The effects of food rationing on phenotypic plasticity in reproductive resource allocation in female wild-type zebrafish, *Danio rerio*

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Abstract

Any resources obtained by an organism must be divided between life history components, namely growth, reproduction and survival. Resources are limited, thus creating trade-offs between competing traits. One trade-off is the relationship between the size and the number of offspring (fecundity) produced by a mother. Although adaptive phenotypic plasticity in this trade-off has been demonstrated across the Animal Kingdom, understanding of the mechanisms behind this is lacking. For example, female zebrafish (*Danio rerio*) subjected to different feeding regimes have shown this phenotypic plasticity, but studies have not determined the mediators of this change. This thesis aimed to examine the phenotypic plasticity in resource allocation to reproduction in wild-type zebrafish and elucidate mechanisms allowing the change. Female zebrafish were fed either 1.5% (Low) or 3% (High) of body weight daily and phenotypic changes in fecundity, offspring size and gene expression of five candidate genes in the ovary were evaluated. Representative ovary samples were also analysed by RNA-Seq. There were trends for increases in *lrp8* and *esr2a* mRNA expression levels in the ovaries of the food-limited females when compared with food-abundant females. This supported previous literature demonstrating increases in vitellogenin in larger eggs. In the food-abundant females, there was an increase in *fshr* mRNA expression levels. This could be a mechanism to increase fecundity, through increased follicles entering vitellogenesis. A second experiment was performed to assess the sensitivity and timing of the resource allocation decisions. Female zebrafish were exposed to an initial feeding regime for four weeks and then switched to the other feeding regime for another four weeks. These females were analysed for differences in resource allocation and the trade-off between follicle size and reproductive investment was analysed regardless of feeding regime. Across both experiments, differences in phenotype were hard to discern due to the effects of maternal condition. There were marked differences in females within the same tanks, possibly due to dominance effects, and this may have obscured the effects of the individual feeding regimes. Overall, there was no adaptive phenotypic plasticity in offspring size or offspring number evident in the experiments presented. Future gene expression studies on females clearly demonstrating differences in phenotype are necessary.

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List of Abbreviations

Zebrafish Nomenclature was used throughout the entirety of this thesis.

actb1/β-actin beta actin

ANOVA	analysis of variance
bp	base pairs
ctsba/ctsla	cathepsin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
eef1a1a	elongation factor 1 alpha 1a
esr2a	estrogen receptor 2a
FDR	false discovery rate
FFA	free fatty acids
FSH, Fsh	follicle-stimulating hormone
Gh	growth hormone
GSI	gonadosomatic index
Igf	insulin-like growth factor
LDL	low density lipoprotein
LH, Lh	luteinising hormone
LMER	linear mixed effects model
Lrp8	vitellogenin receptor in teleost
qPCR	quantitative polymerase chain reaction
PCR	polymerase chain reaction
PGO	primary growth oocyte
RNA	ribonucleic acid
RNA-Seq	ribonucleic acid sequencing
VLDL	very low density lipoprotein
Vtg	vitellogenin
zp	zona pellucida

General Introduction

1.1 Background and History

An organism's ability to acquire resources from the environment and the subsequent allocation of these resources to different areas of life history is a major determinant of its ability to survive and reproduce (Burton et al. 2010, Robinson and Beckerman 2013). Classical life history theory states that traits will become optimised over time to maximise fitness in the local environment (Winemiller and Rose 1992). This can either be achieved through the ability to exhibit different phenotypes in response to different external cues or through long term natural selection resulting in alteration of the underlying genotype (Pigliucci et al. 2006, Crispo 2007). The study of life history theory is a complicated and many faceted discipline. To accurately model the population dynamics and organism phenotypic traits, many different factors must be evaluated including; predation, density-dependence, resource availability, and reproductive strategies (Winemiller and Rose 1992, Reznick et al. 2002, Allen et al. 2008, Marshall and Keough 2009, Marshall et al. 2010). Historically, models which predict optimal endpoints have been relatively simplistic and have failed to take the complexity of a natural system into account (Reznick et al. 2002). Their strength lies however in providing inspiration and a starting point to study the complex interactions of a species' life history and possible evolution (Reznick et al. 2002).

Life history theory at the core evaluates different constraints or trade-offs present between all variables and traits in a species. It also investigates how these trade-offs change or create strategies to enable species to deal with different or changing environments (Winemiller and Rose 1992). There are limited resources available to any organism from the environment in which they reside. These resources are then divided between three main areas; growth, reproduction and survival. Any trait within the same area will be in direct competition for a limited pool of resources and therefore an increase in one will lead to a decrease in another, causing a negative relationship or "trade-off". A trade-off is defined as occurring when an increase in fitness due to change in one trait is opposed by a decrease in fitness due to a concomitant change in another life-history trait (Roff et al. 2006). One such trade-off was modelled by Smith and Fretwell (1974). They proposed that there was a trade-off between offspring number and size in any female individual and that in any environment an optimal offspring size

that maximises maternal reproductive fitness will be produced. Their original classical theory considers that any organism ultimately aims to maximise its total reproductive fitness and produce as many surviving young as possible (Smith and Fretwell 1974). The theory asks the question of how much energy should be invested in offspring on an individual basis? If there is greater individual investment (and therefore larger offspring) there will be an overall reduction in the number of offspring produced due to limited physical space in the mother and limited resources being available for reproduction (Smith and Fretwell 1974, Winemiller and Rose 1992, Winemiller and Rose 1993). Larvae themselves are much more limited in their ability to adapt to a local environment when compared with adults (Winemiller and Rose 1993, Segers and Taborsky 2011). They must therefore rely on the maternal influences such as altered individual provisioning by the mother. In limited environments fish can alter egg-size to produce fewer, larger eggs which allow offspring a greater chance of survival (Bashey 2006, Hassall et al. 2006, Forbes et al. 2010, Segers and Taborsky 2011, Riesch et al. 2012, Closs et al. 2013).

1.2 Adaptive Phenotypic Plasticity and Maternal Effects

Phenotypic plasticity is defined as the ability of individual genotypes to yield distinct phenotypes when exposed to different environments (Pigliucci et al. 2006, Green and Extavour 2014). There are numerous studies which investigate the differences between various phenotypes in different environments. However, even if the differences in phenotype are quite apparent, the underlying cause and benefit of this plasticity can be hard to determine (Bashey 2006, Pigliucci et al. 2006). Phenotypic plasticity is considered adaptive when a clear benefit to the organism or population is demonstrated (Bashey 2003, Bashey 2006, Marshall and Uller 2007, Allen et al. 2008). When resource availability changes, basic life history traits can be highly plastic (Pampoulie et al. 2000). Often when confronted with a resource-limited environment, mothers will produce smaller offspring as less resources are available for reproduction. This is considered to be a passive, physical response with the mother having no control (Bashey 2006). There have been studies which have shown an opposite effect, resource-limited mothers producing offspring which are larger than when in a resource-rich environment. Although this will reduce fecundity in the mother, if the larger offspring has an

advantage in a resource-limited environment then the overall fitness of the mother may be maintained. If larval survival is increased with increased offspring size, then plasticity in this trait is selected for in environments where the larval environment is uncertain or non-favourable (Bashey 2003, Bashey 2006, Bashey 2008, Kuijper and Johnstone 2013).

Anticipatory or adaptive maternal effects involve manipulating the offspring phenotype to be better suited to the local (or larval) environment (Marshall and Uller 2007). These effects are characterised by adjusting offspring phenotypes to match to external environmental cues or by creating inherited environmental effects that ultimately increase offspring survival (Marshall and Uller 2007, Jorgensen et al. 2014, Murphy et al. 2014, Paul et al. 2015). Any organism is informed of future environmental conditions through external cues. If these are consistent, a mother can reasonably predict the environment which her offspring will experience. If a mother can predict unfavourable conditions for her offspring, then she may be able to alter the phenotype (size) of her offspring. Initial offspring size is closely correlated with both offspring and maternal fitness (Marshall and Uller 2007, Fischer et al. 2011). This is because larger offspring are often shown to have higher survival when exposed to harsher environment conditions (Fischer et al. 2011, Kuijper and Johnstone 2013, Murphy et al. 2014, Paul et al. 2015). For this type of plasticity to be selected for the maternal environment must be a good indicator of the potential offspring environment and the cost of the plasticity in the offspring phenotype must be outweighed by the maternal fitness gained through offspring survival (Ware 1975, Marshall and Uller 2007, Jørgensen et al. 2011, Segers and Taborsky 2011, Burgess et al. 2013, Burgess and Marshall 2014, Paul et al. 2015). Reproductive investment is entirely decided by the mother, so the offspring size produced is the size that maximises her fitness in a given environment (Smith and Fretwell 1974, Ware 1975). Often this pattern is true in species which have a limited dispersal as then there is a correlation between the maternal and offspring environments (Kuijper and Johnstone 2013).

1.3 Natural Selection/Speciation

Phenotypic plasticity can provide novel well-adapted phenotypes when a species is subjected to novel conditions (Pigliucci et al. 2006, Crispo 2007). This can occur when species expand into new habitats or are at the edge of a species range (Hassall et al. 2006, Pigliucci et al. 2006, Riesch et al. 2012). As there is a cost to plasticity, this new phenotype being selected for can alter the reaction norm for a species and improve the performance of the population under these new conditions (Hassall et al. 2006, Pigliucci et al. 2006, Crispo 2007).

If there are differences in plasticity in egg size and number between populations then it can be acted on by natural selection. As larger offspring often have a selective advantage in risky or harsh environments then females which produce these larger offspring will have higher reproductive success, producing a population where egg size may be larger than a population in an "easier" environment. If natural selection is acting on offspring size, then risk-spreading tactics and selection at the edges of a species range could allow for radiation of species from geographically distinct ecotypes into new challenging environments (Hassall et al. 2006, Riesch et al. 2012).

There are four different ecological mechanisms which can evoke selection occurring on offspring size, demonstrating clear adaptive reasons for larger offspring being selected for (Jørgensen et al. 2011). 1) Intraspecific competition during early life stages; if offspring develop in a limited habitat where intracohort competition is likely then this pattern is likely to occur. Larger offspring have a competitive advantage to obtain food and other resources (Einum and Fleming 2000). 2) Fertilization success; a larger egg is a better target for sperm in broadcast spawners, so the larger the egg size, the higher the likelihood of fertilisation success (Levitan 1993). 3) Development time; if development time is faster in the egg than when the offspring is feeding alone then larger eggs can sustain yolk-driven development for longer. This allows offspring in the larger eggs to hatch at a more developed stage than their counterparts in smaller eggs. Selection is evoked if a constant mortality rate is assumed, so faster development is directly correlated with higher survival rates (Levitan 2000). 4) Offspring growth rate combined with mortality rate. Mortality is often size dependent, decreasing with increased body size. If mortality is size dependent, then mortality will change as the organism grows (Blueweiss et al. 1978). Growth rate is directly affected by temperature and food

availability; therefore, resource availability can directly play a role in the evolution of offspring size in a population or species. In both fish and butterfly species it has been reported that when it is colder and there is less food available (closer to winter), then offspring size is larger than when the same populations are exposed to warmer, more food-rich environments (spring/summer) (Ware 1975, Fischer et al. 2003).

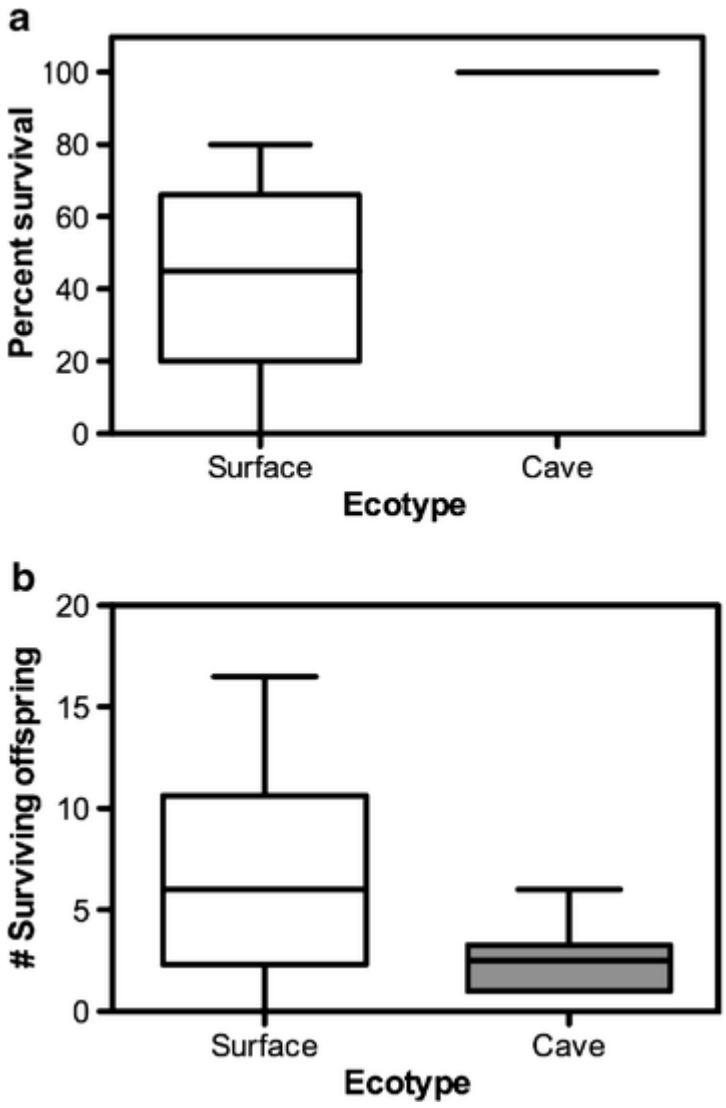

Figure 1.1: Boxplots showing survival rates (%) after 30 days (a) and the number of surviving offspring (b) for *Poecilia mexicana* when exposed to optimal environmental conditions. Fish came from two different habitat types; benign surface, smaller offspring (white) and a toxic cave, larger offspring (grey). (Riesch et al. 2012)

In Atlantic mollies (*Poecilia mexicana*), there are two different reproductively isolated phenotypes; one which inhabits a sulfidic cave environment and another occupying other non-sulfidic habitat in the surrounding sea floor. The mollies present in the more toxic (harsh) sulfidic environment produce fewer large young when compared to the mollies that are living in the non-sulfidic environments. To test whether there was possible speciation occurring, Riesch et al. (2012) tested the survival and the overall fitness of the offspring from both phenotypes under benign conditions. They found that although the larger offspring had a higher innate fitness, there was a higher total number of smaller offspring surviving overall (Figure 1.1). This means that in more benign environments the mollies that produce larger offspring will be selected against due to a lower overall reproductive fitness and vice versa with the mollies from the surface habitats moving into the caves. As these ecotypes appear to be undergoing speciation (multiple divergent traits including behavioural and morphological), the genetic heritability of this difference in egg size and fecundity could promote further reproductive isolation and divergence (Riesch et al. 2012). Similarly, in a population of field grasshoppers (*Chorthippus brunneus*) in Britain, Hassall et al. (2006) showed that at the edges of the range, where conditions were harsher (colder and wetter), females were producing larger offspring and fewer of them when compared to individuals in the optimal areas of the range. This had a significant genetic component and females raised in a laboratory setting continued to produce larger eggs across multiple generations (Hassall et al. 2006).

The phenotype of larger and fewer offspring being produced seems to become genetically fixed in multiple species, when plasticity is no longer selected for. This means that initial plasticity in the offspring size and number trade-off phenotypes could act as a mechanism to allow successful dispersal into new habitats, such as lowland river or sea fish species colonising further upstream (Riesch et al. 2012, Closs et al. 2013). Ultimately this phenotypic plasticity provides a well-adapted phenotype that can be acted upon by natural selection and can help drive reproductive isolation between populations (Hassall et al. 2006, Pigliucci et al. 2006, Crispo 2007). In New Zealand, it has been shown that non-migratory galaxiids have relatively larger eggs and lower fecundity than their closely related amphidromous species. This trait has evolved

separately several times within the genus as the different river systems were colonised (Closs et al. 2013). It is argued that the larger eggs produce morewell-developed larvae which enhances survival in the more resource-limiting freshwater environment (Closs et al. 2013, Vanderpham et al. 2013).

1.4 Zebrafish as a model

To be considered an acceptable research model, an organism must be considered representative of a large range of biological organisms while also having appropriate characteristics that make it easier to study in the laboratory environment than the animals it is used to represent (Ribas and Piferrer 2014). Due to their ability to breed quickly and easily in a laboratory environment, due to the relatively short lifecycle, and due to their genetic similarity to humans and other vertebrates, zebrafish, *Danio rerio,* are increasingly used as a model organism in many different avenues of scientific study (Grunwald and Eisen 2002, Lieschke and Currie 2007, Spence et al. 2008, Lawrence 2012, Ribas and Piferrer 2014). Zebrafish have been used to generate new insights in areas such as toxicology, developmental biology, evolution, disease and genetics research (Haffter et al. 1996, Schier et al. 1996, Grunwald and Eisen 2002, Lieschke and Currie 2007, Lawrence 2012, Ribas and Piferrer 2014).

Danio rerio is a member of the order Cypriniformes, a large group of freshwater fish spread throughout North America, Africa and Eurasia (Northern Hemisphere) (Fang and Douglas 2003, Mayden et al. 2007). Within this order the zebrafish is placed in the family Cyprinidae. The *Danio* genus is one of several genera assigned to the subfamily Rasborinae, which is common in India, Pakistan, Nepal, Bangladesh, Sri Lanka, Thailand, the Malay Peninsula, Sumatra and the Yunnan province of China (Spence et al. 2006, Engeszer et al. 2007, Wang et al. 2007, Spence et al. 2008, Fang et al. 2009). *Danio rerio* is common to South and South-East Asia (Spence et al. 2006, Engeszer et al. 2007, Mayden et al. 2007, Spence et al. 2008).

Zebrafish are highly adaptable and can survive in a wide range of environments. This species has been found inhabiting rivers, small streams, stagnant or slow-moving pools close to streams and rice paddies (Engeszer et al. 2007). In the wild, zebrafish typically

spawn annually. Spawning occurs just before the monsoon season and clutches are laid in shallow water over gravel or plants. Larvae survive on a diet of zoo plankton and organic material (Spence et al. 2008).

Previous studies have demonstrated plasticity in oocyte diameters or fecundity of zebrafish with changing nutritional environments. Forbes et al. (2010) showed that when exposed to two different feeding regimes, a high and a low (40% of the high), fecundity was significantly reduced in female zebrafish subjected to the low feeding regime when compared with the females on the high feeding regime. Conversely the spawned egg diameters were significantly increased in the food-limited females. Lawrence et al. (2012) fed zebrafish at different frequencies to a total of 5% body weight a day and discovered that fish fed once every other day produced significantly fewer oocytes than those fed every day at varying frequencies. But these fish were also significantly smaller (not corrected for body weight) (Lawrence et al. 2012).

1.5 Oocyte Development in *Danio rerio*

Zebrafish are asynchronous batch spawners, which means that oocytes in several stages of development are present in the ovary at the same time. Oocyte growth is dynamic and continuous but key stages are loosely separated and categorised by changes in the morphology of the nucleus and follicle as well as changes in its cytoplasmic composition. Oocyte development has been divided into 5 broad stages in this species (Selman et al. 1993), Figure 1.2).

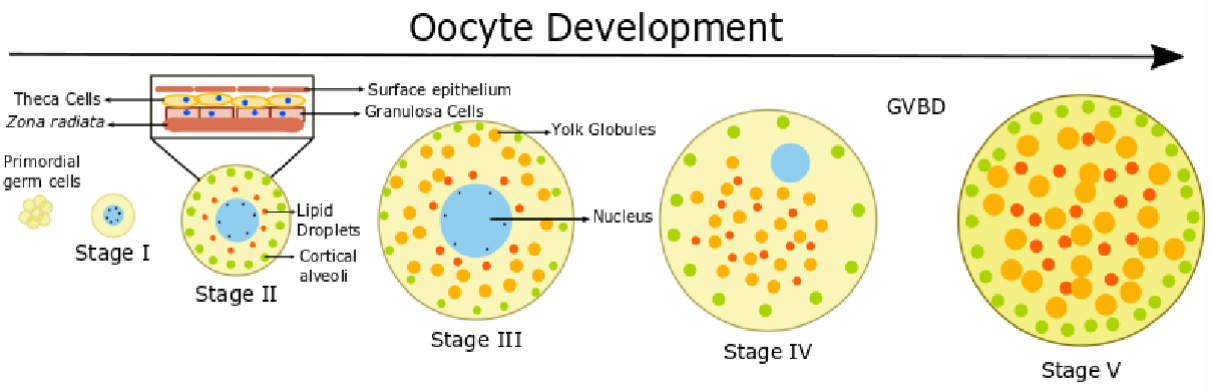

Figure 1.2: Generalised oocyte development in fishes. The sequence of oocyte stages is; Stage I – primary growth (previtellogenic), Stage II – cortical alveoli growth, Stage III – early

vitellogenic oocytes, Stage IV – late vitellogenic oocytes and Stage V – mature oocyte or ovulated egg. Figure is adapted from information obtained from Lubzens et al. (2010) and Urbatzka et al. (2011)

Stage 1 - Previtellogenic

These are classified as primary growth oocytes (PGO) at this stage. PGOs are organised in cell nests alongside oogonia and prefollicular cells. As they begin to grow, PGOs leave the nest and are surrounded by follicle cells (Selman et al. 1993). The layer of follicle cells contains the steroid-secreting granulosa (inner) and theca (outer) cells (Nagahama 1994). Meiosis is arrested at prophase I (Lubzens et al. 2010).

Stage 2 – Cortical alveoli

Stage two is the formation of cortical alveoli. Cortical alveoli are membrane-limited vesicles containing both proteins and carbohydrates. As vitellogenesis continues and the oocyte grows, the cortical alveoli proliferate and move to the periphery. Further downstream (after fertilisation) the contents of the cortical alveoli are discharged into the space between the oocyte and the vitelline envelope. This hardens the vitelline envelope preventing polyspermy after fertilisation has taken place (Patino and Sullivan 2002).

Stage 3 - Vitellogenesis

Stage three is the vitellogenic stage and is the major growth phase with zebrafish oocytes increasing in diameter from 0.3 to 0.7 mm (Selman et al. 1993). This massive size increase is predominantly due to the accumulation of yolk within the oocyte. Vitellogenin and neutral lipids are taken into the ovary mainly through receptor-mediated endocytosis and subsequently processed into yolk proteins (Selman et al. 1993, Patino and Sullivan 2002).

Stage 4 - Maturation

During maturation, meiosis is resumed. The nucleus, or germinal vesicle, migrates towards the periphery of the oocyte and breaks down during the first meiotic division. Meiosis continues until the second metaphase and then it is arrested until fertilisation (Lubzens et al. 2010).

Stage 5 – Ovulation

Stage five is the mature egg, which is ovulated into the ovarian lumen. At this stage these eggs are ready for fertilisation. For a more detailed review of oocyte development in zebrafish, see Selman et al. (1993).

1.6 Vitellogenin

In the majority of oviparous animals vitellogenin is the predominant yolk precursor (Prat et al. 1998). Vitellogenins (Vtgs) are a diverse family of large phosphoglycoproteins. They are produced by the liver and synthesis is mediated by circulating estrogen in vertebrates (Williams 2001, Hiramatsu et al. 2002). As a very high-density lipoprotein, vitellogenin transports the largest proportion of yolk proteins into the developing oocytes. Once vitellogenin is synthesised in the liver, it is secreted into the bloodstream where it is transported around the body. Once it reaches the ovary, developing oocytes sequester vitellogenin from the blood stream and deposit it into yolk granules. This is achieved through receptor-mediated endocytosis. Once vitellogenin is taken up into the oocytes, it is enzymatically cleaved and repackaged into lipovitellins and phosvitins which make up the yolk (Hiramatsu et al. 2002, Hiramatsu et al. 2002).

A member of the low-density lipoprotein (Ldl) receptor family, LR-8 has been designated as the vitellogenin receptor in teleosts (Davail et al. 1998, Prat et al. 1998, Hiramatsu et al. 2004, Johnson 2009, Hiramatsu et al. 2013, Mizuta et al. 2013). The Ldl receptor family are type-1 receptor proteins. All members of this family contain both transmembrane and cytoplasmic domains. They are involved in endocytosis of a variety of ligands including the plasma lipoproteins, such as vitellogenin and the low-density

lipoproteins. The receptors consist of unique configurations of an epidermal growth factor precursor, a Class B repeat and O-linked sugar domains, which defines their identities. They also contain Class A ligand binding (LDLa) repeats, which determines their ligand specificity (Bujo et al. 1995, Prat et al. 1998, Reading et al. 2014, Figure 1.3). Multiple receptors and multiple vitellogenin proteins have been identified in cutthroat trout (*Oncorhynchus clarki*) (Mizuta et al. 2013). Multiple ovarian membrane proteins that specifically bind Vtgs have also been detected in rainbow trout (*Oncorhynchus mykiss*) and white perch (*Morone americana*) (Lancaster and Tyler 1991, Reading et al. 2011).

