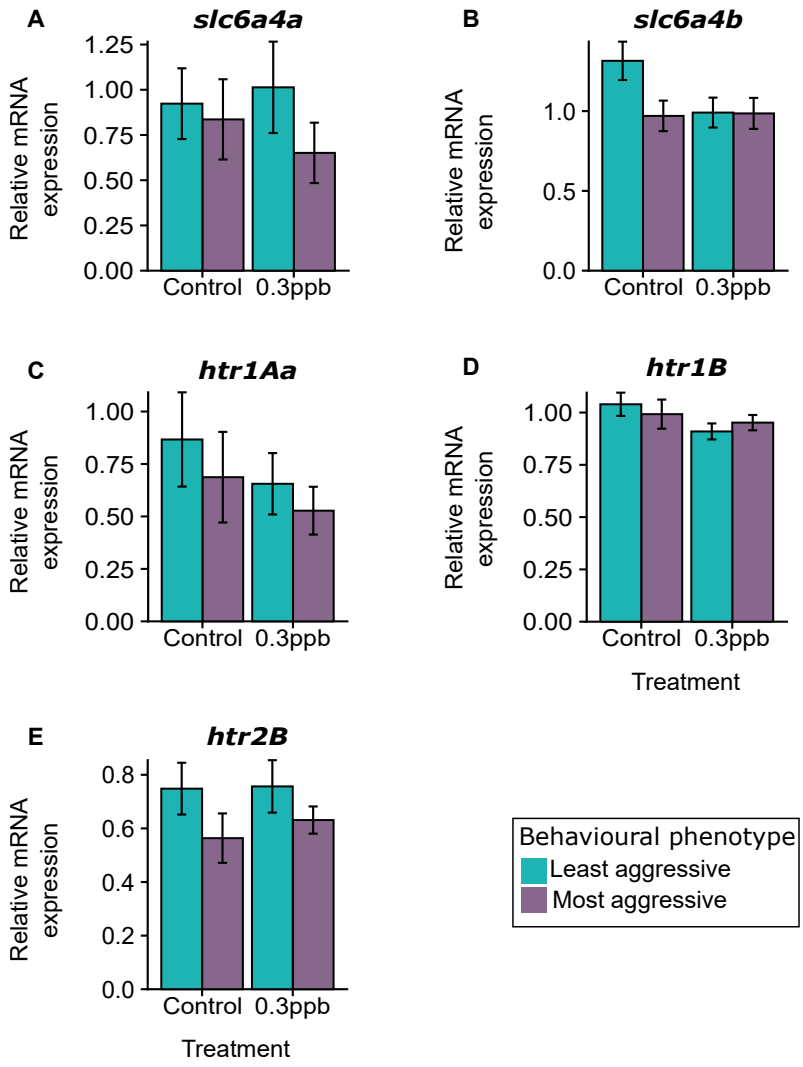


Figure A16.2: Relationship between aggression phenotype (fish categorised as the least and most aggressive) and relative mRNA levels of the candidate genes (A) *slc6a4a*; (B) *slc6a4b*; (C) *htr1Aa*; (D) *htr1B* and (E) *htr2B* of $F_1$ offspring from control and 0.3ppb atrazine treated males noramlised to *b-actin* (from whole brain samples). Sample sizes for control offspring were n=22 (males=10; females=12; progeny per family=6-8; families n=3), and for 0.3ppb offspring were n=24 (males=12; females=12; progeny per family=8; families n=3).