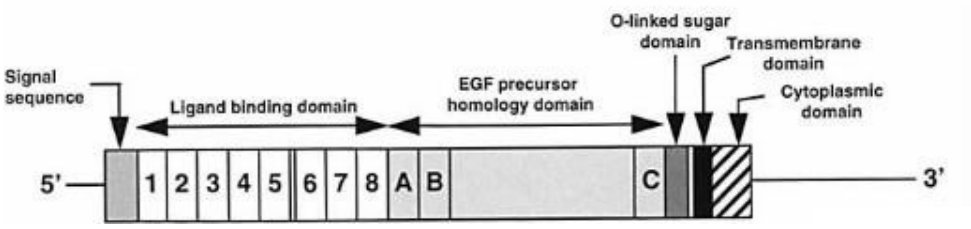

Figure 1.3: Representation of the structure of the very low density lipoprotein and vitellogenin receptor family, modified from a figure in Prat et al. (1998).

Tyler (1991) showed that in rainbow trout, the sequestration and the subsequent packaging of vitellogenin into its yolk derivatives can account for up to 80% of the final oocyte size. As vitellogenesis is the stage during which most of the growth of the oocyte occurs (Prat et al. 1998), changes in the level of uptake of vitellogenin could change the overall size of the resulting oocyte. Vitellogenin and low-density lipoprotein are both yolk precursors found in zebrafish (Wang et al. 2005).

Studies where empirical evidence has been demonstrated for egg size plasticity have shown that the larger eggs have higher amounts of proteins and lipids in the yolk (Guisande and Gliwicz 1992, Hassall et al. 2006, Sun et al. 2015). These extra proteins and lipids in the larger eggs act as more food for the larvae allowing for rapid growth and increasing initial larval size (Johnson 2009, Sun et al. 2015). Vitellogenin expression has been demonstrated to control egg size in birds, locusts and ticks (Williams 2001,

Chen et al. 2015). In zebra finches, *Taeniopygia guttata*, treated with an estrogen blocker, smaller eggs were laid, and clutch size increased when compared to birds which were untreated. This decrease in egg size was accompanied by a 50% decrease in plasma vitellogenin and very low-density lipoproteins (Williams 2001). In migratory locusts there are two distinct density-dependent phenotypes, i.e., high-density gregarious (G) and low-density solitary (S). These phenotypes have differences in the time until maturity and the overall egg size produced. In general, the high-density locusts mature faster and produce larger eggs. This study determined that regulation of vitellogenin expression occurred through a gene called *Syntaxin 1A* (*Syx 1A*). In the wild, *Syx 1A* expression was higher in the G locusts producing larger eggs. When exposed to a *Syx 1A* blocker the G locusts took longer to mature, and the egg size decreased to a similar size as in the S locusts (Chen et al. 2015). These findings suggest that vitellogenin and the upstream regulation of vitellogenin production can play a key role in determining egg size in an individual.

1.7 Very low-density and Low-density Lipoproteins

While the polar lipid and protein components of the yolk are delivered to the developing oocyte by circulating vitellogenins, there is a separate mechanism for the transport of neutral lipids (Bujo et al. 1995, Hiramatsu et al. 2013, Luo et al. 2013, Hiramatsu et al. 2015). Neutral lipids are packaged into very low density (VLDL) and low density lipoproteins (LDL) for transport around the body. These are synthesised in the liver and are made up of primarily triacylglycerides and cholesterol (Hiramatsu et al. 2013, Luo et al. 2013). In birds and mammals, VLDL and LDL are taken up into the oocyte by receptor-mediated endocytosis. This occurs during vitellogenesis in birds and is mediated by the same receptor as vitellogenin. In mammals this endocytosis is achieved via a specific VLDL receptor (Bujo et al. 1995, Johnson 2009). In fish, there are multiple receptors that play a role in the endocytosis of different lipoproteins (Mushirobira et al. 2013, Hiramatsu et al. 2015). The major teleost lipoprotein receptor is an orthologue of the mammalian VLDL receptor and was cloned in cutthroat trout (Luo et al. 2013, Hiramatsu et al. 2015). The abundance of mRNA transcripts for this receptor are highest in

previtellogenic oocytes and decreased during vitellogenesis. This follows the pattern of neutral lipid uptake into the oocyte (Luo et al. 2013).

In fish there are two main pathways of triacylglyceride accumulation in the developing oocyte. 1) Circulating VLDL is processed by ovarian lipoprotein lipase into LDL and the resulting free fatty acids (FFAs) are incorporated into the oocytes and form oil droplets (Hiramatsu et al. 2013). Or 2) VLDL or LDL binds to one or multiple lipoprotein receptors, where it then undergoes endocytosis and is stripped of FFAs. These FFAs are then utilized for oil droplet formation in the oocyte (Hiramatsu et al. 2003, Hiramatsu et al. 2013).

Damsteegt et al. (2015) showed that LDL and not vitellogenin has a major role in lipid deposition and accumulation in previtellogenic eel oocytes. Incubation with specific antisera against the LDL receptor significantly reduced oocyte size. In cutthroat trout VLDL was shown to be the predominant transporter of triacylglycerides while LDL is important in providing cholesteryl ester to the oocyte (Hiramatsu et al. 2015).

1.8 Gonadotropins

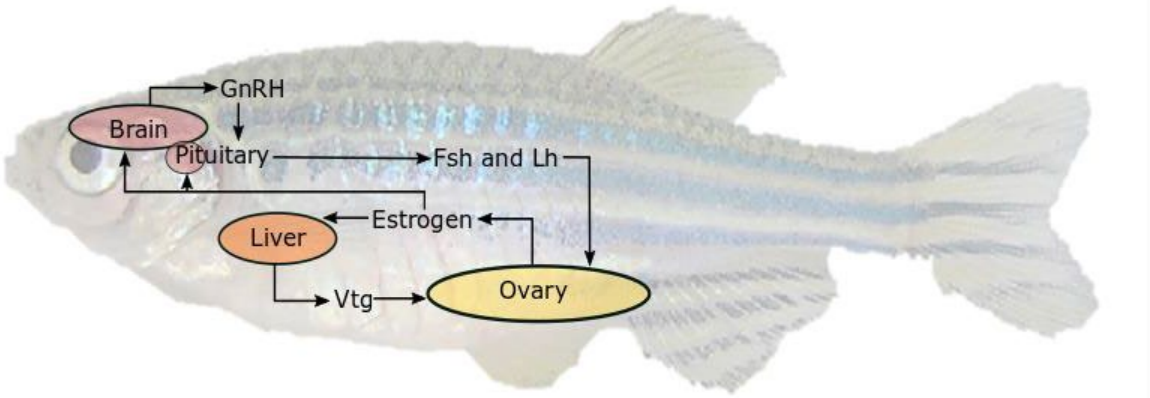

Figure 1.4: Simplified schematic of the brain-pituitary-gonadal-liver axis or the hormonal regulation of vitellogenesis in a generalised teleost. The axis is regulated by negative feedback by estradiol-17β (Estrogen). The brain releases gonadotropin-releasing hormone (GnRH), this stimulates the production of the gonadotropins, follicle-stimulating hormone (Fsh) and luteinising hormone (Lh). These travel through the bloodstream to target tissue (Ovary), in the ovary they stimulate the theca cells that surround developing oocytes to produce androgens. These androgens are converted to estradiol-17β via P450 aromatase in the granulosa cells, that adjoin the theca cells. Estradiol-17β stimulates hepatocytes in the liver to produce vitellogenin (Vtg), this travels to the ovary and is incorporated into the developing oocytes through receptor-mediated endocytosis. This figure was created using information obtained from Nagahama (1994).

In all vertebrates, gametogenesis is controlled by the pituitary through the production of gonadotropins; follicle-stimulating hormone (Fsh) and luteinising hormone (Lh) (Nagahama 1994, Nagahama 1995, Tyler et al. 1997, Patino et al. 2001, Mazón et al. 2015, Figure 1.4). These both stimulate the ovary to produce steroids, which control the process of folliculogenesis. This dual system is well documented, but it is not always clear which parts of the oocyte development pathway is controlled by which hormone as it can differ between species (Swanson et al. 1991, Tyler et al. 1997, Gen et al. 2003, Mazón et al. 2015). Fsh and Lh both consist of a common α-subunit (Cga) which is non-covalently bound to a β-subunit (Fshβ or Lhβ). These distinct β-subunits are what confer the different hormone specificities (Pierce and Parsons 1981, Swanson et al. 1991, Yaron et al. 2003, Levavi-Sivan et al. 2010).

In salmonids, studies suggest that secondary oocyte growth and vitellogenesis are regulated primarily by Fsh while Lh plays a primary role in regulating final oocyte maturation and ovulation (Swanson et al. 1991, Tyler et al. 1997, Campbell et al. 2006).

This is also true in the European sea bass (*Dicentrarchus labrax)*; Fsh is heavily involved in the regulation of growth of the previtellogenic and vitellogenic oocytes whereas Lh is primarily involved in maturation and ovulation (Mazón et al. 2015). This species of bass has a group-synchronous ovary. Although it spawns annually, it carries clutches of oocytes at different stages of development in the ovary which are then successively spawned during the annual season (Mayer et al. 1988, Asturiano et al. 2000, Mazón et al. 2015). Fsh has been demonstrated to enhance uptake of vitellogenin into oocytes during vitellogenesis in cutthroat trout (Tyler et al. 1997). In zebrafish, gene knockout studies investigating the effects of gonadotropins and their receptors have demonstrated that the Fsh receptor is necessary for oocytes to move past the primary growth stage. If *fshb* expression was knocked out in the pituitary, cross reactivity of the Fsh receptor with Lh occurred, this meant small numbers of oocytes entered vitellogenesis but folliculogenesis was still impaired when compared to normal females (Chu et al. 2015, Zhang et al. 2015, Li and Cheng 2018). Changes in the level of Fsh could thus play a role in phenotypic plasticity of oocyte size by increasing vitellogenin uptake.

In mammals, the "fecundity genes" increase the number of follicles ovulated through preventing the down-regulation of FSHr, and therefore increasing sensitivity to FSH (Moore et al. 2004, Moore and Shimasaki 2005). Treatment with exogenous FSH can cause super-ovulation in mammals (Fowler and Edwards 1957). Female bank voles (*Clethrionomys glareolus*) increased the number of pups per litter, when treated with human menstrual urine which contains FSH. Concomitantly, the average size of the pups is also reduced (Oksanen et al. 2002). Similarly, in side-blotched lizards (*Uta stansburiana*) treated with ovine FSH, clutch size was increased, and the size of the eggs was decreased when compared to non-treated lizards (Sinervo and Licht 1991, Sinervo 1999). Sensitivity to circulating gonadotropin levels may therefore play a key role in the determination of the size of clutches brought through to ovulation.

1.9 Growth Hormone and Insulin like growth factor system

The insulin-like growth factor/growth hormone (Igf-Gh) signalling system is conserved across multicellular animals. It coordinates cell growth and proliferation with respect to

physiological condition including the nutritional state (Green and Extavour 2014). In mammals, intraovarian insulin-like growth factors are important for maintaining oocyte development and promoting steroidogenesis (Chandrashekar et al. 2004). This may be the same or similar in fish. Evidence from a variety of fish species suggests that the metabolic system (Igf-Gh system) and somatolactin play roles in the regulation of oocyte development. In a number of species, Igf-1 can induce the oocyte to respond to maturation inducing steroids or can induce final maturation directly (Le Gac et al. 1993, Kagawa et al. 1994, Kagawa et al. 1995, Maestro et al. 1997, Patino and Kagawa 1999).

In studies on *Drosophila* it has been shown that the insulin signalling pathway underlies phenotypic plasticity in ovariole number. Ovarioles are the egg-producing structures of insect ovaries, so numbers of these are directly related to the overall number of eggs produced. It was demonstrated that with a loss of function of the insulin-like receptor, the ovariole number was significantly decreased (Green and Extavour 2012). When flies were fed a poor diet ovariole numbers were lower than in flies fed a nutritionally rich diet, showing that insulin-like growth factor signalling is the molecular mediator of the nutritional state (Green and Extavour 2014).

Campbell et al. (2006) evaluated the interactions between the growth and reproductive systems. In coho salmon, growth during critical periods is important for determining age of maturity and fecundity (Campbell et al. 2006). This study demonstrated that as cortical alveoli accumulated in the oocyte, there were corresponding increases in the plasma levels of Fsh and 17-beta estradiol. Concomitantly, there were increases in the expression levels of *fsh* in the pituitary and the steroidogenic acute regulatory protein in the ovary. There were also corresponding decreases in expression of ovarian growth hormone receptor and somatolactin receptor transcripts. At the lipid droplet accumulation stage, there was a general increase in plasma Igf-1 and components of the Fsh-ovary axis. The components of the Fsh axis that were upregulated; included plasma Fsh, estradiol 17beta as well as ovarian mRNA transcripts for gonadotropin receptors, *star*, *igf1* and *igf2*. This suggests that Fsh signalling may play an important role in transitioning the oocyte into the oil droplet stage. There was a consistent positive relationship between plasma IGF-1, estradiol 17beta and pituitary Fsh during body growth in the spring. This suggests that these factors are important parts of the

mechanism by which oocyte development is influenced by body growth. Body growth was found to be strongly correlated with the rate of oocyte development with the larger salmon possessing oocytes in a further stage of development than the oocytes in smaller fish. Igf1 and Igf2 expression was higher in the ovaries of the fish with oocytes in the oil droplet stage, which were in the fastest growing fish (Campbell et al. 2006). Thus, the Igf-Gh system appears to mediate nutritional status or the nutrient uptake to the reproductive axis and could play a direct role in controlling fecundity in vertebrate species.

1.10 Rationale

Although adaptive phenotypic plasticity in offspring size has been widely demonstrated in a range of vertebrate species, there are few studies which have evaluated the physiological mechanisms by which this occurs. In *Drosophila*, it has been demonstrated that metabolic hormones play a role in informing the reproductive system about the resource availability in the local environment. Insulin-like growth factor-1 levels directly correlate with the number of ovarioles (reproductive structures) produced, which directly reflects adult fecundity in these flies. In teleosts, specifically zebrafish, *Danio rerio*, orthologues of the *fec* gene in mammals, bone morphogenetic protein 15 and growth differentiation factor 9 have been evaluated but have not been found to play a role in this species (Forbes et al. 2010). Wildtype zebrafish have clearly demonstrated adaptive plasticity in the offspring size versus number trade-off and are a model organism with an entirely sequenced transcriptome. This makes them ideal candidates to investigate potential molecular mechanisms involved in the plasticity of maternal reproductive investment in teleosts.

1.11 Thesis aims

This thesis endeavours to shed light on the patterns of resource allocation in zebrafish when exposed to different resource levels in the environment. It also aimed to elucidate the mechanisms that allow for adaptive plasticity in offspring phenotypes when exposed

to changing environmental resource availability. Specifically, it evaluated changes in fecundity and follicle diameter and related changes in mRNA levels of reproductive and metabolic factors in the ovaries of females exposed to different feeding regimes as outlined below.

1.12 Chapter Overview

Chapter Two: Phenotypic plasticity in offspring size in the zebrafish (*Danio rerio*) – investigating expression of candidate genes from the gonadotropic and somatotropic axes

Specific study aims:

1.12.1 To identify expression of candidate genes in relation to differences seen in fecundity and egg size when female zebrafish are exposed to different resource levels in the environment.

Study approach:

For this Chapter, there were two separate, but similar, experiments carried out. Initially a pilot study was carried out; where six tanks of mixed sex zebrafish were randomly assigned to a low (1 % of body weight daily) or high (3 % of body weight daily) feeding regime. Secondly; Phase one (P1) of the experiment outlined in Chapter three below; 24 female fish, 12 exposed to a low (1.5 % of body weight) feeding regime and 12 exposed to a high (3 %of body weight) feeding regime. Both experiments were analysed for differences in reproductive phenotype. Ovarian tissue from the P1 fish was analysed for differences in relative mRNA transcript abundance of the *follicle-stimulating hormone receptor* (*fshr*), the *growth hormone receptor* (*ghrb*), the *insulin-like growth factor one receptor* (*igf1ra*) the *vitellogenin receptor* (*lrp8*) and the *very low density lipoprotein receptor (vldlr*).

Chapter Three: Effects of changing food environments on the phenotypic plasticity in reproductive resource allocation in the zebrafish, *Danio rerio*

Specific study aims:

1.12.2 To use phenotypic measures of follicle size, and fecundity to identify changes in resource allocation to reproductive traits, when female zebrafish are exposed to fluctuating resource levels in the environment

Study approach:

In this Chapter, the experiment was split into two phases. P1: twelve tanks were randomly assigned to either a low (1.5 % of body weight daily) or high (3 % of body weight daily) feeding regime. After four weeks, two tanks from each regime had six fish terminally sampled. Subsequently, the twelve tanks were randomly assigned to a feeding regime for the second phase. Either they remained on the feeding regime from phase one or switched to the other feeding regime. Reproductive investment and follicle diameter was compared between each treatment to elucidate changes in resource allocation.

Chapter Four: Unravelling molecular mediators of maternal effects – using a transcriptomic approach to reveal changes in gene expression between zebrafish, *Danio rerio*, exposed to different resource environments.

Specific study aims:

1.12.3 To compare the complete transcriptomic snapshots of ovaries of zebrafish, displaying different reproductive resource allocation.

1.12.4 Identify differentially expressed genes and pathways between the treatments

Study approach:

For this study representative samples of whole ovary tissue, three from fish that were exposed to a high feeding treatment (3 % of body weight daily) and three from fish that were exposed to a low feeding treatment (1.5 % of body weight daily), were chosen. HiSeq Illumina sequencing technology and later bioinformatics was used to generate and compare transcriptomes of each ovary.

Originally this analysis was planned to occur before the candidate gene research. Unfortunately, due to time constraints and the length of time the analysis takes, there was not enough time to wait for these samples to come back and the candidate genes described in Chapter Two were instead chosen from the literature.

Chapter Five: General Discussion

This chapter collates, summarises and discusses the key findings of the thesis and wider implications. Future research directions are also highlighted.

The chapters of this thesis were designed to be standalone works, therefore some overlap in material of the introductions may occur. There is some cross-referencing between methods and results sections.

Chapter Two

Phenotypic plasticity in offspring size in the zebrafish (*Danio rerio*) – investigating expression of candidate genes from the gonadotropic and somatotropic axes

2.1 Introduction

It can be argued that an organism's purpose is to survive and reproduce, passing on its genetic material to the next generation. In an optimal environment (i.e. constant) there is a trade-off between offspring size and the number of offspring produced, which maximises the reproductive fitness of the parent (Smith and Fretwell 1974). In reality, any environment is heterogenous and therefore this relationship is also variable. The trade-off is dependent on two factors; the resource availability in the environment, and the maternal space for offspring development. Some species respond to a food-limited or harsh environment by producing fewer offspring that are smaller or of "lower quality" than when in ideal conditions (Bashey 2006). There are other species however, that can change reproductive resource allocation when confronted with different environmental conditions, maximising maternal fitness by increasing offspring survival in harsh environments. In these species, when exposed to an environment which has high levels of food then large numbers of small offspring are produced. If the same species are confronted with a food-limited environment then there is a switch in strategy to producing fewer offspring, but these offspring are relatively much larger (Blueweiss et al. 1978, Fox and Czesak 2000, Bashey 2003, Bashey 2006, Forbes et al. 2010). This phenotypic plasticity in offspring size is seen as a way to maximise reproductive fitness, as in limiting environments, larger larvae have a much higher chance of survival (Bashey 2006, Kuijper and Johnstone 2013). If the maternal environment is correlated with the offspring environment, then plasticity in offspring size is favoured to adjust for changes in food availability in different seasons (Kuijper and Johnstone 2013). Few studies however, have investigated the possible molecular mechanisms that may play a role in this strategy.

The relationship between the nutritional environment and reproductive development has been widely documented. In mammals, studies have demonstrated that the growth hormone - insulin-like growth factor (GH – IGF-I) system has roles in maintaining oocyte development and promoting steroidogenesis (Chandrashekar et al. 2004). In *Drosophila,* changes in the level of insulin-like growth factor 1 (IGF-I) directly influences the number of ovarioles present in the ovary which is a direct indicator of the number of offspring that will be produced (Green and Extavour 2014). There is also evidence from a variety

of fish species that the metabolic pathway (growth hormone - insulin-like growth factor system) plays many important roles in regulating and supporting ovarian development. IGF-I directly promotes ovarian development, both through increasing steroidogenesis and through inducing final maturation in the oocytes (Higuchi et al. 2016). The role that the insulin-like growth factors play in ovarian development seems to be species specific with various effects occurring in different fish species (Higuchi et al. 2016). However, despite this variation, this system seems to play a key role in communicating the nutritional and growth status in fish and in regulating ovarian development accordingly (Higuchi et al. 2016). Therefore, this system is a candidate for the signalling of nutritional status and for mediating size and number of offspring in reproductively plastic species.

Other possible systems that could play a role in determining offspring size include the uptake of vitellogenin, very low-density and low-density lipoprotein into the developing oocytes. Vitellogenesis is the major growth phase of an oocyte and maximum oocyte size is often determined during this stage. In rainbow trout, the sequestration and packing of vitellogenin into the yolk derivatives can account for up to 80% of the final oocyte size (Tyler et al. 1991, Tyler et al. 1997). In eels, low density lipoprotein is also associated with oocyte growth (Damsteegt et al. 2015).

Gametogenesis is controlled by the production of gonadotropins in the pituitary in all vertebrates (Tyler et al. 1997, Mazón et al. 2015). The roles of the two gonadotropins, follicle-stimulating hormone (Fsh) and luteinising hormone (Lh), differ between species. In salmonids and the European sea bass, *Dicentrarchus labrax*, Fsh is the hormone that is mainly responsible for regulation of secondary oocyte growth and vitellogenesis (Tyler et al. 1997, Mazón et al. 2015). Secondary oocyte growth is the stage of most growth in the oocyte, it involves the cortical alveoli stage, and the uptake of both vitellogenin and neutral lipids into the oocyte (Lubzens et al. 2010, Lubzens et al. 2017).

Across all the vertebrate species that exhibit plasticity in offspring size and number, very few studies have looked at the molecular mediators of the change. To gain a better understanding of the possible links between the metabolic and reproductive axes in teleost species, female zebrafish (*Danio rerio*) were subjected to high and low feeding regimes. It has previously been demonstrated that zebrafish exhibit adaptive plasticity in reproductive resource allocation when exposed to different nutritional environments

(Forbes et al. 2010). Females placed on a low feeding ration produced fewer and larger eggs than females that were fed more (Forbes et al. 2010). This study aimed to elucidate changes in the ovarian environment when female zebrafish are exposed to different feeding regimes. To achieve this, the expression levels of messenger RNA in the ovary for five candidate genes were investigated. These included the *follicle stimulating hormone receptor* (*fshr*), the *growth hormone receptor* (*ghrb*), the *insulin-like growth factor one receptor* (*igf1ra*) the *vitellogenin receptor* (*lrp8*) and the *very low density lipoprotein receptor (vldlr*).

2.2 Methods

2.2.1 Husbandry

All zebrafish used in the following experiments were purchased from an aquarium distributor, Brooklands Pet Products, New Plymouth. The fish were shipped overnight using standard tropical fish transporting procedures. Fish were randomly assigned to tanks measuring 40 cm x 40 cm x 70 cm, which were filled to around three quarters of the total volume with purified water. The experiments were carried out in a temperature-controlled facility which was kept at an ambient temperature of 25 °C, with a light cycle of 14 hours light, 10 hours darkness. Each tank contained internal submerged filters which had cycled the water for two weeks before any fish were added, allowing bacterial biofilms to form. Tanks were cleaned and siphoned with accompanying water changes every seven days. All water used in the tanks had been pre-warmed and all chlorine was removed through evaporation. All experiments were carried out in accordance with the guidelines of the University of Otago Animal Ethics Committee.

2.2.2 Experimental Design

2.2.2.1 Experiment 1 -

This was designed to recreate the work done by Forbes et al. (2010). Upon arrival, fish were separated into seven mixed sex tanks, six containing ten fish each and a final tank containing fourteen fish (Figure 2.1). Six tanks were exposed to the experimental feeding treatments whereas the seventh tank was kept on a permanent intermediate feeding ration and males from this tank were used for any spawning trials. This was an attempt to avoid any possible differences in male quality that may occur between the different feeding regimes becoming a confounding factor in the experiment.

Prior to the experimental period, all fish were allowed an acclimation period of seven days, during which time a predetermined amount of food was fed to every tank (roughly 1.5% of total bodyweight (see below), Salmon starter, NRD 5/8, INVE Aquaculture). Following this acclimation all experimental tanks were starved for a day to allow total evacuation of any food present in the digestive tract. Fish were weighed to the nearest mg. This was done by using a tared beaker of water to find the total fish weight of each tank which was then used to calculate the average weight for each tank and an overall average weight (around 1 g per fish). To determine the experimental feeding levels, satiation feeding was carried out. Fish were starved for a day to allow complete evacuation of any previous food. The total amount of food consumed by each tank over the course of five minutes was measured, and the results across all six experimental tanks were used to determine an average maximum feeding amount. These results were used to assign a high feeding regime of 300 mg per tank per day which corresponded to around 3 % of body weight. The low feeding regime was set at a third of this value (100 mg per day) which was equivalent to 1 % of the total bodyweight. Tanks were assigned to treatments using a random number generator (Figure 2.1). The tank containing 14 fish was maintained at an intermediate regime and was fed 160 mg of food per day (1.5% body weight).

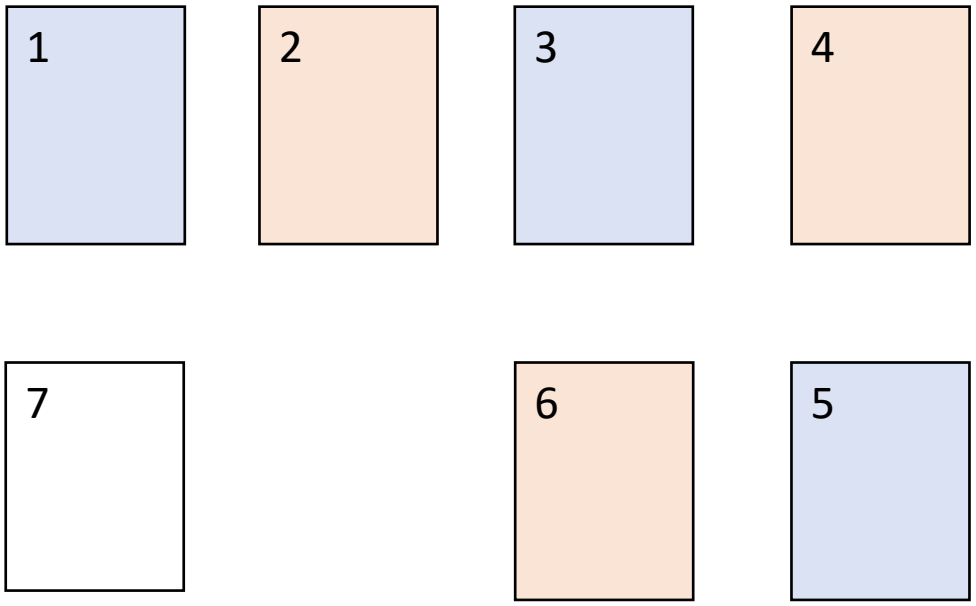

Figure 2.1: The layout of the experimental tanks in Experiment 1, blue colour indicates the low feeding treatment, red indicates the high feeding treatment, white indicates a tank maintained on an intermediate feeding treatment. All tanks contained ten zebrafish, *Danio rerio*, of mixed sex except tank seven which contained fourteen fish.

Feeding was carried out twice per day, at 9 am and 5 pm. In the third week, spawning trials were attempted with females from tank 1 and 2. One gravid female was placed overnight in a standard spawning tank with two males from the intermediate tank (Tank 7). In zebrafish, spawning usually occurs the next morning close to daybreak. If spawning occurred, eggs were collected the next morning and photographed (Section 2.2.4). Terminal sampling was carried out to evaluate differences in gonadosomatic index (GSI), oocyte size and overall ovary composition between the two treatments.

Due to unsuccessful spawning attempts, only two tanks were terminally sampled at the end of three weeks (Tank 1 and Tank 2). The other four tanks remained on the experimental treatment for another four weeks while continuing to carry out breeding trials. These remained unsuccessful and all remaining tanks were terminally sampled after seven weeks total.

2.2.2.2 Experiment 2a - Timing and Plasticity of Fecundity Changes

This experiment is split and analysed across this chapter and Chapter Three. This is due to the results of the initial dissections after four weeks closely relating to the results found in Experiment 1, which assesses the differences between fish subjected to two feeding regimes, high food or low food. These initial dissection results have therefore been titled Experiment 2a. In Chapter Three, I investigate the results obtained after the full 8 weeks of the experiment and explore the changes in plasticity when the feeding regimes are switched. For the purposes of this chapter, Experiment 2a consists of four tanks on two different feeding regimes.

As in Experiment 1, fish were initially fed roughly 1.5% body weight until the experiment began. To determine the experimental feeding doses, satiation feeding over five minutes was used accordingly to the method outlined in Experiment 1. For this experiment, satiation feeding was carried out twice and the average of the two measurements was combined. The high feeding dose was set at a total of 30 mg per fish per day and the low dose was set at half of this value. This corresponds to around 3 and 1.5 % of the average fish body weight. All tanks were fed twice a day, at 9 am and 5 pm.

Table 2.1: Experimental endpoints for the female zebrafish, *Danio rerio*, terminally sampled at the halfway point of Experiment 2a (Phase 1). Fish were kept on one of two feeding regimes; Low: 1.5% of body weight or High: 3% of body weight for four weeks .Non – fish which had completely non-ovulated ovaries. Cohorts taken refers to the separation of a section of the un-ovulated ovary tissue into three distinct follicle size classes for more targeted qPCR analysis.

Tank ID	Treatment	Total number of fish	Spawned	Ovulated	Non	qPCR	Cohorts taken	Transcriptome
A7	High	6	-	4	2	4 ovulated, 1 non	6	yes, 3 ovulated
A8	Low	6	3	-	3	2 non	3	no
A10	Low	6	-	4	2	4 ovulated, 1 non	6	yes, 3 ovulated
A11	High	5	2	-	3	2 non	3	no

After four weeks, six female fish from each tank were placed in a spawning tank containing males from an intermediate tank (one female from each tank per day). Fish were checked at daybreak and if spawned, eggs were collected, counted and yolk diameters measured (Section 2.2.4). Regardless of whether or not spawning occurred, fish were euthanised in a lethal dose of benzocaine and sampled as outlined below. This resulted in three experimental endpoints; fish that spawned eggs, fish that had ovulated eggs in the body cavity and fish that did neither (Table 2.1).

2.2.3 Dissection Protocols

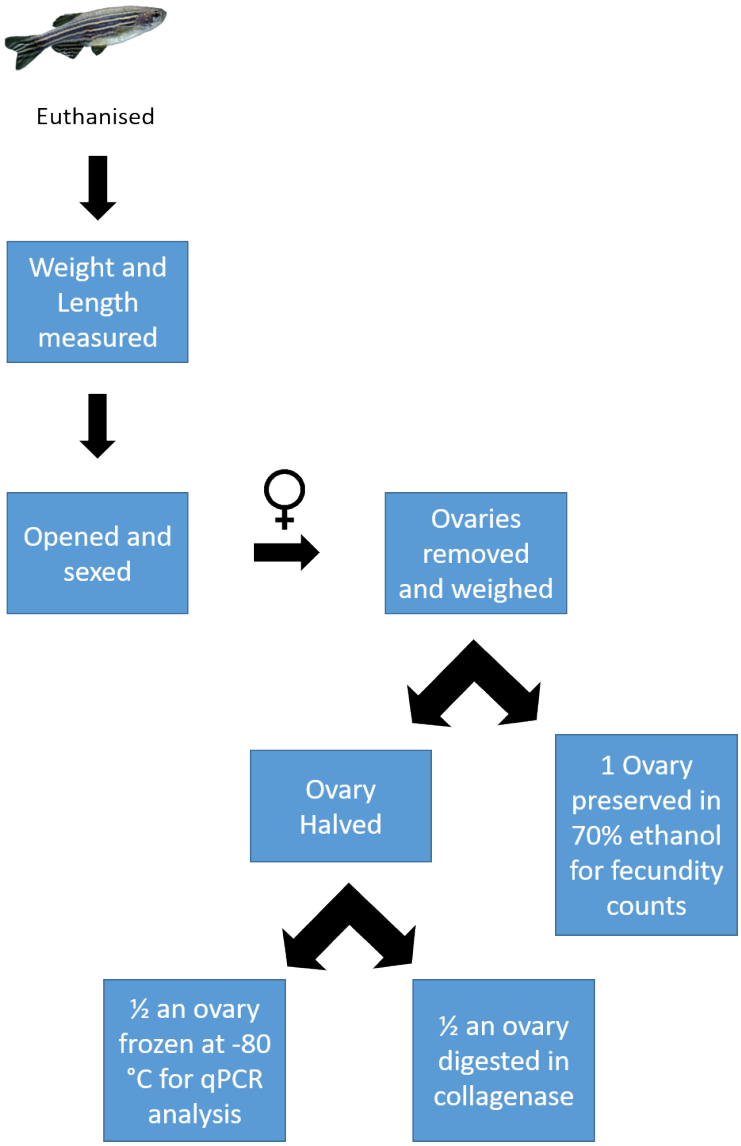

Figure 2.2: Simplified dissection and sampling protocol for all female zebrafish, *Danio rerio*, in Experiment 1.

Fish were individually placed in a beaker containing a lethal dose of benzocaine (1 ml in 200 ml). After fish went limp, they were removed and patted dry with a paper towel. Fish were then measured to the nearest mm using callipers, for both the standard length (from snout tip to beginning of the caudal fin) and the total length (entire fish). Prior to dissecting, total body weight was measured to the nearest mg.

After outside measurements were taken, the spinal column was severed, the body cavity opened, and each fish was sexed by eye. Males were discarded. Ovaries were then removed from females and total ovary weight measured. A single ovary was weighed and preserved in ethanol for total fecundity estimates.

In Experiment 1, for the first three females of each tank the second ovary was cut into two, half was placed in collagenase (1 mg/ml) for digestion. This was used later for size frequency analysis. The second half of the ovary was frozen on dry ice and stored at -80 °C for later qPCR analysis. Any females which were not analysed for size frequency distribution of follicles had the entire second ovary frozen on dry ice and stored at -80 °C for later qPCR analysis. (Figure 2.2)

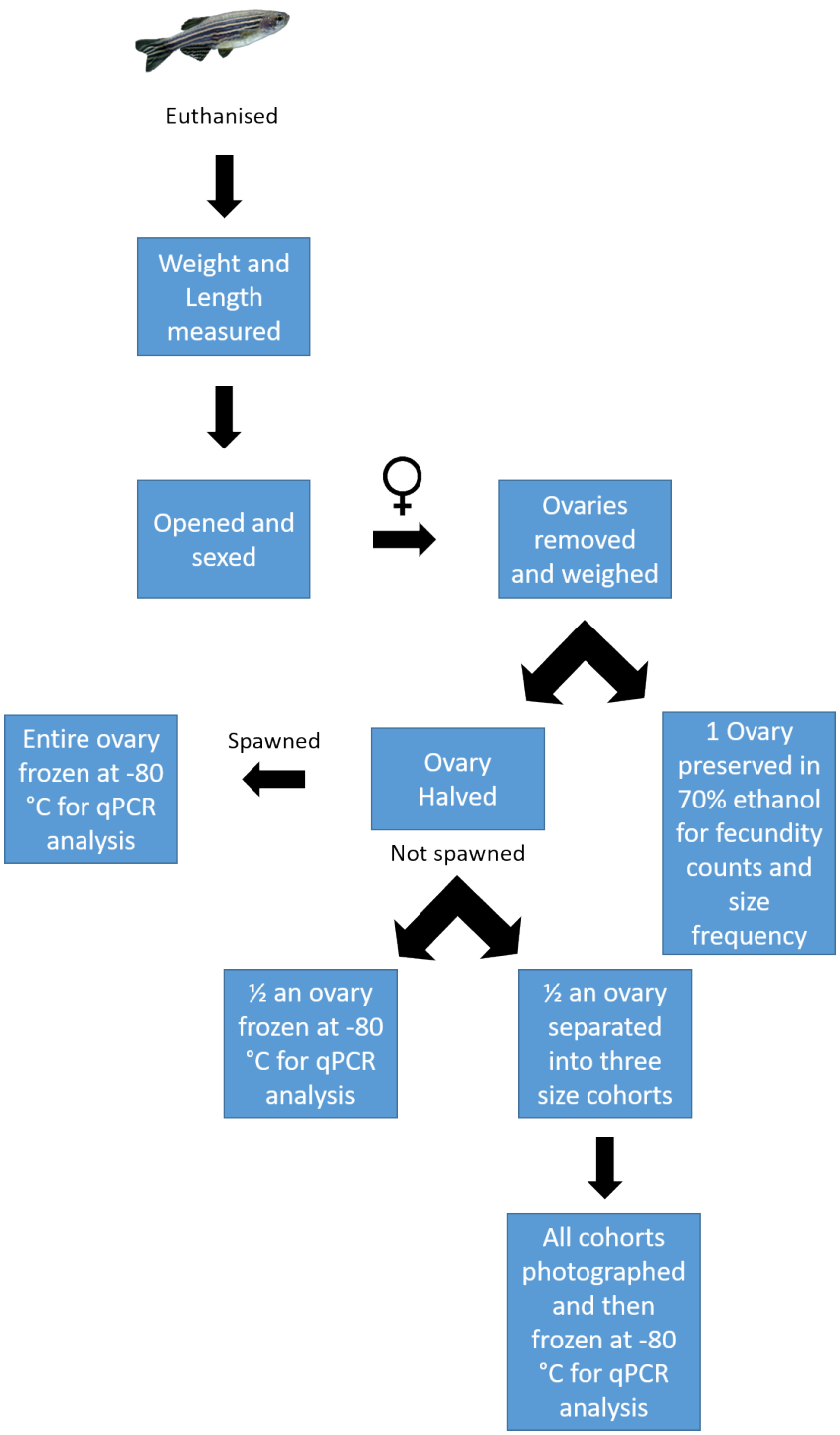

Figure 2.3: Simplified dissection and sampling protocol for all female zebrafish, *Danio rerio*, in Experiment 2a.

For Experiment 2a sampling was carried out as above (Experiment 1), except for the following changes. Collagenase digestion was not carried out. When fish had large mature oocytes, collagenase digestion was too vigorous, resulting in inaccurate representations of the true size frequency distribution of follicles in the ovaries (data in

Appendix – Figure A.1). Instead, after the ovary weights were measured, one ovary was preserved in 70% ethanol and used for both size frequency analysis and for estimation of fecundity. This ovary was gently broken apart, using forceps to preserve the integrity of the follicles.

The second ovary was divided in half, one half was frozen on dry ice and stored at -80 °C for qPCR analysis (in spawned fish this was one whole ovary). The second half of the ovary was then separated into different follicle size classes or cohorts. This was done as zebrafish have an asynchronous ovary containing multiple stages of follicle development at any one time (Selman et al. 1993). This means that all follicles are exposed to the same hormonal environment. Separation into different size classes will illuminate stage specific changes in the mRNA expression levels of the receptors that may be diluted in the whole ovary. The ovary was gently separated into three distinct size classes by eye using forceps, placing different sized follicles into a chilled glass viewing plate to preserve the tissue. All size classes were then photographed for each fish and the cohorts were frozen on dry ice and stored at -80 °C for later qPCR analysis. Only non-ovulated ovary tissue was used for qPCR analysis. (Figure 2.3)

When the cohorts were compared between treatments, there were no differences between average oocyte diameter for any of the different size classes. This meant that there was a direct comparison between follicles of the same size and developmental stage between feeding regimes. (Figure 2.4)

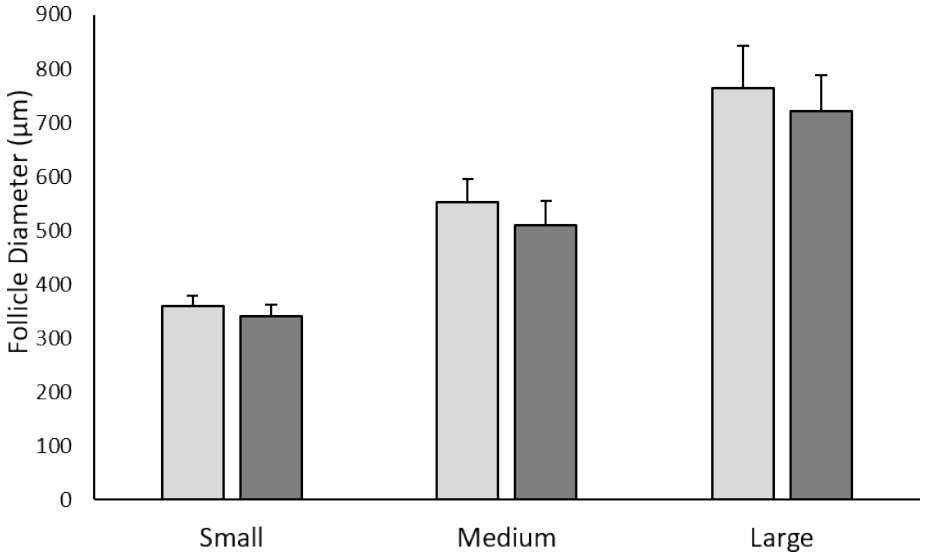

Figure 2.4: The average follicle diameters of zebrafish, *Danio rerio*, when ovaries separated into three separate follicle size classes, Small, Medium and Large. The fish were exposed to two different feeding regimes, low (light grey) which was 1.5% of body weight fed daily, or high (dark grey) which corresponds to 3% of body weight fed daily. N = 5 fish for low and 6 fish for high. Error bars are ± one standard error of the mean.

2.2.4 Macroscopy and Microscopy

All photography of ovary tissue was carried out using an Olympus SZS2 microscope with an Olympus Sc100 camera attached. The Olympus Cellsens program was then used to calculate follicle diameters and numbers of vitellogenic follicles for each ovary sample.

All spawned or ovulated eggs in Experiment 2a were photographed. Yolk diameters and numbers of eggs were counted using the Olympus Cellsens program.

2.2.5 Sample analysis

2.2.5.1 Size Frequency and Total Fecundity

For total fecundity, each ethanol-preserved sample was placed in a petri dish and gently separated using forceps. All follicles with a diameter above 140 µm were counted, as any larger than 140 µm were assumed to have been recruited into vitellogenesis and it is likely they will eventually be spawned (Selman et al. 1993). As these counts were only from one ovary, each value was adjusted to obtain a total follicle count per individual. This was then used as a proxy for the fecundity of each fish. Relative fecundity was

obtained by dividing the total number of vitellogenic follicles by the total body weight of each fish. Gonadosomatic index (GSI) was calculated by dividing the total ovary weight by the total body weight for each individual fish. Total body weight was used as a proxy for the condition of each fish, with heavier fish assumed to have had more access to food resources.

In Experiment 1, three fish from each tank were analysed for size frequency distribution of the follicles in the ovary. Three random separate areas of the digested ovary were photographed under a dissecting microscope. The Olympus Cellsens program was used to obtain average diameters for every visible follicle above 140 µm. To compare "offspring size" between the feeding regimes, the largest 20% of the follicles measured were averaged.

In Experiment 2a, all fish sampled were analysed for the size frequency distribution of follicles in the un-ovulated ovary tissue. Five separate random areas of the ethanol-preserved ovary were photographed under a dissecting microscope. The Olympic Cellsens program was again used, and all follicles with a diameter of 140 µm and above were measured. The ovulated and non-ovulated size frequency distributions were separated before analysis. To evaluate differences in the mean follicle diameters between feeding regimes the largest 20% of the measured follicles were compared.

2.2.5.2 Quantitative Polymerase Chain Reaction

RNA Extraction and cDNA Synthesis

Samples were retrieved from storage at -80 °C for analysis of mRNA expression differences between treatments. RNA was extracted using the TRIzol reagent (Invitrogen) following the protocol designed by the manufacturer. Any genomic DNA was removed through incubation with DNA-free DNase I (Turbo DNA Free Kit, Ambion) for 30 minutes at 37 °C. Quantity of RNA was obtained by using a Nanodrop ND-1000 spectrophotometer (Labtech). All cDNA was synthesised via reverse transcription using the ABI, High Capacity cDNA Reverse Transcription Kit. This was carried out in a 10 µl reaction volume, with 500 ng of RNA and the master-mix (prepared according to the

manufacturer's instructions) added to a 200 µl PCR tube. Tubes were then placed in a PCR machine and subjected to the following program; incubation at 25 °C for 10 minutes, amplification at 37 °C for 120 minutes and heat inactivation at 85 °C for 5 minutes. Any cDNA was then stored at -30 °C until needed for further analysis.

Primers

Five primers were designed to quantify the expression of genes that represent both the metabolic and reproductive pathways: the *follicle stimulating hormone receptor* (*fshr*), the *growth hormone receptor* (*ghrb*), the *insulin-like growth factor one receptor* (*igf1ra*) the *vitellogenin receptor* (*lrp8*) and the *very low density lipoprotein receptor (vldlr*). Two housekeeping genes were also chosen; *β-actin (actb1)* and *eukaryotic translation elongation factor 1 alpha 1a (eef1a1a)*. These were designed to be used to filter out any potential background differences in transcript quantity not caused by the treatment effects. All primers except those for the amplification of *β-actin*, were designed using Primer-BLAST (NCBI). All primer pairs, except *eef1a1a* and *lrp8*, were designed to have at least one primer of the pair crossing an exon-exon boundary. Similar annealing and melting temperatures were also chosen. The primer pair for *actb1* was obtained from a previous study (Tang et al. 2007). The primers were then purchased from Integrated DNA Technologies (Table 1). Each primer was resuspended in distilled water to 100 µM as per the manufacturer's instructions. All primers were further diluted to 10µM before use in the q-PCR assays.

Table 2.2: Specific primer sequences 5'-3' for all genes used for quantitative PCR, *follicle stimulating hormone receptor* (*fshr*), *vitellogenin receptor* (*lrp8*), *insulin-like growth factor 1 receptor* (*igf1ra*), *growth hormone receptor beta* (*ghrb*), *very low-density lipoprotein receptor* (*vldlr*), *β-actin* (*actb1*) and *eukaryotic translation elongation factor 1 alpha 1a* (*eef1a1a*). All q-PCR primers were designed to span intron boundaries.

Gene	Direction	Sequence	Amplicon size
fshr	Forward	TGA CCA ACG CCA CCA TAA CT	179
	Reverse	GGA GAT ATC CAG AAC GAC GGG	
lrp8	Forward	GTG AAG GAG TGC GGA CTG AA	97
	Reverse	AGG GGC ATT GAC ACT CGA AG	
igf1ra	Forward	TGG GAG ATC GCC ACA TTA GC	108
	Reverse	GGG GCA GTT GTC CGG TTT AT	
ghrb	Forward	GCT TCC TAC AAC ACA GGG TCA	129
	Reverse	AGA TCC TCG AGT TCT GTG CG	
vldlr	Forward	AGA AAA ACT GCT CTG CCC GT	123
	Reverse	TGC ACT CTC CAC TTC GAC AC	
actb1	Forward	CGA GCT GTC TTC CCA TCC A	88
	Reverse	TCA CCA ACG TAG CTG TCT TTC TG	
eef1a1a	Forward	CTC CTT CAA GTA CGC CTG GG	195
	Reverse	GCA ACA ATC AGC ACA GCA CA	

Standards

An initial qPCR was carried out on a single sample and the qPCR products were used to create a standard curve for each gene. Using a Nanodrop ND-1000 spectrophotometer (Labtech) the concentration of cDNA was measured in each sample. The cDNA for each gene was diluted to 100 ng/µl and then this was used to create seven standards ranging from 100 pg/µl to 0.0001 pg/µl after a series of 10-fold serial dilutions.

Quantitative real time PCR

Gene expression levels were determined using qPCR for all target genes. All qPCR assays were carried out using a QuantStudio™ 5 – 96 well quantitative PCR machine

(Thermofisher Scientific). All results were analysed using QuantStudio™ Design and Analysis software. All plates were run using the following reaction per well; 1 µl cDNA (10 ng/µl), 5 µl of Takara SYBR Green, 0.5 µl each specific forward and reverse primers and 3 µl of distilled water. All primers annealed at 62 °C.

The following thermal profile was used across all reactions; initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of denaturation (95 °C for 5 seconds), annealing (62 °C for 10 seconds) and extension (72 °C for 5 seconds). In the final step, a dissociation curve analysis was run. This consisted of a 95 °C (60 seconds) denaturation step, a 55 °C (30 seconds) annealing step and then incremental 1 °C temperature increases (30 seconds for each degree) to reach a final temperature of 95 °C (30 seconds). To ensure the specificity of the primers the dissociation curves were analysed for the presence of only one peak of fluorescence. This indicates that only one product has been amplified in each qPCR reaction. For every gene analysed, two no-template-controls (distilled water) and a six-step standard curve were also included on the plate alongside the two replicate wells for each sample. The standard curves were used to quantify the relative abundance of all the unknown samples.

2.2.5.3 Statistical Analysis

All statistical analyses were carried out using R software, version 3.4.2 (R Core Team 2017). Data distributions were checked for assumptions of normality (exploratory boxplots and histograms) and homogeneity of variances (Levene's test). If either assumption was violated then data was log transformed and assumptions were re-checked, all mRNA data were log transformed before analysis.

For Experiment 1, one-way analysis of variance models (ANOVA) were used to evaluate differences in mean follicle diameters, relative fecundities and GSI between feeding regimes.

Experiment 2a, consisted of a nested design, with multiple tanks present in each treatment. The results for this experiment were analysed by nested ANOVA with tank identity as a factor within feeding regime. Any significant interaction effects between

tank and treatment ($p < 0.05$ are reported). A Linear Mixed Effects model with tank as a random effect was used to analyse the relationship between follicle diameter and body weight.

There was high variation in both housekeeping gene expression levels when tested using a nested ANOVA between the two feeding regimes. For *eef1a1a* relative expression levels between the two feeding regimes were significantly different and there was high variation in relative expression seen between individuals within the individual feeding regimes ($F_{3,12} = 4.17$, $p = 0.02$, treatment*tank: $p = 0.05$). The relative expression levels of *actb1* were not significantly different between feeding regimes but there was a trend for an increase in expression in the low treatment and a strong trend for differences between individuals in the same feeding regime ($F_{3,12} = 1.27$, $p=0.31$, treatment*tank: $p = 0.08$). Due to these results relative expression levels of the candidate genes were normalised over total RNA as quantified by Nanodrop.

2.3 Results

2.3.1 Experiment 1:

2.3.1.1 Fecundity and GSI

Relative fecundity was around 0.6 vitellogenic follicles per mg of body weight, regardless of feeding regime (t_4 = -0.49, p = 0.65, Figure 2.5 A). GSI also showed no difference when females on the low feeding regime were compared to females on the high feeding regime, remaining at around 10% (t_{11} = -0.73, p = 0.47, Figure 2.5 B).

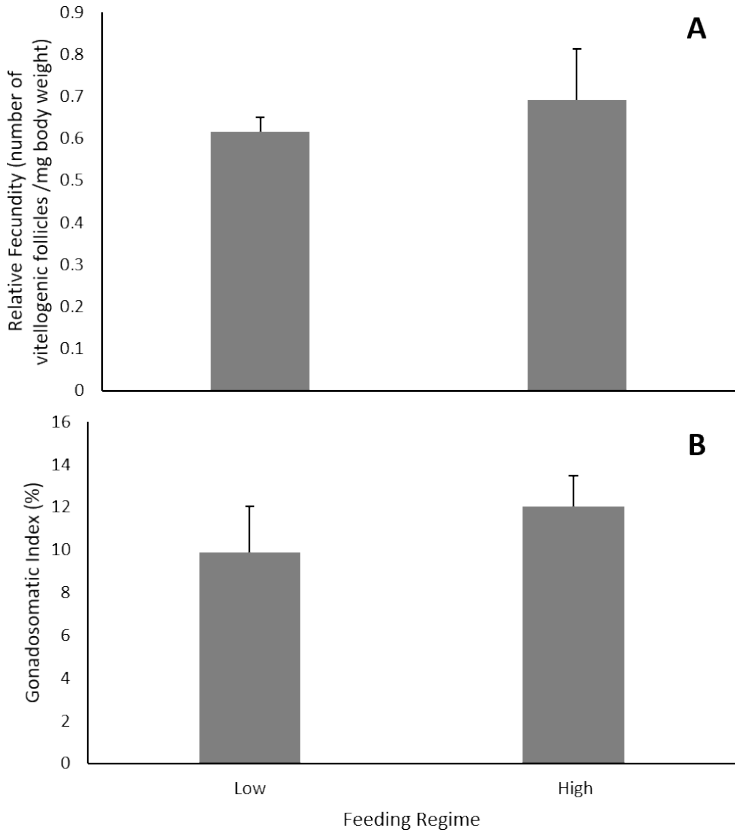

Figure 2.5: The average relative fecundity (A) and the average gonadosomatic index (GSI) (B) in female zebrafish, *Danio rerio*, when fed 1% (Low) or 3% of body weight daily (High) during Experiment 1. *N* (number of fish) = 3 for fecundity and *N* (number of fish) = 6 for GSI, each bar represents the average value for the feeding regime ± 1 standard error.

2.3.1.2 Follicle Diameter

There was no difference in follicle diameter between females exposed to either high feeding or low feeding regimes in Experiment 1 ($F_{1,5}$ = 0.23, p = 0.61, Figure 2.6). When the overall average follicle diameters across all size classes in the ovary were compared, then there was a significant difference found between the two treatments with the females on the high feeding regime appearing to have a lower follicle diameter overall ($F_{1,5}$ = 13.4, p < 0.001). This was due to the large number of smaller follicles present in the ovary, compared with fewer larger follicles (see section 2.3.1.3 Size Frequency).

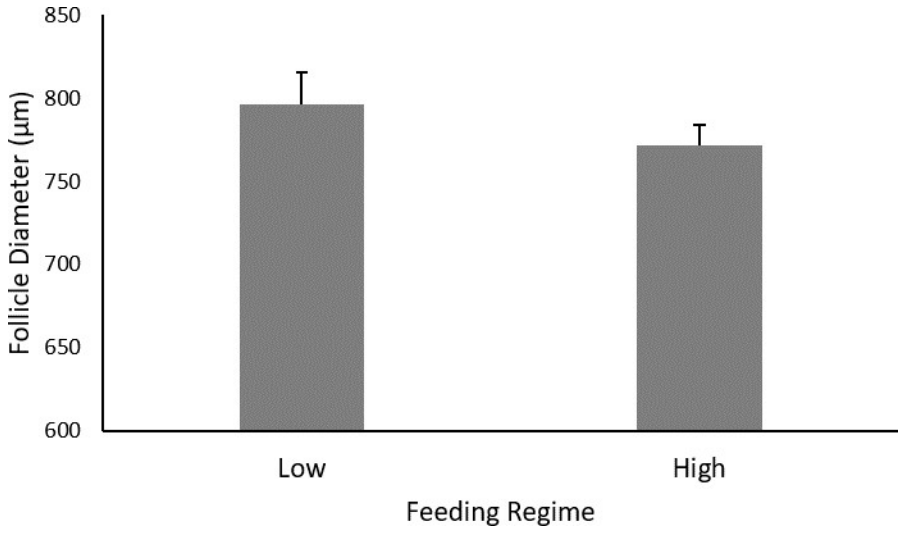

Figure 2.6: The average follicle diameter of the top 20% of the total follicles in the ovaries of female zebrafish, *Danio rerio*, when fed 1% (Low) or 3% of body weight daily (High) during Experiment 1. N (number of fish) = 3 for each feeding regime, each bar represents the average follicle diameter for the feeding regime, error bars are ± one standard error of the mean.

2.3.1.3 Size Frequency

The size frequency distribution of the oocytes in the ovaries of the females on the low feeding regime, showed two distinct clutches of follicles, one centred approximately around 350 µm and another centred approximately around 750 µm. In contrast the distribution from the higher feeding regime had no clear separation into distinct clutches, although there were still small peaks around 300 µm and 750 µm. There was a

higher proportion of oocytes in the larger size range in the ovaries of the females on the lower feeding regime (around 60-70% of all oocytes found between 600 – 800 µm). The higher feeding regime had a more even distribution of follicles present across the size classes, with a slightly higher proportion at the lower end (Figure 2.7).

Unfortunately, due to over-digestion by collagenase, after the second sampling, I was unable to construct an accurate size frequency distribution for the last four tanks. Everything above 500 µm was digested, skewing the results. Therefore, the distribution of the oocytes was only compared between Tank 1 and 2.

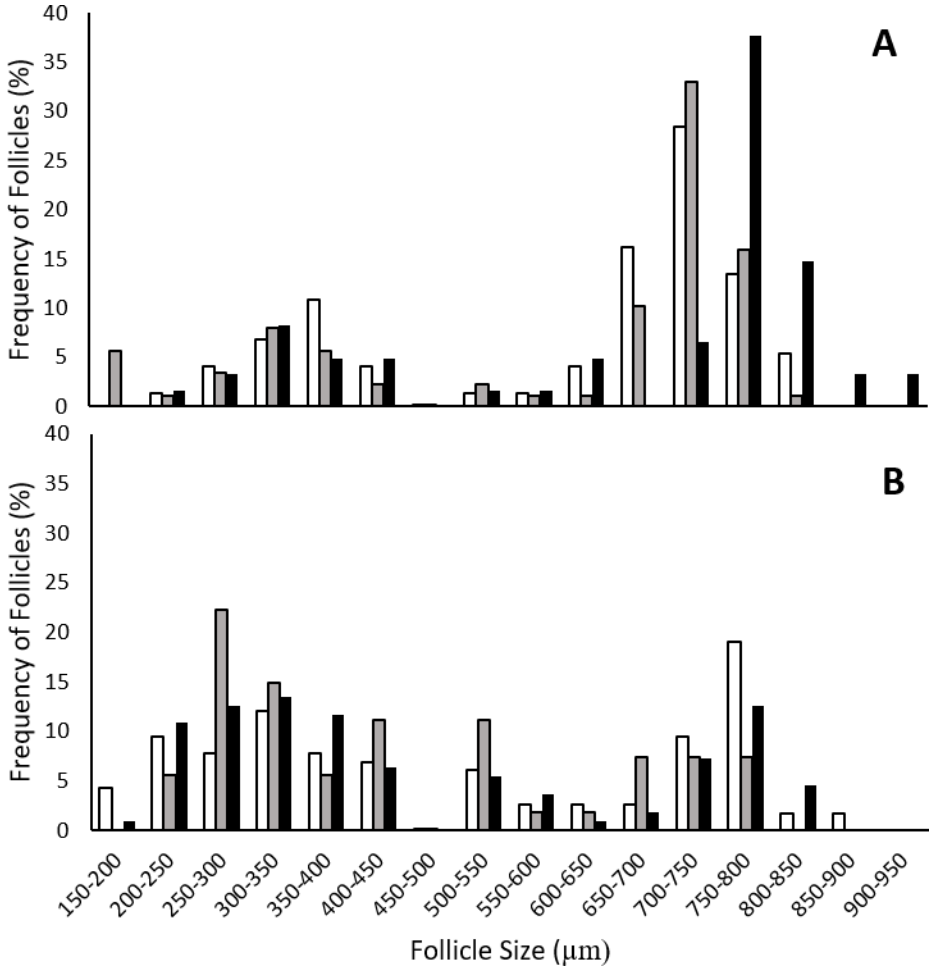

Figure 2.7: The size frequency distribution of oocytes in the ovaries of female zebrafish, *Danio rerio*, when fed 1% (A) or 3% of body weight daily (B) in Experiment 1. Each different coloured size frequency distribution represents the ovary of a different female (three females were sampled in each regime).

2.3.2 Experiment 2a:

2.3.2.1 Fecundity and GSI

In females exposed to the higher feeding regime there was a tendency for an increase in fecundity (1.00 ± 0.23 vitellogenic follicles/ mg body weight) when compared to the females fed lower amounts (0.57 ± 0.17 vitellogenic follicles/ mg body weight) (Figure 2.8 A). This was not significant (Nested ANOVA: $F_{3,6} = 1.45$, $p = 0.27$). GSI was very similar across both feeding regimes at around 13% (Nested ANOVA: $F_{3,6} = 0.10$, $p = 0.76$, Figure 2.8 B).

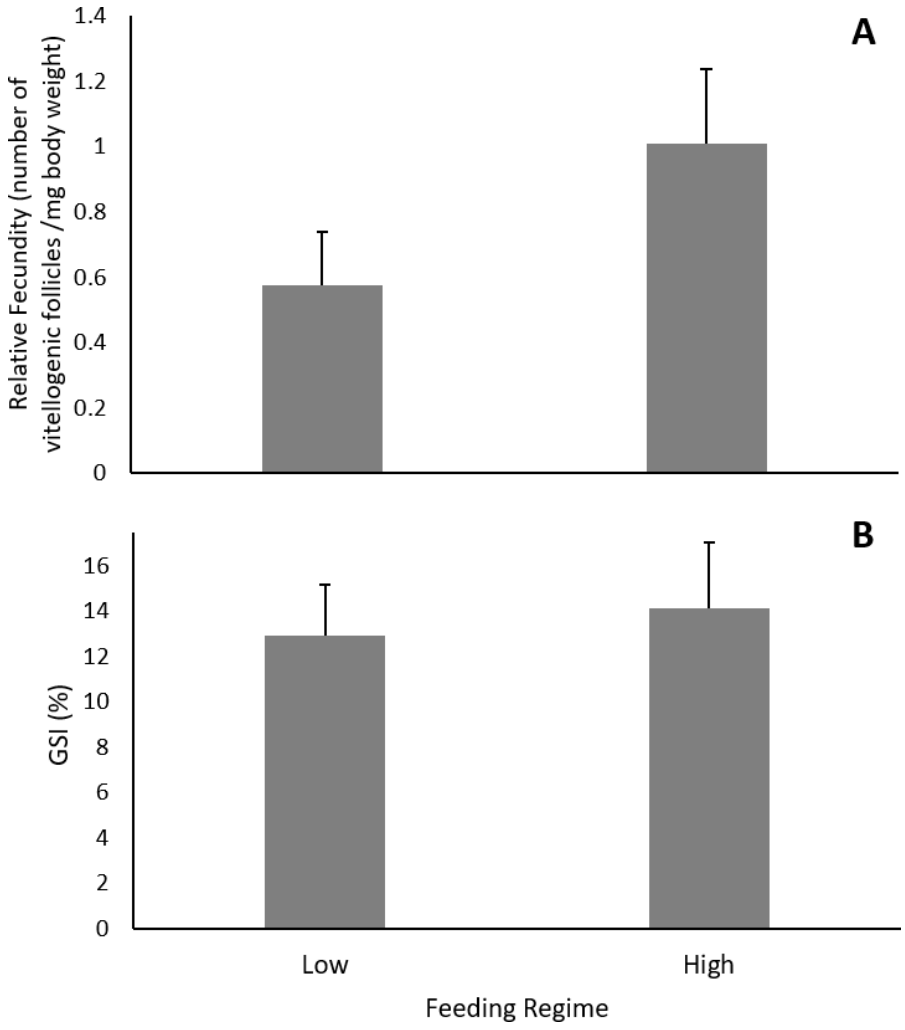

Figure 2.8: The average relative fecundity (A) and the average GSI (B) for non-ovulated female *Danio rerio*, when fed 1.5% (Low) or 3% (High) of bodyweight daily during Experiment 2a, *N*

(number of fish) = 5 for each regime. Bars represent the average value for each feeding regime, error bars are ± one standard error.

When the top 20% largest follicles present in the ovaries were compared between feeding regimes in non-ovulated fish, average follicle diameters were found to be around 760 µm regardless of the amount fed (Nested ANOVA: $F_{3,6} = 0.13$, $p = 0.77$, Figure 2.9 A). There was a tendency towards a decrease in follicle diameter in the remaining ovary tissue in ovulated females fed a higher amount of food when compared to the low feeding regime (Nested ANOVA: $F_{3,5} = 1.93$, $p = 0.31$, Figure 2.9B)

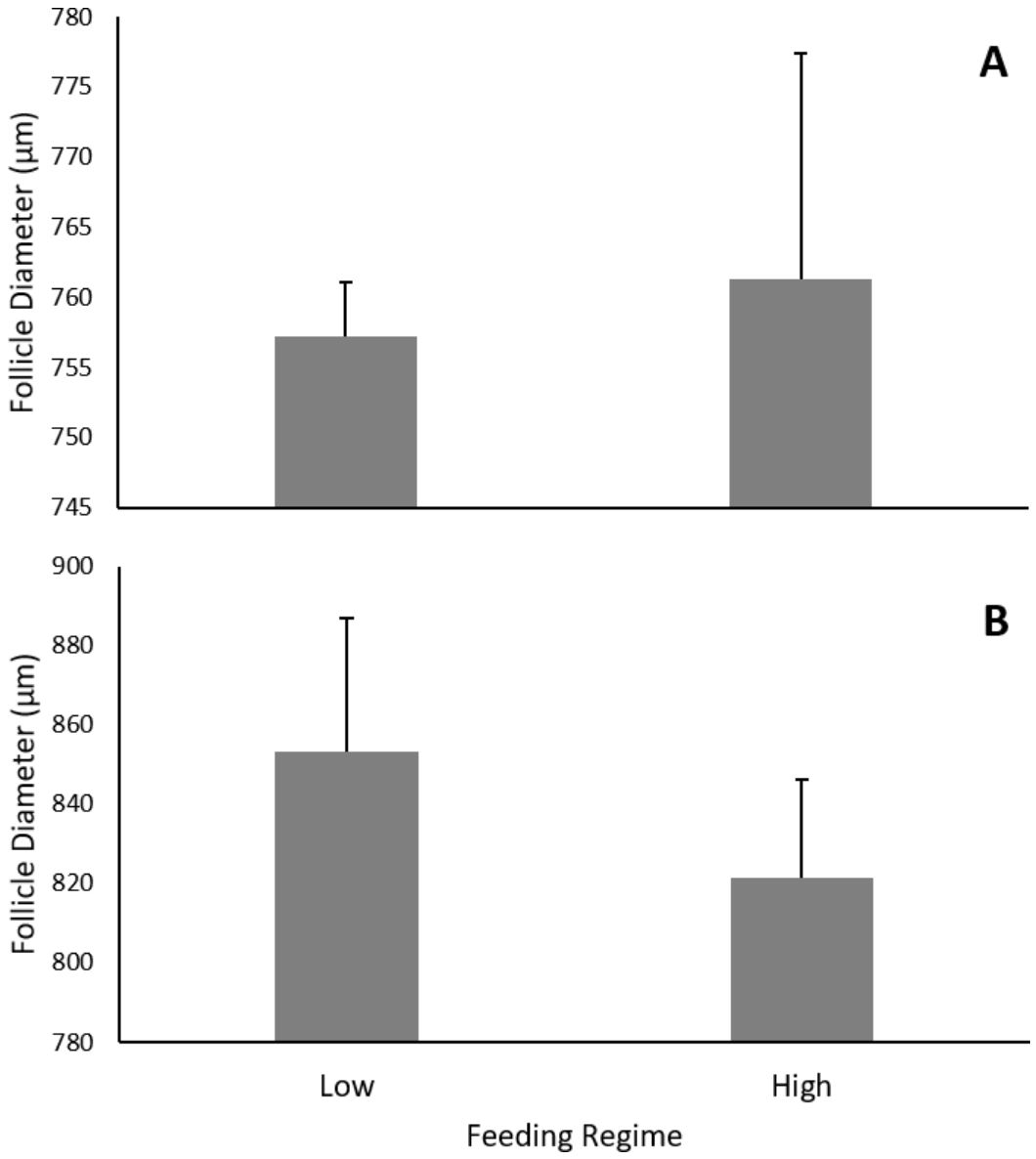

Figure 2.9: The average follicle diameter (µm) of the largest 20 % of follicles present in the ovaries of non-ovulated (A) and ovulated (B) female zebrafish, *Danio rerio*, when fed 1.5% (Low) or 3% (High) of body weight daily during Experiment 2a. For non-ovulated females, n = 5

for each feeding regime. For ovulated females, n = 3 for each feeding regime. The bars represent the average follicle diameter for each feeding regime ± 1 se.

2.3.2.2 Body Weight and Oocyte Diameter

If feeding regime was removed from the analysis, there was an increase in follicle diameter as body weight increased. The larger fish were producing larger oocytes compared to the smaller ones regardless of the amount they were fed (LMER: $p = 0.02$, $t_8 = 5.6$, $R^2 = 0.50$) (Figure 2.10).

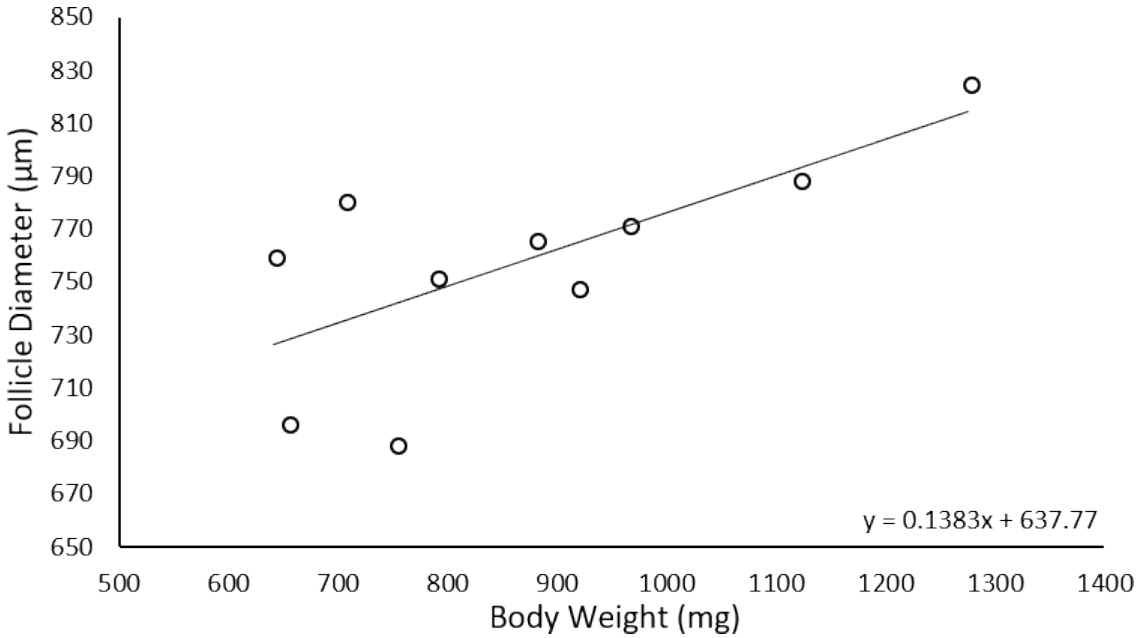

Figure 2.10: The relationship between the average follicle diameter (of the largest 20 % of follicles in the ovary, µm) and the body weight (mg) of individual female zebrafish, *Danio rerio* in Experiment 2a. $R^2 = 0.50$.

Due to the positive correlation between body weight and oocyte diameter, relative oocyte diameters were used to compare between treatments, removing the effect of body condition. There was a tendency towards larger relative follicles in the females which were fed lower amounts of food when compared to females that were fed more. This was true for both ovulated females and the females that did not spawn or ovulate. It was not a significant relationship however ($F_{3,14}$= 2.66, p = 0.12) (Figure 2.11).

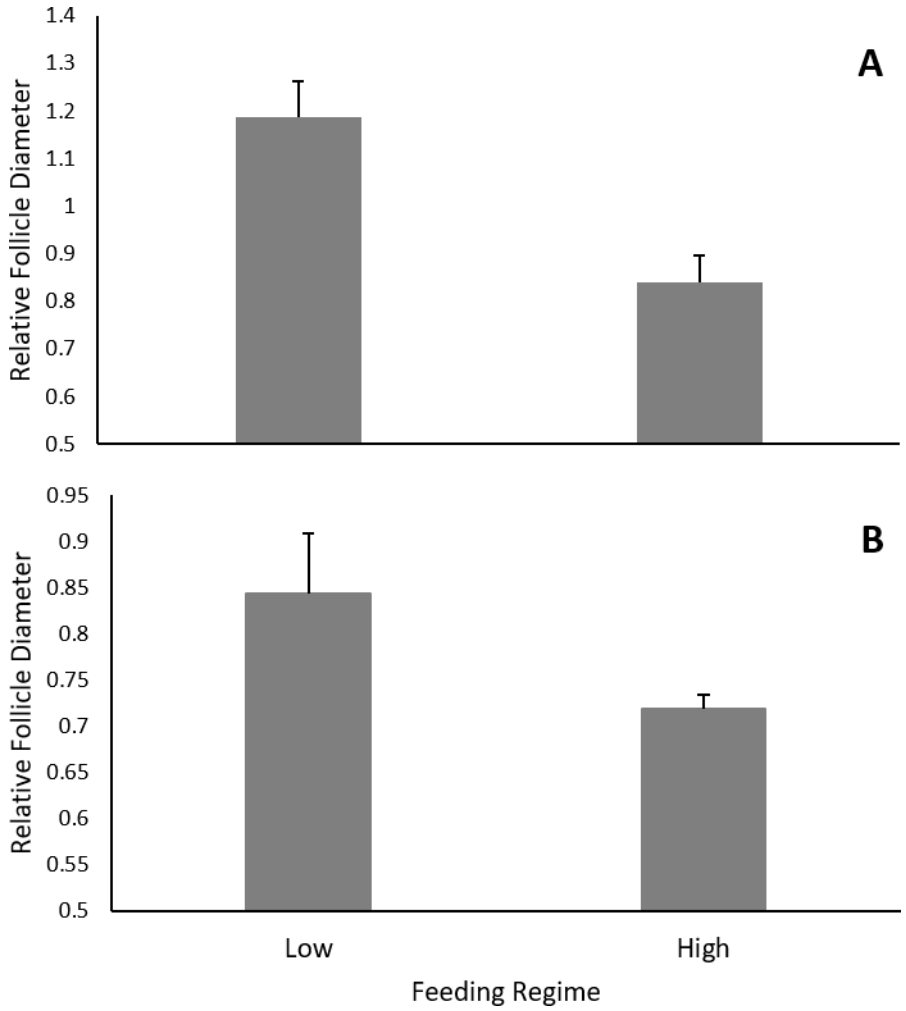

Figure 2.11: The relative follicle diameter (largest 20% (µm) / mg body weight) for female zebrafish, *Danio rerio*, when fed 1.5% (Low) or 3% (High) of bodyweight daily during Experiment 2a. A is females who did not spawn or ovulate, *N* (number of fish) = 5 for both feeding regimes. B is ovulated females, *N* (number of fish) = 3 for both feeding regimes. The bars represent the average relative follicle diameter for each treatment ± 1 standard error.

2.3.2.3 Size Frequency

For Experiment 2a, in both treatment groups when fish neither spawned nor ovulated, there were two distinct clutches of oocytes found in the ovaries. As in Experiment 1, these are centred around 350 µm and 750 µm. However, unlike Experiment 1 there was no clear difference between the treatment groups, with around 60 % of the total oocytes measuring between 140 µm and 540 µm, with the remaining 40 % between 620 µm and 860 µm (A and B). When fish that had spawned were compared between feeding regimes there was also no difference in size frequency distribution. When the ovulated fish were compared, then the females in the low food treatment seemed to have a higher proportion of larger oocytes than the high treatment (C and D), which is consistent with the trends found in Experiment 1. These results are only comparing two tanks and each bar represents a fish, whereas graph A and B are comparing tank averages, variation in the responses is clearly seen between fish even in the same tank (Figure 2.12).

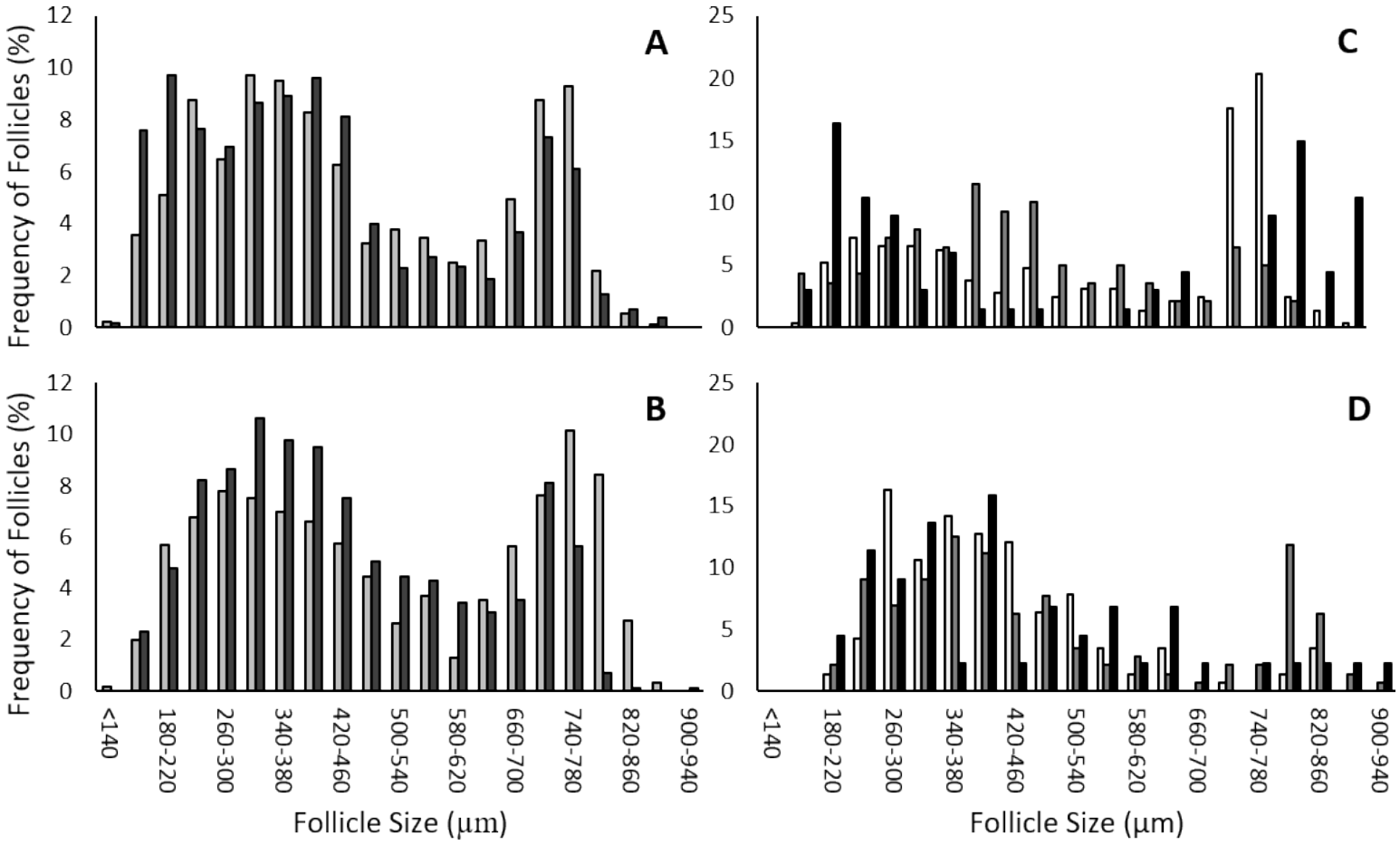

Figure 2.12: The size frequency distribution of follicles in the ovary in female zebrafish when fed a low feeding regime (1.5 % of bodyweight), non-ovulated (A) and ovulated (C) and females fed a high feeding regime (3% of bodyweight), non-ovulated (B) and ovulated (D). *N* (number of fish) = 5 for both non-ovulated graphs (different coloured distributions are tank averages) and *N*= 3 for both ovulated graphs (different bars represent individual fish).

2.3.2.4 Quantitative PCR

Table 2.3: The F and p values obtained through statistical analysis of the differences in ovarian transcript abundance for *follicle stimulating hormone receptor* (*fshr*), *vitellogenin receptor* (*lrp8*), *insulin-like growth factor 1 receptor* (*igf1ra*), *growth hormone receptor beta* (*ghrb*) and *very low-density lipoprotein receptor* (*vldlr*) when female zebrafish, *Danio rerio*, are subjected to two different feeding regimes, low (1.5 % body weight) and high (3 % of body weight). Ovaries were also separated into three different follicle size classes for more targeted analysis (Small, Medium and Large). Black dots represent strong trends ($p < 0.1$)

	Whole Ovary	Small	Medium	Large
fshr	$F_{3,10}$= 0.98 p = 0.35	$F_{3,8}$ = 4.42 p = 0.06 •	$F_{3,9}$ = 3.46 p = 0.09 •	$F_{3,7}$ = 2.30 p = 0.17
igf1a	$F_{3,10}$ = 1.51 p = 0.24	$F_{3,10}$ = 0.15 p = 0.71	$F_{3,10}$ = 0.39 p = 0.55	$F_{3,9}$ = 0.03 p = 0.86
lrp8	$F_{3,10}$ = 3.75 p = 0.08 •	$F_{3,10}$ = 0.04 p = 0.84	$F_{3,10}$ = 0.30 p = 0.60	$F_{3,9}$ = 0.50 p = 0.50
vldlr	$F_{3,10}$ = 0.60 p = 0.46	$F_{3,10}$ = 0.05 p = 0.83	$F_{3,10}$ = 0.34 p = 0.57	$F_{3,9}$ = 2.24 p = 0.17
ghrb	$F_{3,10}$ = 0.06 p = 0.81			

There were no effects of feeding regime on the relative abundance of either *vldlr* transcripts or *igf1ra* transcripts. This was true across the three different follicle size classes as well as in the whole ovary sample. The relative transcript abundance of *ghrb* was also unaffected by feeding regime (Table 2.4, Figure 2.12).

There was also no effect of feeding regime on the transcript abundance of *lrp8* (the vitellogenin receptor) in any of the separated follicle cohorts, small, medium or large. There was a trend for a higher transcript abundance in the low feeding regime ($1.18 \times 10^{-7} \pm 3.7 \times 10^{-8}$) compared to the high feeding regime ($4.44 \times 10^{-8} \pm 1.65 \times 10^{-8}$) when the whole ovary samples were compared ($F_{3,10} = 3.75$, $p = 0.09$).

There was a trend for an increase in the transcript abundance of *fshr* in the high feeding regime (Small: $8.69 \times 10^{-8} \pm 2.69 \times 10^{-9}$, Medium: $6.46 \times 10^{-8} \pm 2.24 \times 10^{-8}$) compared to the low feeding regime (Small: $2.25 \times 10^{-8} \pm 9.2 \times 10^{-9}$, Medium: $2.91 \times 10^{-8} \pm 1.74 \times 10^{-8}$), for both the small and medium cohorts (Small: $F_{3,8} = 4.42$, $p = 0.06$, Medium: $F_{3,9} = 3.46$, $p = 0.09$). There were no differences in transcript abundance between the feeding regimes in the largest oocytes or in the whole-ovary sample comparison. In the whole

ovary samples there was large variation between tanks in the same treatment with a lower transcript abundance of *fshr* found in Tank Seven compared with Tank Eleven ($F_{2,10}$ = 17.04 p-value < 0.01). This was not the case with the small and medium follicle size classes, there were no differences found between the tanks in the same treatment. (Table 2.4, Figure 2.13)

For all genes, there was a decreasing level of relative transcript abundance seen as the follicles matured with the largest expression levels seen in the smallest oocytes (Figure 2.13).

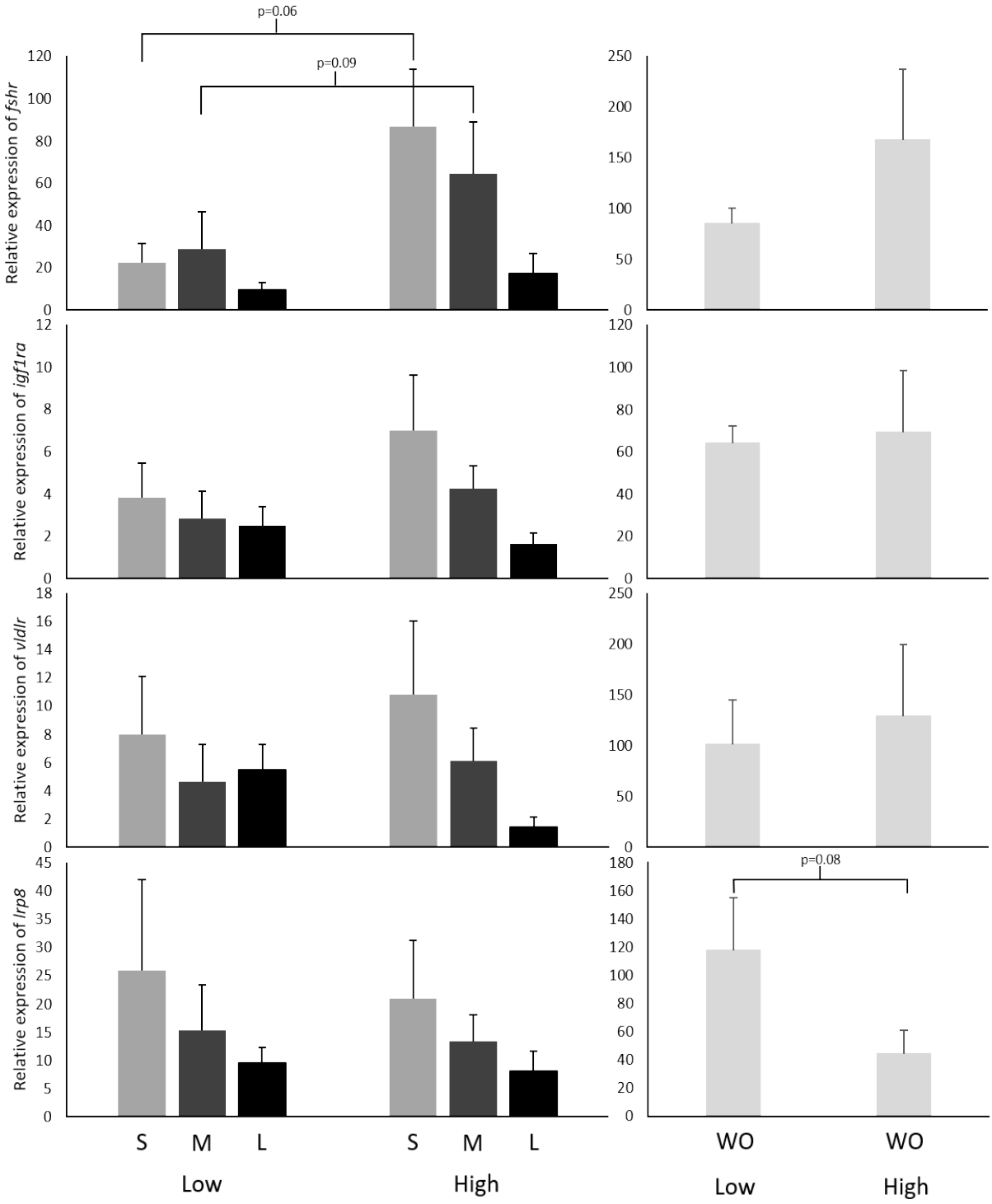

Figure 2.13: Relative expression levels of the target genes; *fshr*, *igf1ra*, *vldlr* and *lrp8* in the ovaries of female zebrafish exposed to high (3% of bodyweight) or low (1.5% of bodyweight) feeding regimes. Ovaries were separated into different cohorts containing different size classes of oocyte, small (S), medium (M) or large (L) (Graphs A, C, E and G). Sections of whole ovary from the same fish were also analysed (Graphs B, D, F and H). *N* (number of fish) = 6 for all low treatments and *N* (number of fish) = 7 for all high treatments. Bars represent the average relative expression levels for each feeding regime ± 1 standard error, strong trends are indicated.

2.4 Discussion

2.4.1 Reproductive Investment and Oocyte Diameter

In Experiment 1, all fish regardless of the feeding regime, had a similar relative gonad weight and number of vitellogenic oocytes present in the ovary. In Experiment 2a, there was also no increase in GSI in fish on the high regime (HF) when compared to fish on the low regime (LF). There was a weak trend for an increase in the number of vitellogenic follicles seen in HF when compared to LF. This was not significant but is supported by previous work carried out by Forbes et al. (2010). My experiment was designed as a pilot study to recreate the work done by Forbes et al. (2010) but did not show the same level of phenotypic plasticity that was found in that study. In Forbes et al (2010), zebrafish were exposed to four different levels of feeding (5.33%, 2.67%, 1.34% and 0.67%) and the two highest treatments had significantly increased fecundity when compared to the two treatments fed lower amounts.

For the original design of the experiment, all fish were meant to spawn as an experimental endpoint to evaluate the differences in egg and offspring size. Unfortunately, there were very low success rates in the spawning trials across all the treatments. When spawned diameters were taken from the females which did spawn in Experiment 2a and females that spawned in a closely related experiment, there was a very strong trend for a decrease in yolk diameter seen in the HF fish compared to the LF fish. This trend was carried through to hatchling lengths as well with smaller offspring hatching from the eggs spawned from HF females when compared to the LF females. These results were not statistically analysed due to low sample size and the combination of experiments (Appendix- Figure A.2 and A.3). This suggested that there was an effect of feeding regime on egg and offspring size in this experiment. This is supported by the study carried out by Forbes et al. (2010), where fish exposed to lower feeding regimes produced larger spawned eggs (0.72 mm, yolk diameter) than fish which were fed higher amounts (0.64 mm yolk diameter). After the failed spawning attempts, to evaluate differences in follicle diameter between feeding treatments the top 20% of the diameters measured for size frequency analysis were analysed for each fish. There was no effect of feeding regime on the average of the top 20% follicle diameters for either experiment. Follicle diameter is therefore potentially not directly comparable to yolk

diameter after spawning has occurred. As zebrafish are asynchronous spawners, taking the top 20% of follicles in the ovary could be mixing different clutches, especially in the LF fish which produce smaller clutches than the HF fish. Therefore, the proxy used in these experiments for offspring size may not be entirely accurate.

A study on killifish (*Nothobranchius furzeri*), has shown that as the fish age/ grow larger there is a lessening (or even complete loss) of the egg size vs fecundity trade-off. They state that this is due to the energetic constraints being higher during periods of rapid growth in early life (Vrtílek and Reichard 2015). The zebrafish used in the current study were already on average 1000 mg when they arrived, in contrast the fish used in by Forbes et al. (2010) were only 500 mg. At larger sizes, there is more space to grow oocytes that are larger and still have a high fecundity. There is potentially a physiological limit on the number of offspring able to be produced at any one time, perhaps related to the number of primordial germ cells. Once this limit is reached, any surplus resources are devoted to egg size rather than number (Vrtílek and Reichard 2015). Also, when there are few resources being redirected towards growth, there is a potential surplus of energy allocated to reproduction, allowing for both large eggs and high numbers of them (Auer 2010, Vrtílek and Reichard 2015).

In Experiment 1, when all the measured oocytes were combined in an average, the LF females had significantly larger oocyte diameters than the HF females. This was due to a larger proportion of oocytes found in the larger size classes of the overall ovary. As zebrafish are asynchronous batch spawners, there are multiple clutches and multiple oocyte sizes present in the ovary (Selman et al. 1993, Gothilf et al. 1997). When the size frequency distribution of follicles in the ovaries were broken down into size classes and compared between the feeding regimes, there was a clear difference in the distribution seen in Experiment 1. In the LF fish there was a higher proportion of oocytes (around 60-70%) present in the larger size classes (around 650 - 850 µm) compared with only around (30-40%) present in the HF fish. This then creates a larger oocyte diameter overall in the LF. This could point to a lower size for maturation in the oocytes present in the HF fish ovaries which results in an overall smaller yolk diameter on average when the eggs are spawned (Crean and Marshall 2009, Forbes et al. 2010).

This difference in the size frequency distributions of the oocytes in the ovaries is not found in Experiment 2a when fish that neither spawned or ovulated are compared. However, when fish had ovulated the largest clutch into the body cavity the remaining oocytes in the ovary followed a similar trend to Experiment 1. This could be due to higher recruitment into the secondary growth phase in the HF fish, resulting in larger numbers of oocytes coming through the ovary in each clutch.

In conclusion, neither experiment indicated adaptive phenotypic plasticity in female zebrafish when they are exposed to different feeding regimes. There was no increase in offspring size found under food-limited conditions, although the proxy for offspring size may not have been entirely accurate.

2.4.2 Molecular Mediators

In both the smallest and medium cohorts of oocytes, previtellogenic or vitellogenic (based on size 250 – 500 µm (Selman et al. 1993)), there was a strong trend for an increase in relative transcript abundance of *fshr* in the ovaries of HF females. Follicle stimulating hormone (Fsh) plays key roles in the early development of oocytes and mediates recruitment into vitellogenesis. When rainbow trout, *Oncorhynchus mykiss*, were subjected to a unilateral ovariectomy during early vitellogenesis, an increase in the plasma concentration of Fsh occurred when compared to control fish that had both ovaries (Tyler et al. 1997). This increase in plasma Fsh coincided with recruitment of a secondary batch of primary oocytes into vitellogenesis indicating that Fsh plays a direct role in mediating whether oocytes enter the secondary growth phase (Tyler et al. 1997). A similar pattern of increased fecundity has been demonstrated in sheep, with mutations in bone-morphogenetic protein 15 (BMP-15) and growth differentiation factor 9 (GDF-9) causing an increased sensitivity to FSH in the developing follicles. This increased sensitivity causes earlier development into preovulatory follicles, which increased the overall number of follicles ovulated (Moore et al. 2004, Moore and Shimasaki 2005).

In synchronous spawning fish, such as salmonids, it has been widely demonstrated that Fsh is the main gonadotropin throughout vitellogenesis and early oocyte development

before declining and Lh induces maturation and therefore the levels of Lh only increase later in development. (Prat et al. 1996, Tyler et al. 1997, Breton et al. 1998, Prat et al. 1998, Yaron et al. 2003). In asynchronous spawners, including zebrafish, there is a much less distinct hormonal profile, both Fsh and Lh are present simultaneously in the ovary. This is due to there being multiple clutches of oocytes at different developmental levels (Gothilf et al. 1997, Jackson et al. 1999). Therefore, regulation of the individual oocytes is likely to occur at the receptor level (Chu et al. 2015, Zhang et al. 2015). Gene knockout studies in zebrafish have demonstrated that without *fshr* expression oocytes do not develop past the primary growth stage (previtellogenic) (Chu et al. 2015, Zhang et al. 2015, Li and Cheng 2018). As there is a higher abundance of *fshr* transcripts found in the previtellogenic and early vitellogenic follicles in the HF fish, changes in the receptor could be a mechanism for a change in fecundity. The increase in *fshr* transcript expression could correspond to larger numbers of primary follicles being recruited into vitellogenesis in each clutch, resulting in more follicles per clutch overall (Campbell et al. 2006, Chu et al. 2015). As maternal ovary space is a limiting factor in both the size and number of follicles produced, this increase in the number of follicles per clutch could result in a less room and smaller follicles overall (Uller and Olsson 2005). This is demonstrated in the size frequency distribution profiles of the different feeding regimes. Females on the LF regime have a much larger proportion of follicles in the larger size classes, possibly reflecting smaller clutch sizes, whereas the fish on the HF regime have higher levels of follicles present in the early vitellogenic stages.

In multiple oviparous species, *vtgr* expression is found to be highest in previtellogenic oocytes and expression decreasies as development progresses until it is barely detectable once they have entered vitellogenesis (Bujo et al. 1995, Prat et al. 1998, Hiramatsu et al. 2004, Mizuta et al. 2013). Conversely, the Vtgr protein is predominantly found in the periphery of vitellogenic oocytes. This suggests that the main de novo *vtgr* transcription occurs during pre-vitellogenesis and the protein is continuously recycled during vitellogenesis (Mizuta et al. 2013, Damsteegt et al. 2015). In my study, there was an increase in *lrp8* transcript abundance in the whole ovaries of the female on the LF regime when compared to the females on the HF regime. Lrp8 has been designated as the main vitellogenin receptor in teleosts (Prat et al. 1998, Hiramatsu et al. 2004, Mizuta

et al. 2013). These differences in gene expression between the feeding regimes were not seen in the size-separated follicle samples. Even the smallest follicles separated from the ovaries were above 300 µm, meaning that they were all at least early vitellogenic (Selman et al. 1993). As stated above, *lrp8* is only expressed in previtellogenic oocytes, therefore the increased trend was only able to be discerned in the whole ovary sample. In future studies, better separation of the ovary into smaller cohorts even lower than 300 µm may be able to further elucidate any changes in *lrp8* expression between treatments as then the previtellogenic oocytes can be analysed. The increase seen in the mRNA expression of the *lrp8* receptor in LF females could correlate with an increased vitellogenin uptake into the follicles, increasing oocyte size overall. Previous studies have shown that when adaptive plasticity in egg size is present, larger eggs have higher amounts of proteins and lipids in the yolk (Williams 2001, Hassall et al. 2006, Chen et al. 2015).

These experiments provided no link between the metabolic hormone pathway and the adaptive plasticity in follicle size found in zebrafish. This is regardless of previous literature clearly showing a clear relationship between nutritional environment and fecundity in many fish species (Le Gac et al. 1993, Kagawa et al. 1994, Kagawa et al. 1995, Maestro et al. 1997, Patino and Kagawa 1999, Campbell et al. 2006). The trend for an increase in the vitellogenin receptor suggests that there could be regulation at the level of the liver. Vitellogenin is primarily synthesised in the liver and production is mediated by circulating estrogens (Peyon et al. 1996, Peyon et al. 1998, Prat et al. 1998, Hiramatsu et al. 2015). Previous studies on the European eel found that level of vitellogenin produced by hepatocytes was increased when growth hormone (GH) was administered alongside 17-beta estradiol (Peyon et al. 1996). Future studies evaluating vitellogenin expression in the liver between different feeding regimes could be valuable.

Expression levels of all the target genes regardless of feeding regime showed a decrease in relative transcript abundance as the follicle size increased. This is likely due to a dilution of the mRNA population occurring as the oocytes grow. As an oocyte develops maternal mRNA and ribosomal RNA accumulates in the cytoplasm, diluting the overall RNA population (Selman et al. 1993, Lubzens et al. 2010). This trend further complicates

comparison of gene expression between feeding regimes, as the relative expression level of each gene will change as the oocyte grows.

Conclusions

There was no evidence for phenotypic plasticity in offspring size in female zebrafish when exposed to two different feeding regimes, low (1.5 % of body weight daily) and high (3 % of body weight daily). There was a trend for higher fecundity in female fish kept on the high feeding regime compared to the females fed lower amounts.

Although there was no support for adaptive plasticity in offspring size found, there were strong trends in the relative abundance of *fshr* and *lrp8*. This suggests these could play a key role in determining the offspring size and number in zebrafish, but future studies are needed to evaluate the true nature of this relationship.

Chapter Three:

Effects of changing food environments on the phenotypic plasticity in reproductive resource allocation in the zebrafish, *Danio rerio*

3.1 Introduction

The energy and resources available to any given organism are finite and must be allocated between the different activities the organism undertakes to complete its lifecycle. These activities can be broadly divided into growth, reproduction and survival and an organism must "decide" how to allocate resources to each activity. The availability of resources and how they are allocated to reproduction will ultimately determine reproductive success (Auer 2010, Burton et al. 2010).

Due to the finite nature of resources available to a mother, maternal provisioning of one component of reproduction must reduce the resources available to another (Auer 2010, Burton et al. 2010, Lim et al. 2014). Many studies have examined the "trade-off" between offspring size and the number of offspring produced and due to the fact that offspring size and number are competing for a limited pool of resources, a negative relationship has been expected (Smith and Fretwell 1974, Lim et al. 2014). Classical theory predicts that a mother is constrained by the optimal offspring size for the local environment and the trade-off between offspring size and offspring number will reflect this. In high quality environments, a mother will produce large numbers of small offspring. Conversely, in low quality environments a mother will produce fewer numbers of larger offspring (Smith and Fretwell 1974). Given that environments will vary over the course of a mother's lifetime, mothers must rely on external cues to determine the optimal offspring phenotype which will maximise maternal fitness.

In a constant environment, a mother has long-term external cues available that can help to accurately predict an optimal offspring phenotype. In environments that vary unpredictably, any one "optimal" offspring phenotype is unlikely to perform well in every situation and in these cases, it is predicted that a mother will "hedge her bets" by producing a range of offspring sizes. This strategy allows her to maintain reproductive fitness regardless of the environmental conditions of the offspring (Crean and Marshall 2009).

Phenotypic plasticity is defined as a single genotype having the ability to produce alternate phenotypes depending on environmental cues (Nylin and Gotthard 1998, Pigliucci et al. 2006). Adaptive plasticity is demonstrated when the changes in

phenotype between different environments have a measurable adaptive advantage to the organism. Phenotypic plasticity can be expressed at multiple different levels; behavioural, biochemical, physiological and developmental. While biochemical and physiological plasticity can be reversed or changed over relatively short time scales, developmental plasticity takes longer to be reversed or can even be irreversible (Pigliucci et al. 2006). Phenotypic plasticity can be a mechanism that allows a single organism to maximise fitness in an uncertain environment, or allows an entire species to thrive in a variety of habitats (Vrtílek and Reichard 2015). It is easy to state that environment can effect changes on variables related to life history, but it is harder to demonstrate that these changes are adaptive. To determine whether any phenotypic plasticity is adaptive, a clear enhancement of an organism's fitness needs to be demonstrated.

If a mother can predict an unfavourable offspring environment and alters the resulting offspring phenotype in response, i.e. producing larger eggs, then she can increase her fitness through greater offspring survival. The phenotypic plasticity of the mother is likely to be physiological, with changes occurring at a metabolic or hormonal level, directly changing the number or size of the offspring produced. The phenotypic plasticity of the offspring is developmental, as changing the size of the egg will change the initial size of the offspring. Larger eggs ultimately mean larger initial offspring size. The growth rate of all organisms is affected by temperature and food availability and studies have linked changes in temperature and food availability to changes in the size of the offspring produced (Fischer et al. 2003). In pelagic fish species, eggs produced in winter are larger than eggs produced by the same species in spring and summer when conditions are more favourable for larval growth (Ware 1975).

A female's sensitivity to environmental changes depends on how the overall resources are allocated to reproduction. If clutches are provisioned from stored reserves, then there will be limited response to changing resource availability in the short term. If oocytes are directly provisioned from resources obtained straight from the environment then the system is likely to be highly sensitive to environmental change (Reznick and Yang 1993).

Offspring phenotypes are variable at all levels: among different species, different populations within species, different females within a population, between different clutches of oocytes in a single female and even between different oocytes within the same clutch. Previously, it has been demonstrated that zebrafish (*Danio rerio*) exhibit adaptive plasticity in offspring size, producing larger offspring in food-limited environments (Forbes et al. 2010). To investigate the sensitivity of this adaptive response, female zebrafish were exposed to changing food conditions, through experimentally switching feeding regimes. To investigate when in oogenesis decisions are made about offspring size and fecundity, changing feeding regimes allowed for assessment of any changes in patterns of resource allocation. Follicle diameters were compared between females exposed to a short period (phase) of high or low feeding regimes, a long period of high or low feeding regimes (constant) or changing feeding regimes. The trade-off between follicle size and reproductive investment was also assessed.

3.2 Methods

3.2.1 Husbandry

All non-experimental parameters were kept as described in Section **2.2.1** in Chapter Two. The wildtype zebrafish, used in this experiment were also obtained from Brooklands Pet Products, New Plymouth and shipped overnight using standard tropical fish shipping procedures.

For this experiment 180 mixed sex zebrafish of around 4 cm were ordered, and once they had arrived were divided between 12 tanks at random with 15 fish per tank. Unfortunately, over the ensuing week there was a high level of mortality (50 fish died) and to maintain sample size another 140 fish were ordered as replacement stock. This resulted in a final total of 190 zebrafish distributed between the 12 experimental tanks. Fish were randomly separated into tanks, four containing the original fish (Cohort 1, 13 fish per tank), and eight containing the new group of fish (Cohort 2, 17 fish per tank). To avoid differences between the different age groups all fish were kept on a feeding regime of around 12 mg per fish (roughly 1.5% bodyweight) until fish reached an average

body weight of 1 g in all tanks. Fish were weighed weekly using a tared beaker filled with water until the desired weight was reached.

3.2.2 Experimental Design:

For the entirety of the experiment, fish were separated between two feeding regimes, one with a high ration (H) and one with a low ration (L). As in the previous experiment (Section 2.2.2.1, Chapter Two), satiation feeding was used to determine a maximum feeding dose. In this case, two satiation feedings were carried out and then the maximum dose (H) was taken as an average of both. For this experiment H was set at 30 mg per fish per day (around 3% of bodyweight) and L was set at 15 mg per fish per day (1.5% of bodyweight). Feeding was carried out twice a day at around 9 am and 5 pm with the overall daily amount split over the two feedings. All uneaten food was siphoned out to maintain water quality and 25% water changes were carried out every seven days.

This experiment (Experiment 2, part of which is described in Chapter 2, Section 2.2.2.2), was separated into two phases; phase 1 (P1) and phase 2 (P2). During P1, the twelve tanks were divided randomly between the high or low regime and remained on these for three weeks. At the three-week mark, four tanks were randomly chosen (from the tanks containing the younger fish, two per regime) to have six females terminally sampled (Low: L-0, High: H-0). To create a 'naïve' ovary without mature follicles that represent the previous feeding history, females were spawned over the period of a week, one female per tank per day. A female was randomly selected from each tank and placed overnight in a spawning tank containing a male fish. In the morning, the female was euthanised in a lethal dose of benzocaine and sampled according to the protocols explained in the previous chapter (Figure 3.2, Chapter Two, Section 2.2.3). Any spawned eggs were photographed for later diameter and fecundity analysis, using an Olympus SZS2 dissecting microscope with an Olympus SC100 camera attached. Females were euthanised whether they had spawned or not. At the end of this week all tanks were assigned to the second phase of treatment. The majority of the P1 data is analysed in Chapter 2 (Section 2.3.2, Experiment 2), the 8 tanks that were not sampled after P1 were

still split evenly between the high and low feeding regimes during P1. When presenting data from P1 in this chapter fish are referred to as either L-0 or H-0.

During P2, tanks either remained on the same feeding regime as in the first four weeks or were switched to the other feeding regime. This resulted in a total of four treatments (Low – Low: L-L, Low – High: L-H, High – High: H-H and High – Low: H-L) as well as the earlier sampled fish (P1: Low: L-0 and High: H-0). The second phase continued for another four weeks and then all females were spawned and terminally sampled as in P1. When sampling was carried out after P2 it was discovered that there were no remaining females in Tank 8 after the sampling for P1 (Table 3.1). This means that there were only two tanks with females present in treatment L-L. (Figure 3.1)

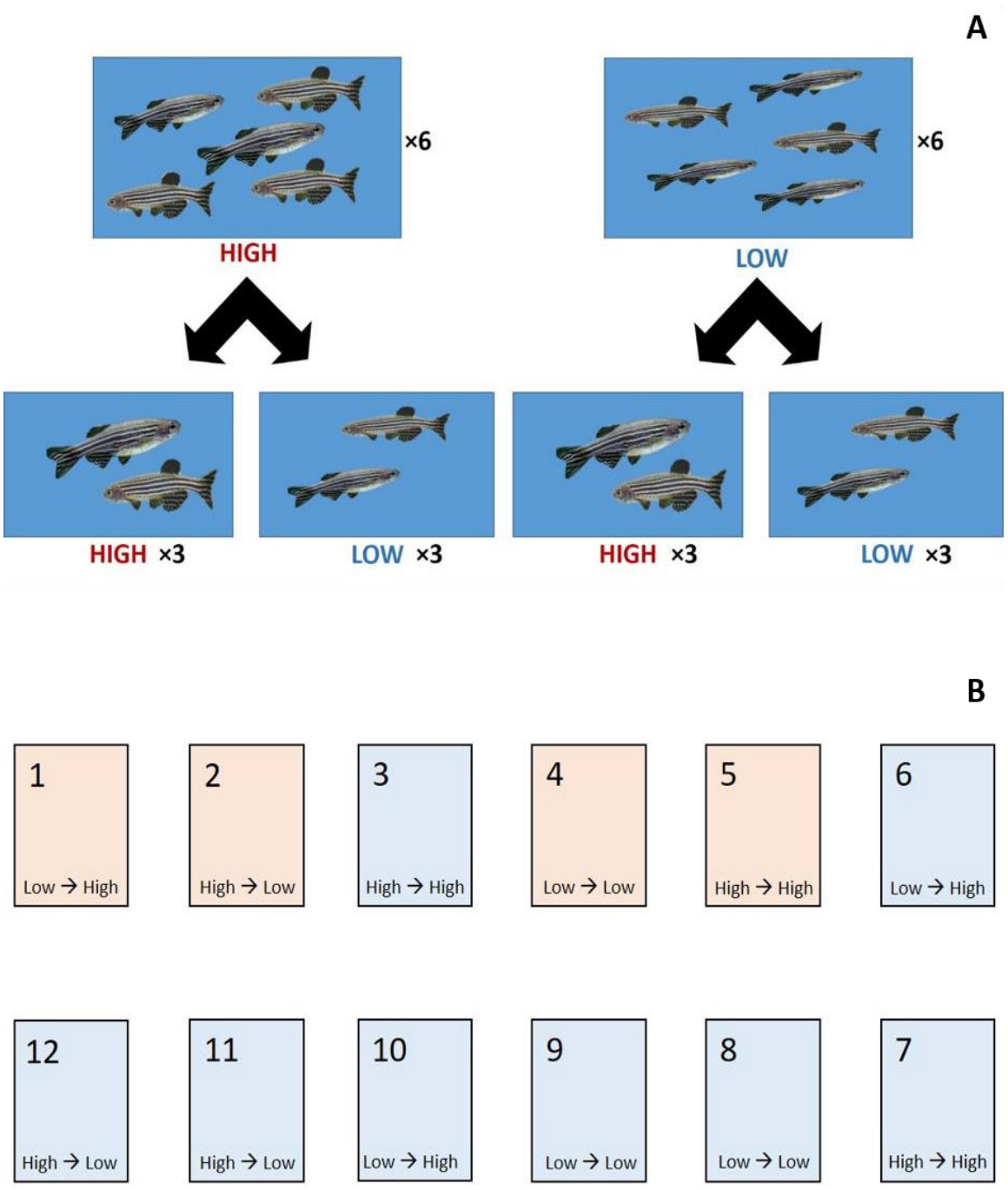

Figure 3.1: Experimental design for Experiment Two. Twelve tanks containing female zebrafish, *Danio rerio*, were placed on two different feeding regimes (high and low, Phase one), then for Phase two each feeding regime was split again producing four total treatments. Each phase lasted four weeks (A). The layout of the tanks in Experiment Two, all tanks were randomly assigned to treatments, orange tanks correspond to the tanks containing Cohort 1, treatment is outlined at the bottom of each tank (B).

3.2.3 Dissections

All dissections were carried out according to the protocols outlined in Chapter Two, Section 2.2.3 – 2.2.4.1. For reference the sampling protocol is outlined in brief in Figure 3.2.

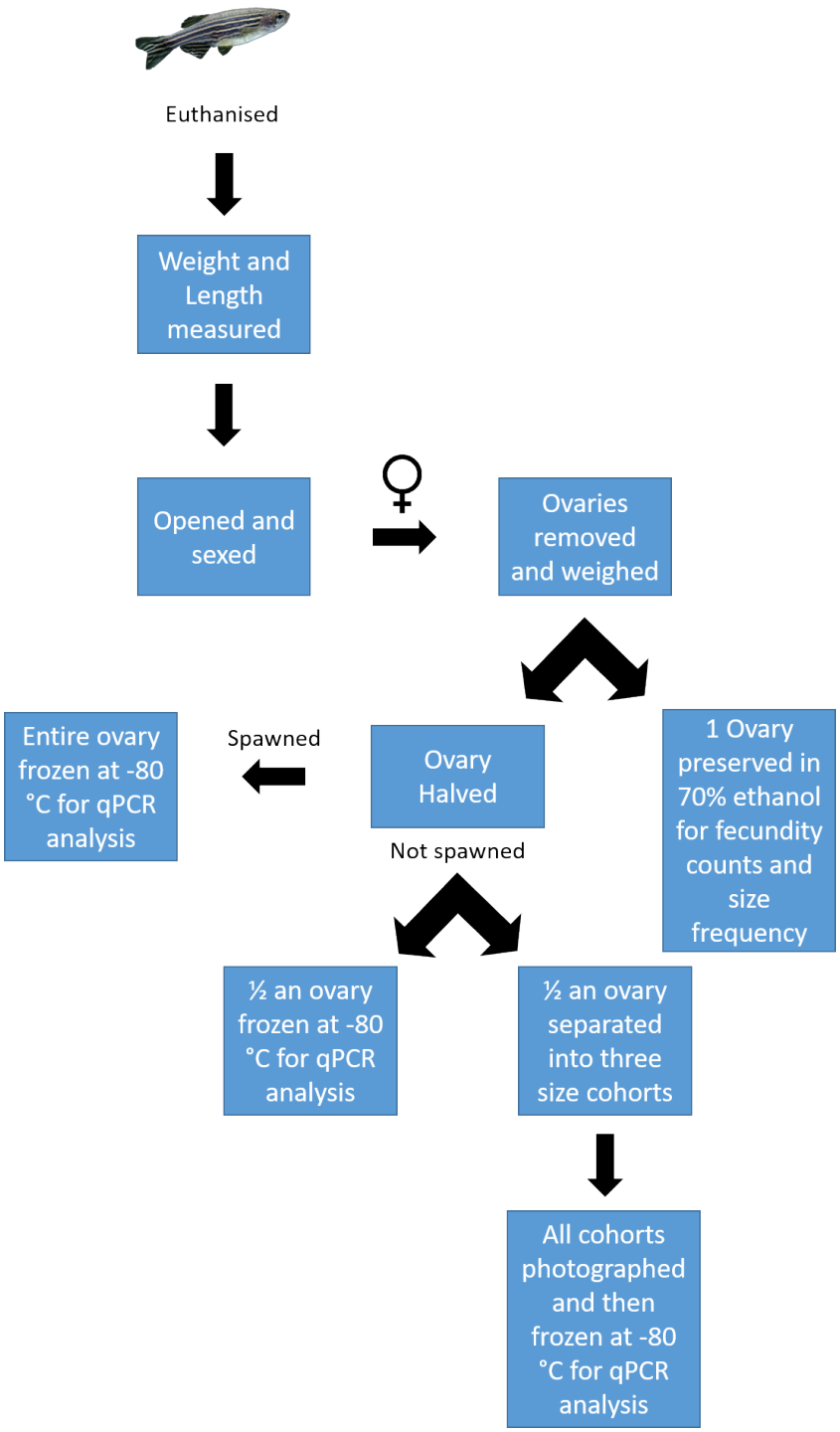

Figure 3.2: Dissection protocols for all female zebrafish terminally sampled during Experiment Two.

Table 3.1: Sampling endpoints on a per tank basis for all female zebrafish, *Danio rerio*, in the final dissection (Phase 2) round of Experiment Two. Treatments were a combination of feeding regimes; Low: 1.5% of body weight and High: 3% of body weight, in four week phases. Non – all females which did not spawn or ovulate before being sacrificed. Cohorts taken – follicles were separated into size classes from non-ovulated tissue present in the ovary and frozen for later qPCR analysis.

Tank ID	Treatment	Total number of fish	Spawned	Ovulated	Non	Cohorts taken
A1	Low-High	4	-	1	3	3
A2	High-Low	6	1	1	4	4
A3	High-High	8	-	1	7	6
A4	Low-Low	7	2	2	3	7
A5	High-High	3	1	-	2	1
A6	Low-High	6	1	2	3	4
A7	High-High	3	1	-	2	1
A8	Low-Low	-	-	-	-	-
A9	Low-Low	8	1	3	4	7
A10	Low-High	5	2	-	3	2
A11	High-Low	6	2	1	3	4
A12	High-Low	6	1	2	3	5

Although ovary tissue was taken for potential qPCR analysis, for this chapter only morphological data were considered; molecular analyses were carried out on P1 samples in Chapter 2 (Section 2.3.2.4). Also due to low statistical power (for both spawned and ovulated fish), only fish which did not spawn or ovulate were analysed (Non, Table 3.1).

3.2.4 Within-clutch Variation Analysis

As the food level in the environment was not constant (changed between phases), it was important to check for any bet hedging strategy. This was achieved through analysing variation in follicle diameter within the largest clutch in the ovary, using the coefficient of variation of the largest 20 % of oocytes for each fish. The coefficient of variation was calculated by dividing the standard deviation of the largest 20 % and then dividing it by the average largest follicle diameter of each fish. This was then compared between treatments.

3.2.5 Statistical Analysis

All statistical analyses were performed using the R software, version 3.4.2, and assumptions were checked as in the previous chapter (Section 2.2.4.3).

A Linear Mixed-Effects model using the '*lme4'* package, (LMER, Pinheiro and Bates 2000, Bates et al. 2015) was used to determine the influence these fixed effects, i.e feeding regime, had on follicle diameter. Tank identity was included as a random factor in all analyses to account for dependency within the tanks. All data is expressed as averages of tank values. Model selection was carried out according to the protocol outlined by Zuur et al (2009).

Originally the fixed section of the model was saturated with parameters and the model with the lowest AIC was chosen. Following this, non-significant components were sequentially removed, starting with the highest interactions. Significance of differences between treatments were determined post hoc. Response variables included, follicle diameter, GSI, fecundity, body weight, and relative follicle diameter, these were combined in different combinations depending on the model. Feeding regime was always a fixed effect when used.

For follicle diameter, the model containing feeding regime, GSI and body weight as factors as well as tank identity as a random effect, had the lowest AIC value. Any significant interaction effects between fixed parameters ($p < 0.05$) are reported.

Initially all six treatments were analysed together for all variables, but due to significant differences between the phases, L-0 and H-0 (P1) were compared and the four treatments in P2 were compared (L-L, L-H, H-L and H-H) in separate statistical analyses. All six treatments were analysed together for variation in follicle diameter within the largest clutch, as this had no relationship with body weight.

3.3 Results

3.3.1 Fecundity, GSI and Oocyte Diameter

Body weight dramatically increased between phase one and two of the experiment. P1 had an average body weight of 870 ± 84 µg, compared to an average body weight of 1288 ± 76 µg in P2 ($F_{1,44}$ = 25.49, p = 8.0 x 10^{-6}, Figure 3.3). Due to this marked difference in condition, all variables were analysed separately for P1 and P2.

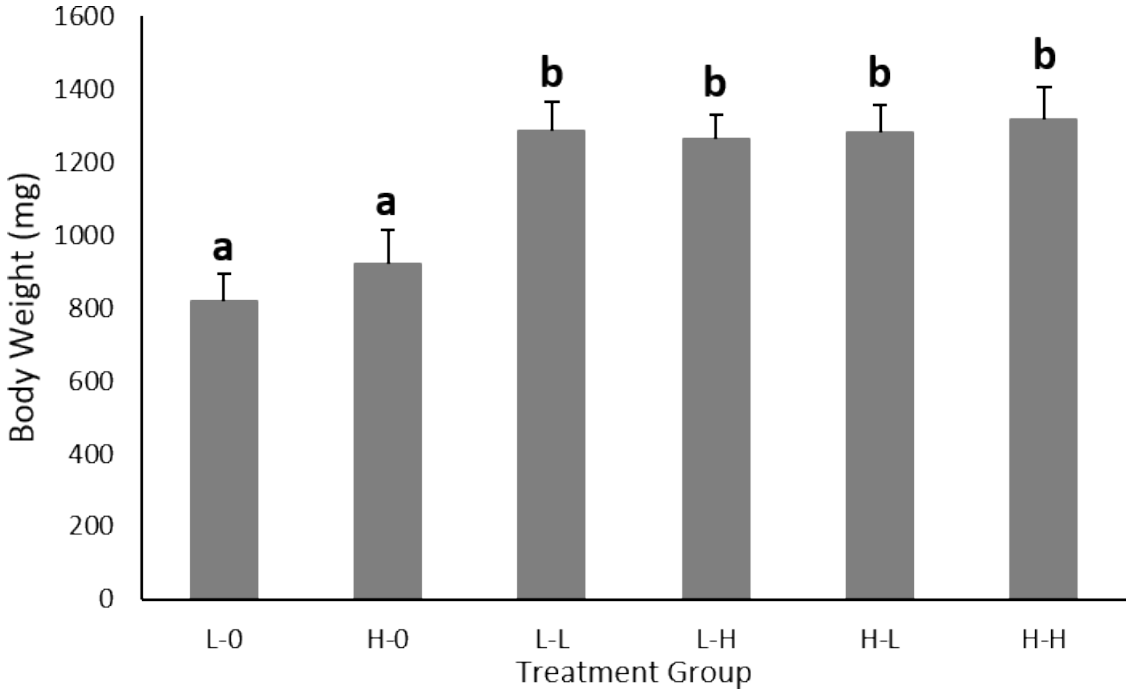

Figure 3.3: The effect of different feeding regimes on body weight of female zebrafish, *Danio rerio*. There were six different treatments present all consisting of a combination of two different feeding regimes; Low (L): 1.5% of body weight or High (H): 3% of bodyweight, in four-week phases. Fish were sacrificed after phase one (L-0 and H-0) or after phase two (L-L, L-H, H-L and H-H). Bars represent the average body weight ± 1 se in each treatment, n (number of tanks) = 2 for L-0, H-0 and L-L and 3 for the other treatments. Different letters refer to treatments that were significantly different from one another.

3.3.1.1 Phase 1

The majority of the results for P1 were presented in Chapter Two in Section 2.3.2, and so will be briefly summarised here as the differences between the phases are talked about in the discussion. For further detail refer to Chapter Two.

Body weight did not differ between feeding regimes for P1 (LMER: $F_{1, 1.84} = 0.532$, $p = 0.54$, Figure 3.3, L-0 and H-0). GSI was similar at around 1.3 % of total body weight for females fed on both the low and high regimes. Relative fecundity tended to increase in fish fed more food (H-0) with around twice as many vitellogenic follicles per mg of body weight when compared to fish fed lower amounts (L-0). Follicle diameters did not differ between feeding regimes in non-ovulated females and were around 760 µm. When "quality" (body weight) was controlled for, there was a tendency for larger 'relative' follicle diameter in females on the low feeding regime (L-0) when compared with the high feeding regime (H-0).

3.3.1.2 Phase 2

Body weight was comparable across all treatment groups and the average was around 1300 mg (LMER: $F_{3, 7.2} = 0.04$, $p = 0.99$, Figure 3.4). However, there was high variation in body weight seen both within individuals in the tanks and between the tanks in each treatment group (treatment*tank interactions: $F_{10,26} = 4.9$, $p = 0.001$, Figure A.5 in Appendix 4). When coefficients of variation were calculated for each treatment (L-L, L-H, H-L and H-H), they were all above 12% (Table 3.2).

There was no difference in GSI across all four treatments, it was all around 16% on average (LMER: $F_{10,26} = 1.01$, $p = 0.4$). High variation in GSI between individuals in each tank was also present, some fish in the tank displaying a GSI of 8% and some a GSI of 20% (treatment*tank interactions: $F_{10,26} = 2.61$ $p = 0.03$, Figure A.6 in Appendix 4). All treatments except L-L had high coefficients of variation (Table 3.2).

Table 3.2: Coefficients of variation (%) calculated for both body weight and GSI for female zebrafish, *Danio rerio*, both within each tank and for the entire treatment groups after phase two dissections. Fish were exposed to four different combinations of feeding regime. Feeding regimes were set at 1.5% of body weight daily (Low) or 3% of body weight daily (High).

	Body weight Coefficient of Variation		**GSI Coefficient of Variation**	
	Tank	*Treatment*	*Tank*	*Treatment*
Low-Low	4.6	14.6	35.4	1.7
	7.2		19.4	
Low-High	2.0	13.8	2.0	20.5
	8.2		15.3	
	9.4		22.5	
High-Low	13.2	13.9	7.2	13.6
	7.9		7.5	
	13.2		27.5	
High-High	17.2	12.6	33.1	21.5
	20.8		58.7	

Follicle diameter was slightly smaller in the females kept on the high feeding regime for both phases (H-H) when compared to females who spent any time fed a lower feeding regime (L-L, L-H and H-L). The largest difference was between H-L and H-H; 754 ± 16 µm compared to 806 ± 9 µm (LMER: $F_{3,5.93} = 1.26$, $p = 0.37$, Figure 3.4 B).

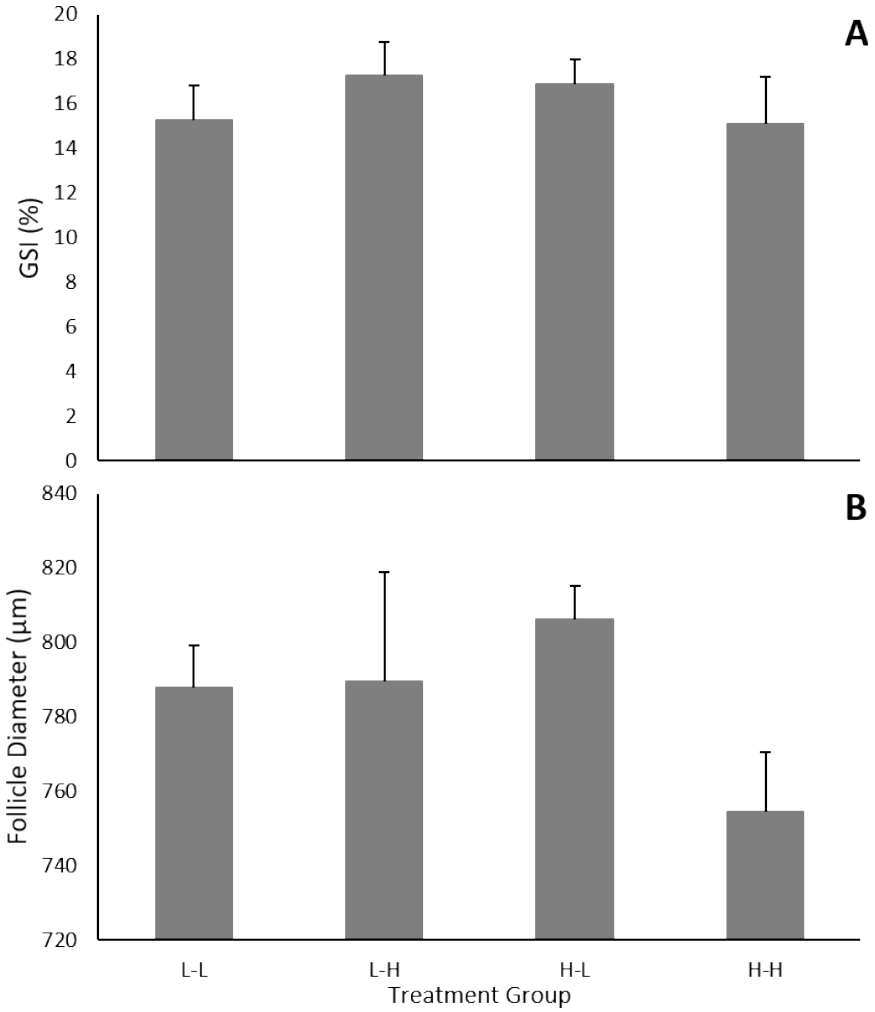

Figure 3.4: The average gonadosomatic index (GSI) (A) and average follicle diameter (largest 20%, µm) (B) for non-ovulated female zebrafish, *Danio rerio*. There were four different treatments present all consisting of a combination of two different feeding regimes; Low: 1.5% of body weight or High: 3% of bodyweight, in four-week phases. Fish were sacrificed after phase two (L-L, L-H, H-L and H-H). Each bar represents the average value in the treatment ± 1 se, n (number of tanks) = 2 for L-L, 3 for the remaining treatments.

3.3.2 Maternal – Offspring Correlations

Follicle diameter was strongly dependent on body weight in P1, with larger fish producing larger follicles (LMER: $t_8 = 2.817$, $p = 0.02$, $R^2 = 0.50$, Figure 3.5, blue triangles). This correlation was not significant in phase 2, but there was still an observable weak positive relationship (LMER: $t_{25.7} = 1.205$, $p = 0.24$, $R^2 = 0.04$, Figure 3.5, black circles).

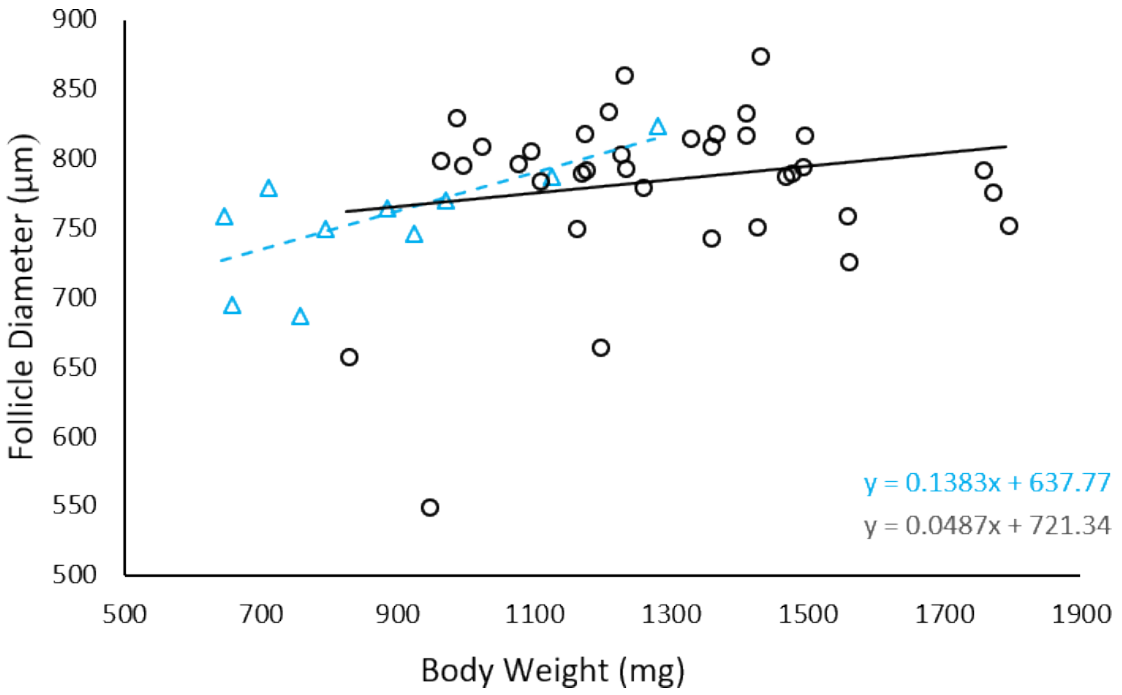

Figure 3.5: The relationship between the average follicle diameter (of the largest 20 % of follicles present in the ovary, µm) and the body weight (mg) of individual female zebrafish, *Danio rerio,* after phase 1 (blue triangles) and phase 2 (black circles).

When feeding regime was removed from the analysis in P1; relative fecundity was strongly correlated with follicle diameter (LMER: $t_8 = 3.683$, $p = 0.006$, $R^2 = 0.55$). The females with the highest relative number of vitellogenic oocytes produced the largest follicles (Figure 3.6).

Body weight is often used as a proxy for body condition, as the larger the body weight/ body size, the more access to resources in the environment (Lim et al. 2014). Due to the high variation seen in body weight within the treatments, oocyte diameter was corrected over female body weight to look for the trade-off between number and size of offspring. In P1, there was a strong negative relationship between relative follicle diameter and relative fecundity (LMER: $t_8 = -5.40$, $p = 0.0006$, $R^2 = 0.58$, Figure 3.7).

In P1, females with the largest relative ovary size (GSI) were also producing more vitellogenic follicles (LMER: $t_{5.78} = 7.145$, $p = 0.0004$, trend line; Relative Fecundity = 7.396 (GSI) – 0.2428, $R^2 = 0.82$) (Figure 3.7). Since GSI and relative fecundity were

strongly correlated, and both are an indication of reproductive investment, only GSI was used to describe follicle biometrics for P2.

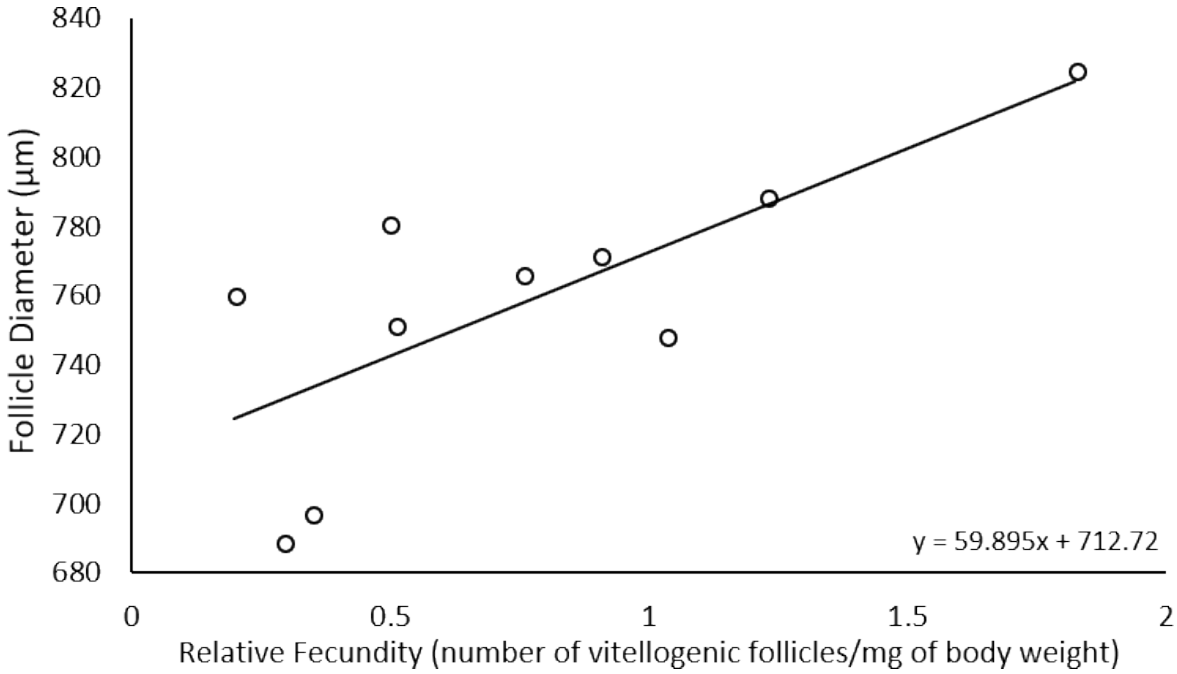

Figure 3.6: The relationship between average follicle diameter (of the largest 20 % of follicles in the ovary, µm) and the relative fecundity (number of vitellogenic follicles per mg of body weight) in female zebrafish, *Danio rerio*, sacrificed after phase one, $R^2 = 0.55$.

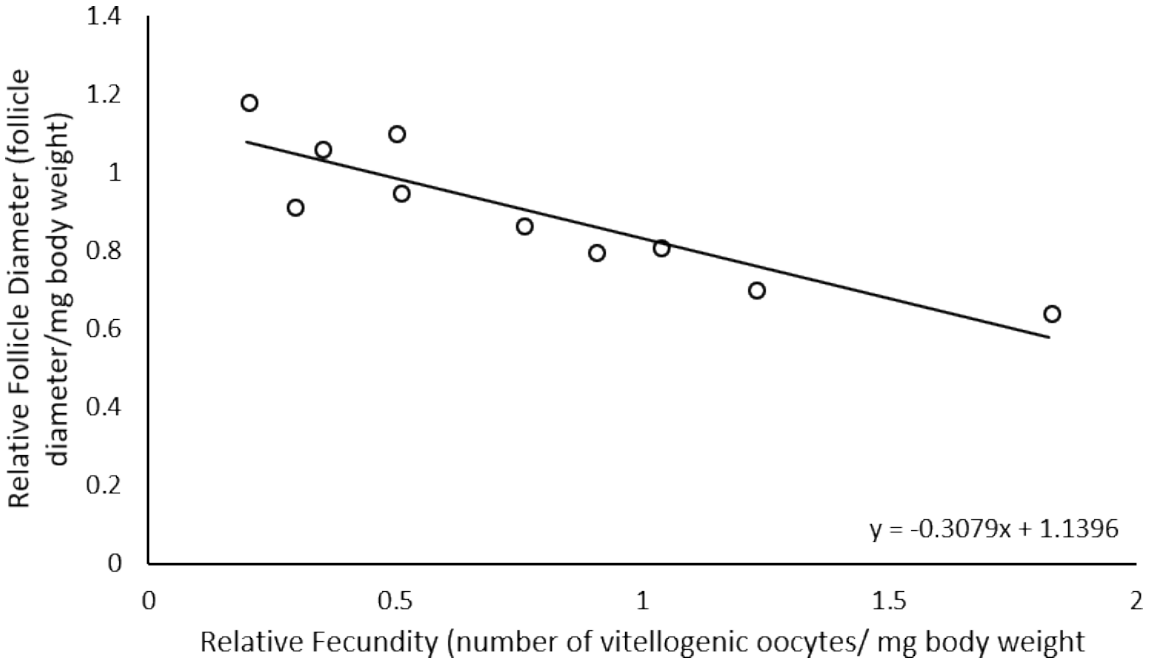

Figure 3.7: The relationship between relative follicle diameter (follicle diameter, µm / body weight, mg)) and the relative fecundity (number of vitellogenic follicles per mg of body weight) in female zebrafish, *Danio rerio*, sacrificed after phase one. $R^2 = 0.58$.

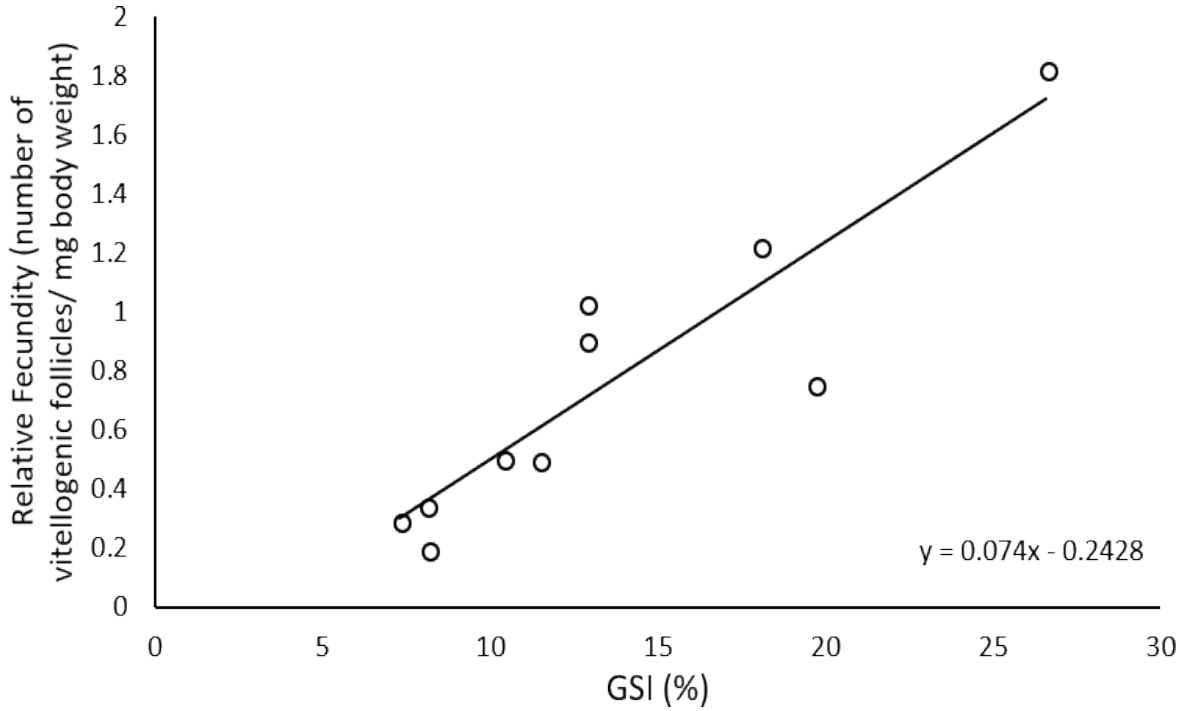

Figure 3.8: The relationship between the relative fecundity (number of vitellogenic follicles per mg of body weight) and the gonadosomatic index (% of body weight) in female zebrafish, *Danio rerio*, sacrificed after phase one. $R^2 = 0.82$.

In fish sacrificed in both P1 and P2, females with a higher GSI had smaller relative follicle diameters (LMER: Phase 1: $t_8 = 2.817$, $p = 0.01$, $R^2 = 0.58$, Phase 2: $t_{32} = 2.733$, $p = 0.01$, $R^2 = 0.20$, Figure 3.9). In other words, as females increased their investment in reproduction (relative gonad size, GSI), the diameters of the follicles produced decreased.

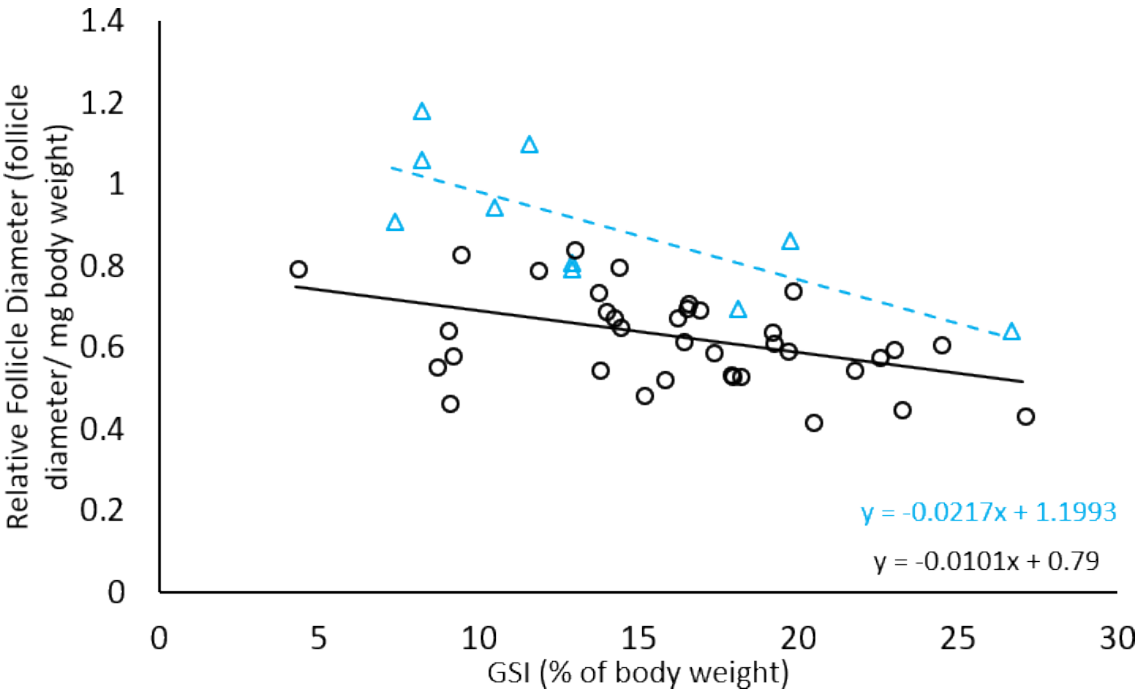

Figure 3.9: The relationship between the relative follicle diameter (follicle diameter, µm / body weight, mg) and the gonadosomatic index (%) in female zebrafish, *Danio rerio*, sacrificed after phase one (blue triangles, $R^2 = 0.58$) and phase two (black circles, $R^2 = 0.20$).

3.3.3 Size Frequency Distributions

The following results are subjective, comparing the frequencies of follicle size classes in the ovaries of non-ovulated fish between each different treatment without any statistical analysis of the differences (Figure 3.10).

For the P1 treatments (Figure 3.10; L-0 and H-0) there were no differences in the profile of the size and frequency of the follicles. The clutches appeared to blend together, although the largest clutch was centred around 750 µm, the smaller clutches were not distinctly visible. This contrasted with all the P2 treatments (Figure 3.6; L-L, L-H, H-L and

H-H) which had clearly bimodal distributions containing a distinct larger clutch centred around 750 µm, and a wider distribution of smaller follicles centred around 350 µm.

In P2, the three treatments which were exposed to a low feeding ration had a similar size frequency distribution pattern. There was an even spread of follicles from around 140 µm to around 500 µm and a smaller cohort of oocytes centred around 750 µm (Figure 3.10, LL, LH and HL). The L-L treatment had a slightly higher frequency of follicles sized 700 - 800 µm than the other two treatments exposed to low feeding rations, around 20 % compared to around 15 %. The largest clutch in the L-L treatment also had a narrower distribution with no follicles larger than 860 µm. The size frequency distribution profile for the tanks that were only exposed to the high feeding regime seemed different to that of the other three treatments. Most of the follicles were smaller than 420 µm with a smaller proportion of follicles present in the larger size classes, around 10 % on average (Figure 3.6, HH).

The large distribution spread of the largest clutch in P1 is supported by the variation in diameters of the largest 20 % of all follicles in the ovary. There was large variation in follicle diameters seen in both feeding regimes and the individual regimes were not different from each other (LMER: $F_{3,7} = 0.144$, $p = 0.74$).

In P2, there was no overall effect of treatment on variation in the follicle diameters of the largest clutch (LMER: $F_{3,32} = 1.12$, $p = 0.38$). There was a tendency for less variation in follicle diameter within the largest clutch seen in the fish which were kept on the low feeding regime for both phases, when compared to the other three treatments that had at least one phase of high rations.

When both phases were compared there was a significant decrease in the follicle diameter variation within the largest 20% of follicles in the older, larger fish (P2) when compared to the younger, smaller fish (P1) (LMER: $t_{8.73} = -8.25$, $p = 2.09 \times 10^{-5}$). (Figure 3.11)

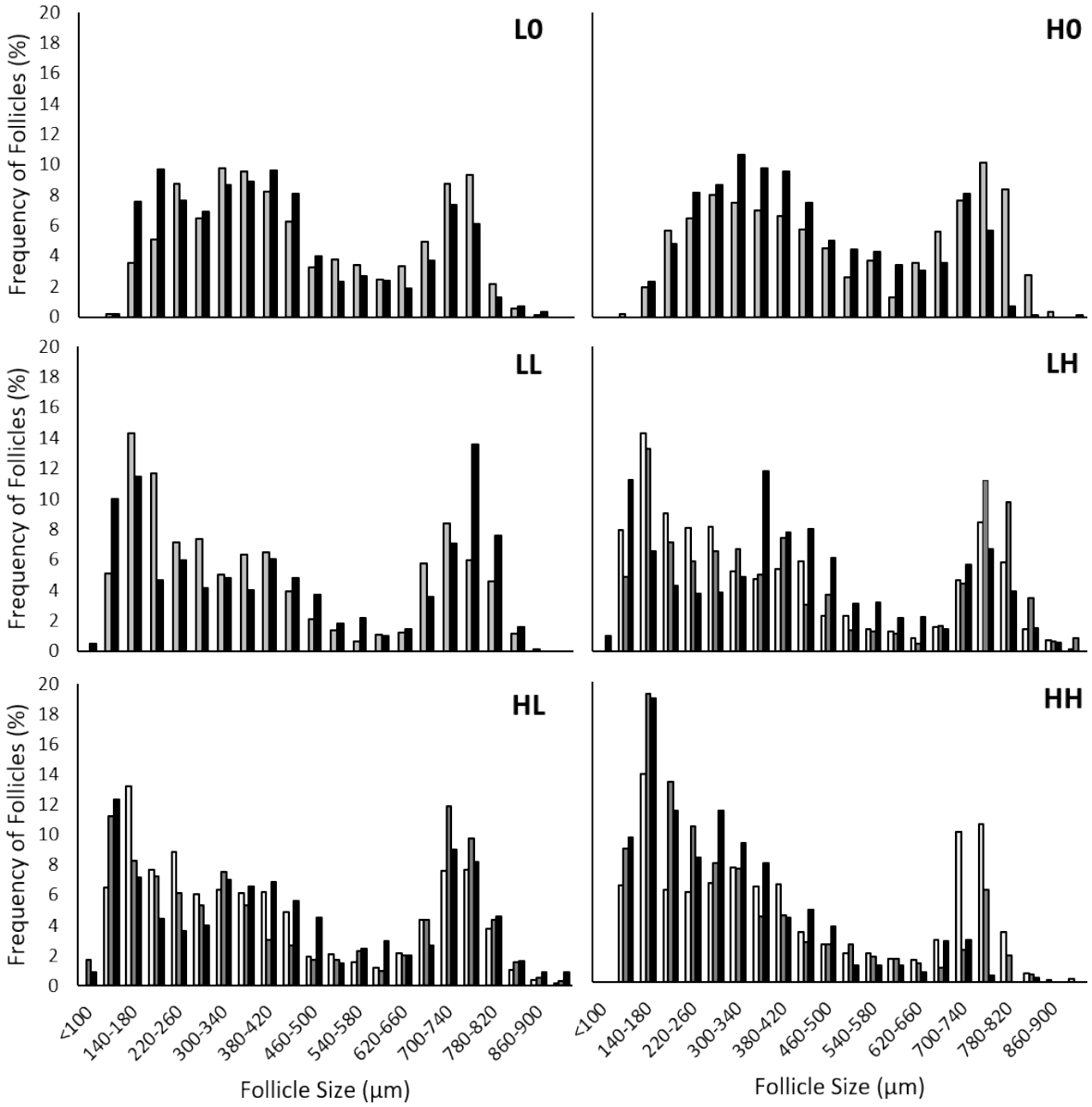

Figure 3.10: The size frequency profiles of follicles present in non-ovulated ovaries in female zebrafish, *Danio rerio,* when exposed to six different food environments. There were six different treatments present all consisting of a combination of two different feeding regimes; Low: 1.5% of body weight or High: 3% of bodyweight, in four-week phases. Fish were sacrificed after phase one (L0 and H0) or after phase two (LL, LH, HL and HH). Each different coloured profile represents the average distribution of follicles in the different size classes for each tank in the treatment, n (number of tanks) = 2 for L0, H0 and LL, 3 for the remaining treatments.

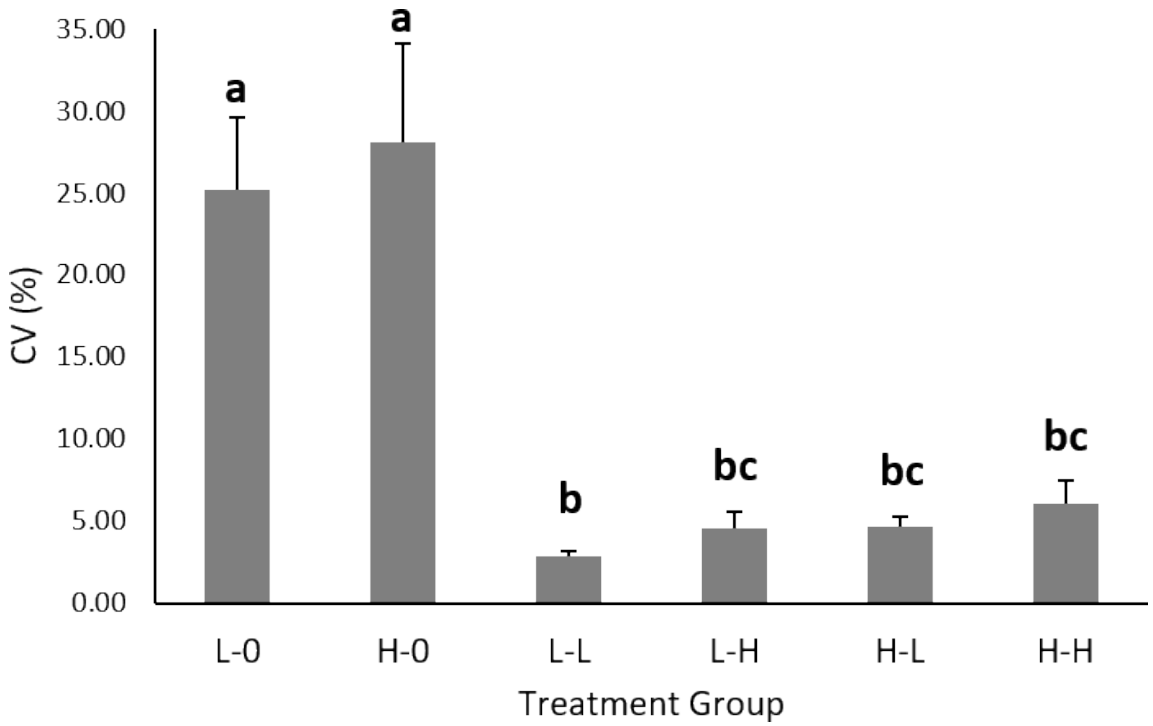

Figure 3.11: The effect of different feeding regimes on the follicle diameter variation within a clutch, in female zebrafish, *Danio rerio*. There were six different treatments present all consisting of a combination of two different feeding regimes; Low: 1.5% of body weight or High: 3% of bodyweight, in four-week phases. Fish were sacrificed after phase one (L-0 and H-0) or after phase two (L-L, L-H, H-L and H-H). Bars represent the average coefficient of variation ± 1 se in each treatment, n (number of tanks) = 2 for L-0, H-0 and L-L and 3 for the other treatments. Different letters refer to treatments that were significantly different from one another.

3.4 Discussion

The sensitivity of the maternal effects in changing environmental conditions, depends on when during oogenesis decisions about size and number of offspring are made. To evaluate the sensitivity of the phenotypic plasticity in offspring size displayed by *Danio rerio*, females were placed on changing feeding regimes. The effects of changing ration size were investigated through phenotypic changes in the mother.

3.4.1 Oocyte Diameter (Size vs Number Trade-off)

There was no effect of changing the feeding regime, on either reproductive investment (either relative fecundity or GSI) or follicle diameter, even when corrected for female body weight. A study by Filby et al. (2010), investigating access to food, mates and shelter in zebrafish, demonstrated that dominance behaviour can affect the physiology of individuals in a tank. Subordinate fish are shown to have higher cortisol levels and slower growth than dominant fish due to reduced access to food. In my experiment, there were large differences between the body weights of individual fish in each tank regardless of feeding regime. This observation could reflect the possibility that the more aggressive fish within the tanks were limiting the access to food for subordinates. There were one or two larger females present in most tanks, creating large coefficients of variation (CV) in body weights. Anecdotally, these larger females were more food aggressive, rising to feed before the smaller fish. In Filby et al.'s (2010) study, dominant females were also shown to have larger GSI when compared to subordinate fish in the same tank. In my experiment, there was high variation in GSI within tanks (large CV). This means dominant/subordinate effects could be the cause of heterogeneity in phenotypic traits within feeding regimes, blurring the effects of food limitation.

In a previous study by Forbes et al. (2010) adaptive maternal effects were observed in zebrafish, with fish exposed to lower feeding regimes producing larger spawned eggs (0.72 mm, yolk diameter) than fish which were fed larger amounts (0.64 mm yolk diameter). The fish studied by Forbes et al. (2010) also displayed higher fecundity in the treatments which were fed higher amounts of food when compared with fish fed lower amounts. This result is not evident in the current study. No adaptive maternal effects

were demonstrated when females were food-limited. This lack of a response could be due to an age effect. In killifish (*Nothobranchius furzeri*), the negative trade-off between egg size and number is lost as the fish ages. In larger, older fish, larger mature oocytes are produced without losing fecundity if resources are abundant (Vrtílek and Reichard 2015). In the current study, the trade-off seen between relative oocyte diameter and reproductive investment (GSI) was less steep in older fish, though still present. In Forbes' study, there was a clear trade-off without needing to correct for female body weight (Forbes et al. 2010). The zebrafish used by Forbes were around half the size of the fish used in the current study, where the fish had obtained full adult size. A possible explanation for the loss or relaxing of the trade-off between egg size and number as the fish age, is that the trade-off is only maintained strongly during energetically demanding periods, such as rapid growth in younger fish or extremely food limiting environments (Vrtílek and Reichard 2015).

When follicle size and fecundity were corrected over female body weight and modelled without feeding regime, a significant negative relationship was demonstrated. This shows that the classical trade-off that is theoretically predicted is still occurring at the individual level (Lim et al. 2014). If body weight was not considered, then the opposite relationship occurred showing an increase in follicle diameter when fecundity was increased. Previous studies have shown that correlations between competing traits may appear in opposition to the predicted trade-off when there is heterogeneity in two different characteristics; resource availability and individual resource allocation strategy (Van Noordwijk and Dejong 1986, Reznick et al. 2000, Lim et al. 2014). Therefore, when quantifying a trade-off at a phenotypic level, individual quality needs to be controlled for (Lim et al. 2014).

Resource availability to individuals is rarely constant and there are many factors that mean that no two individuals obtain resources in the same way. This can be due to heterogeneous environments as well as inherent differences in the way individuals obtain food (Cam et al. 2013). This intraspecific variation in obtaining resources is often referred to as "quality". Individuals with more resources or "high-quality" are able to display a higher level of investment into all competing traits, than "low-quality"

individuals with fewer resources (Wilson and Nussey 2010). In my experiment, there was a high heterogeneity of individual quality within feeding regimes.

A positive relationship was observed between maternal body weight and follicle diameter, suggesting that the larger fish had more access to resources overall regardless of treatment and were able to invest more in both oocyte diameter and number of oocytes (GSI). This pattern is seen to be the 'norm' across most taxa (Lim et al. 2014), and can be explained if optimal offspring size increases as maternal size increases. This can occur in environments where there are high levels of intraspecific competition (Kindsvater et al. 2012). As *Danio rerio*, clearly demonstrates this positive correlation, it may not be the best candidate species for studying maternal resource allocation strategies. Females exposed to higher levels of food tend to grow faster, therefore obscuring any species (or population) wide adaptive phenotypic plasticity (Filby et al. 2010, Kindsvater et al. 2012, Lim et al. 2014).

In conclusion, the heterogeneity of quality seen in the female zebrafish within each feeding regime, is likely to be blurring any phenotypic signs of trade-offs present (Lim et al. 2014). To determine if a trade-off between follicle size and fecundity was occurring at the individual level, these parameters had to be corrected over maternal body weight. The trade-off was also seen to decline as the females aged (grew larger). The positive relationship between maternal body weight and follicle diameter, may also mean that zebrafish are only suitable to investigate maternal reproductive strategies at a younger age.

3.4.2 Variation in Follicle Size Within Clutches

Another way in which a mother can ensure her reproductive fitness and manipulate offspring phenotype, is through varying the size of offspring within a clutch. If a mother can predict the environmental conditions of her offspring, then it makes sense that she invests in an average oocyte size that is close to optimum for the environment and maximises her fitness (Smith and Fretwell 1974, Reznick and Yang 1993, Marshall and

Uller 2007, Crean and Marshall 2009). In guppies, it has been shown that there is an adaptive switch from large numbers of small offspring to a few large offspring when exposed to food limited conditions. In their natural habitat, there are clear rainy vs dry seasons which have a major effect on resource availability and are long enough to provide stable environmental cues (Reznick and Yang 1993). If the offspring's environment is unpredictable however, then the mother cannot determine which offspring size will maximise her fitness. In this situation, theory states that a mother should increase the within-clutch variation in offspring size (or phenotype), or in other words, bet hedge (Crean and Marshall 2009). This ensures that at least some of the offspring have the correct phenotype regardless of environmental conditions, and as a result the average oocyte size will be further from optimal and may appear larger (Crean and Marshall 2009). (Figure 3.12)

In my study the younger fish, P1, (L-0, H-0) had higher variation in follicle size within the largest clutch of oocytes, than older fish in P2. This was also supported by the size/frequency distribution plots for each treatment. The P1 fish had a much broader spread of oocytes without any distinct clutches visible. The largest clutch appeared to spread from 500 to 900 µm. The P2 fish had a narrower distribution spread for the largest clutch, from around 660 – 900 µm, with L-L having the narrowest of all 660 – 820 µm. In soil mites, there is a decrease in egg size variation within clutches as the females age. It was suggested that this was due to older females 'experience', and a more predictable environment later in life (Plaistow et al. 2007, Crean and Marshall 2009). The high variation in follicle size within the clutches seen in P1, could be due to a lack of long term predictability in the environment as they were moved from an intermediate feeding regime on to the experimental feeding regimes.

In unpredictable environments, mothers will often produce a range of offspring sizes to take advantage of a range of potential offspring environments (Crean and Marshall 2009). The two changing feeding treatments, L-H and H-L could be therefore expected to show a higher variation in follicle diameter within the largest clutch. This was not the case. Females in the L-L feeding regime, tended to have lower within-clutch variation compared to the other three P2 treatments, but there was no overall treatment effect. The reason L-L may have had a narrower distribution of oocyte size, is that the minimum

size for offspring survival will be larger in a resource limited environment. This narrows the overall distribution as both the minimum and optimal size of offspring is larger when compared to a more resource rich environment (Ware 1975, Crean and Marshall 2009, Figure 3.12).

3.4.3 Conclusion

In conclusion, on the basis of this experiment there was no evidence that zebrafish, *Danio rerio,* demonstrate adaptive plasticity in the number or size of their offspring when exposed to different resource levels. It has been demonstrated before that adaptive plasticity is present in this species (Forbes et al. 2010), and the lack of it in the current study may be due to differences in the strength of the trade-off between egg size and number at different stages in life. In killifish, the trade-off is only demonstrated when fish are exposed to energetically demanding periods in life, such as rapid growth during the younger phase (Vrtílek and Reichard 2015). The fish used in this experiment were adult size and future experiments investigating the changes in this relationship from juvenile growth until full size may further illuminate changes in this important life history parameter.

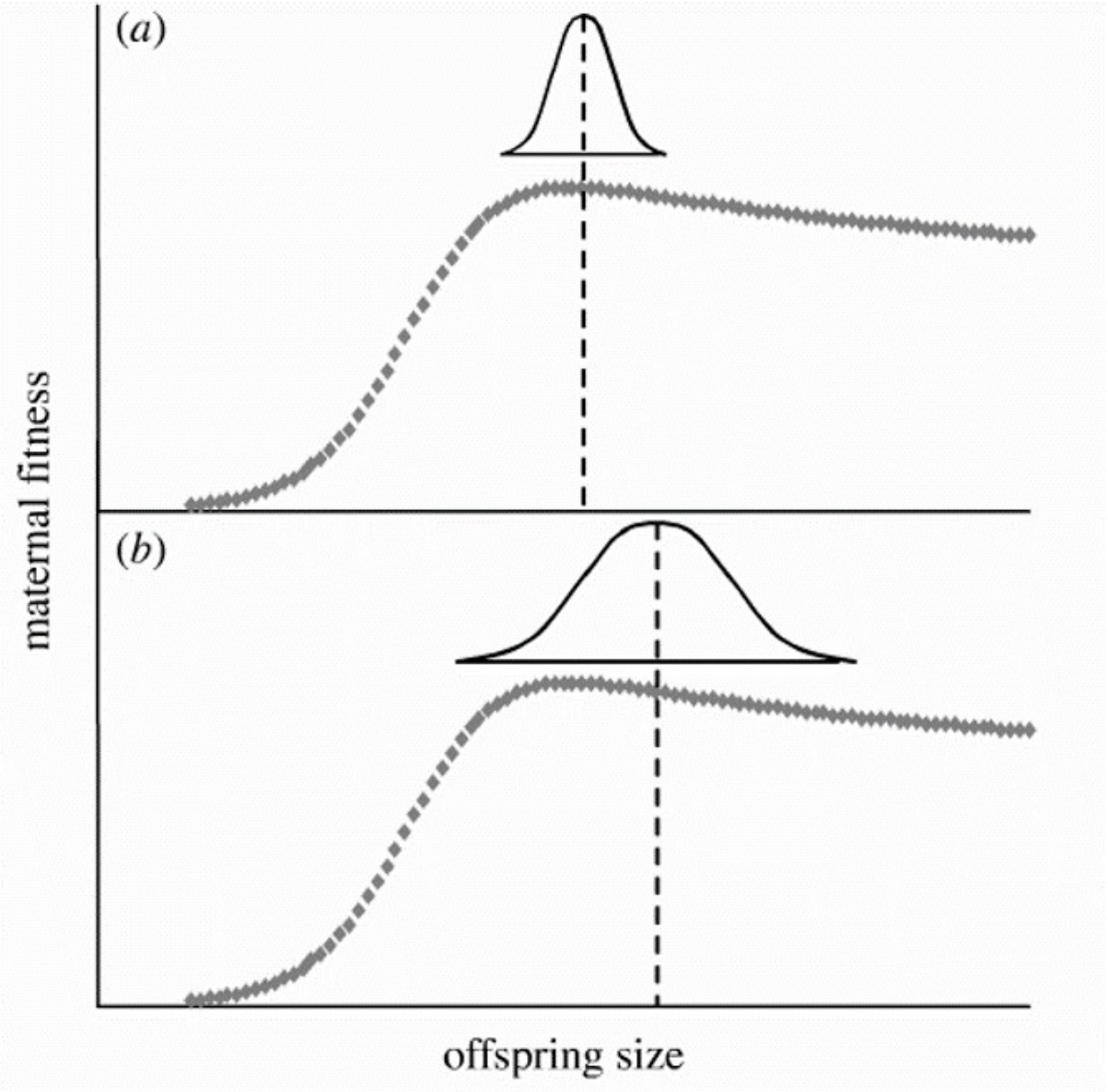

Figure 3.12: Schematic showing the effect of increasing offspring size variability on the average size of offspring that a mother produces. Panels show the relationship between offspring size and maternal fitness (dotted curves) and the normal distributions indicates the distribution of offspring sizes that the mother produces. (*a*) Mothers produce a small range of offspring sizes; the average offspring size is close to both the minimum offspring size threshold for survival and the optimal size for the environment. (*b*) Mothers produce a larger range of offspring sizes; the average offspring size produced is further from the minimum offspring size threshold for survival and the optimum offspring size. (Crean and Marshall 2009)

Chapter 4

Unravelling molecular mediators of maternal effects – using a transcriptomic approach to reveal changes in gene expression between zebrafish, *Danio rerio*, exposed to different resource levels in the environment

4.1 Introduction

Understanding how a mother changes provisioning of her offspring when exposed to different levels of resources in the environment will have wide-reaching implications across many fields of study. Nutritional state is a key indicator of the quality of the outside environment and can have important effects on many life history traits, including reproduction (Green and Extavour 2014). Although adaptive plasticity of offspring size is a clearly established life history strategy and there are examples across the animal kingdom (Fox and Czesak 2000, Bashey 2003, Forbes et al. 2010, Riesch et al. 2010, Green and Extavour 2014), there are very few studies that investigate the molecular mediators of this change.

A study on *Drosophila,* has found that the insulin-like growth factor system plays a key role in determining the reproductive output of an individual by determining the number of ovarioles produced in the ovary (Green and Extavour 2012). In mammals, such as sheep and cows, it has been demonstrated that bone morphogenetic protein-15 and growth differentiation factor-9 play a key role in determining fecundity of an individual. Mutations in the genes coding for these proteins can result in increased fecundity (Moore et al. 2004, Moore and Shimasaki 2005). Forbes et al. (2010) looked at whether these mammalian 'fecundity' genes played a key role in the adaptive plasticity displayed by zebrafish, *Danio rerio*, and found that these were not the molecular mediators of fecundity in this species. Due to the lack of knowledge about the molecular mechanisms that play a role in offspring size plasticity in vertebrates, a method allowing for detailed sequencing of entire mRNA populations could be highly informative. RNA-Seq is a technique which allows for sequencing and quantification of the relative abundance of mRNA transcripts in a tissue.

A transcriptome is a complete set of mRNA transcripts present in a cell at a specific developmental stage or physiological condition (Lokman and Symonds 2014). The study of transcriptomics aims to distinguish and categorise the transcripts present in a transcriptome (Marioni et al. 2008, Lokman and Symonds 2014). This is often done using a high throughput sequencing method such as Illumina, which enables the RNA species in a sample to be sequenced to reasonable depth and coverage (Bennett et al. 2005, Marioni et al. 2008, Xuan et al. 2013). This is useful in expression studies and can reveal differences between mRNA species and abundance in different samples when exposed to different treatments or

between developmental stages (Smith et al. 2013, Brinkmann et al. 2016). These techniques are powerful as they are untargeted and can give a much more detailed glimpse into changes in developmental pathways and cascades than simple quantitative PCR or other more targeted techniques (Hansen et al. 2011, Lokman and Symonds 2014, Brinkmann et al. 2016).

RNA-Seq is a next generation sequencing technique under the umbrella of transcriptomics. RNA is isolated in the samples and is then reverse-transcribed into complementary DNA (cDNA). This resulting cDNA is replicated to form double-stranded DNA and the nucleotide sequence is obtained for all RNA transcripts in the population via next generation sequencing (i.e. Illumina) (Bennett et al. 2005, Xuan et al. 2013). The sequenced population of mRNA is considered to be representative of the entire RNA population present in the tissue, however some genes with very low expression levels may not be sequenced. To increase the validity of the assumption that the sequenced population is representative, greater sequencing depth can be used (Todd et al. 2016). The difference between RNA-Seq and other sequencing techniques is that RNA-Seq also quantifies the relative abundance of each RNA species contained in a sample, and this allows global RNA abundances to be compared between samples or treatments (Lokman and Symonds 2014).

This study aimed to use RNA-Seq and bioinformatic analyses to take a comprehensive look at the molecular changes that may occur in the ovaries of female zebrafish, when exposed to different levels of resources in the environment. Females were kept on two different feeding regimes, either low or high, and after the conclusion of the experiment, representative fish ovary samples, three fish from each feeding regime were sent for analysis.

4.2 Methods

4.2.1 Experimental Design

The six fish used in this analysis were chosen from fish sacrificed after P1 of Experiment Two (Chapter 3, Table 3.1, Section 3.2.2). Fish were kept at 25 °C on two different feeding regimes, a low (1.5% of body weight daily) or a high (3% of body weight daily) regime. Twelve fish per regime were sampled after four weeks. These were placed in spawning tanks with a male overnight, then terminally sampled. Three endpoints were reached, i.e., fish that spawned eggs, fish that had ovulated oocytes in the body cavity and fish that did neither. See Chapter Three, Section 3.2.2 for more detail.

All the biometric data from females which did not spawn were compared using a multidimensional scaling analysis (MDS) performed in R, using the package stats and the *cmdscale* function (Figure 4.1). These data included gonadosomatic index (GSI), body weight, standard body length, and total ovary weight. Ovary state (ovulated or not) was also considered. For this selection only females which had ovulated were chosen, to avoid any effects caused by differences in ovarian composition between ovulated females and the females that did not ovulate. The size distribution profiles of follicles in the individual ovaries were also added to the analysis. Females with similar profiles were chosen, to directly compare between ovaries in similar developmental stages. With all the above constraints, females from within each regime which were closely clustered together in the MDS analysis were chosen (Figure 4.1).

Ovarian tissue was therefore obtained from six ovulated females, only taking the remaining ovary with no ovulated eggs (Table 4.1). Due to budget constraints only these 6 samples were sent away for RNA-Seq analysis.

Table 4.1: Summary of the treatment (feeding regime; high = 3 % of body weight, low = 1.5 % of body weight), individual fish identification, ovarian state, GSI and body weight of the six female zebrafish, *Danio rerio*, chosen for transcriptomic analysis.

Fish	Treatment	Transcriptome ID	Ovarian state	GSI (%)	Body weight (mg)
A7F2	High	HF1	Ovulated	7.3	1068
A7F3	High	HF2	Ovulated	12.1	1008
A7F4	High	HF3	Ovulated	12.0	1247
A10F3	Low	LF1	Ovulated	9.3	1091
A10F5	Low	LF2	Ovulated	6.5	926
A10F6	Low	LF3	Ovulated	5.9	824

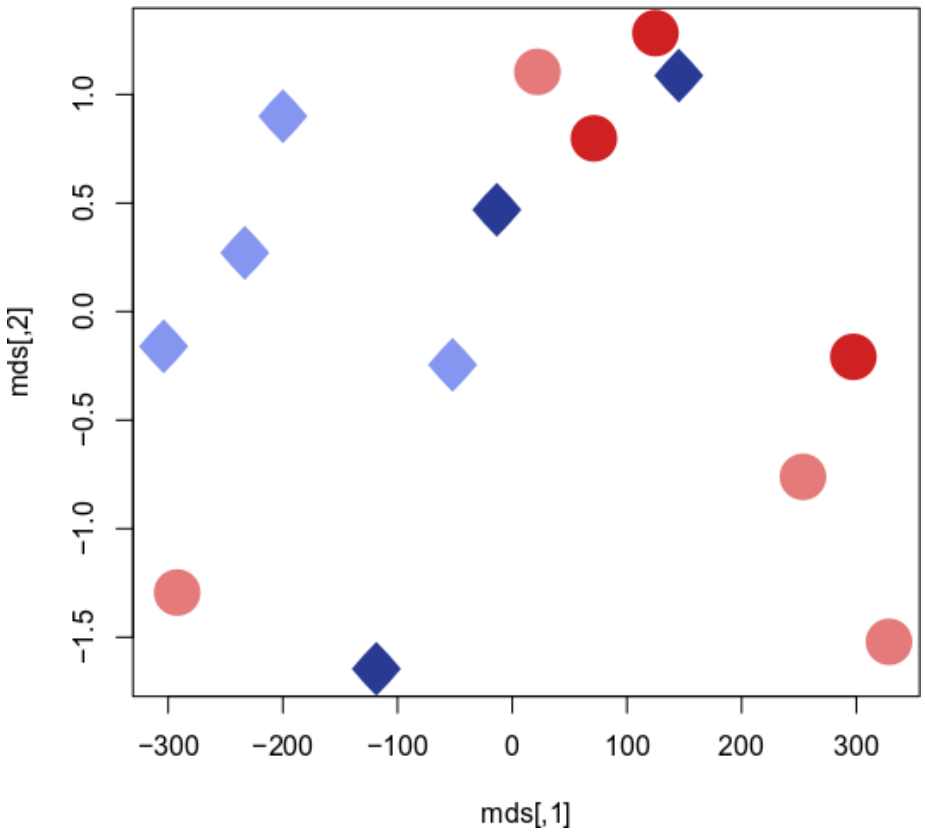

Figure 4.1: Multidimensional scaling plot of the female zebrafish, *Danio rerio,* in Phase 1 of Experiment 2. Factors included in the analysis are outlined in Section 4.2.1. Blue diamonds indicate females on the low feeding regime, red circles indicate females on the high feeding regime, darker symbols represent the samples sent for RNA-Seq analysis.

4.2.2 RNA Extraction

Total RNA from each ovary sample was extracted using Trizol reagent (Invitrogen Life Technologies), following the protocol laid out by the manufacturer. Genomic DNA was removed through incubation with DNA-free DNase I (Turbo DNA Free Kit, Ambion, USA) at 37°C for 30 minutes. The concentration of the RNA was initially obtained by measuring the absorbance at 260 nm/280 nm using a Nanodrop ND-1000 spectrophotometer (LabTech, USA). The quality of the RNA was verified through measuring RNA integrity and concentration with an RNA Pico chip on an Agilent Bioanalyzer. Samples, each containing 3 µg of freeze-dried RNA, were sent to an independent contractor, BGI Tech Solutions (Hong Kong), for sequencing.

4.2.3 Illumina Sequencing

The samples containing RNA were sent to BGI Tech Solutions, where cDNA library construction, next generation sequencing and bioinformatic analyses were performed. Sequencing was carried out using an Illumina HiSeq 4000 and samples were sequenced to a depth of 1 Gb.

4.2.4 Library Construction

Sequence data were sorted into individual samples and adapters were trimmed by the service provider prior to the analysis. Internal software from BGI Tech Solutions was used for filtering and removed any reads that were adapter polluted, any reads in which the number of unknown bases were above 5% and finally any low-quality reads (which are defined as any reads in which the number of bases, with a quality lower than 15, are above 20%). After filtering was carried out, all clean data were stored in a FASTQ format.

4.2.5 Transcriptome Assembly and Annotation

The alignment program used to map the RNA-seq reads to the reference genome was HISAT, due to the large number of reads spanning multiple exons (Kim et al. 2015). Any novel transcripts identified were reconstructed using StringTie and then compared to reference annotation using Cuffcompare (Trapnell et al. 2014, Pertea et al. 2015). CPC was then used to predict the coding potential of these novel transcripts (Kong et al. 2007). Novel transcripts were classed as either new isoforms of known genes or completely unknown transcripts.

4.2.6 Gene Expression Analysis

For gene expression analysis, all clean reads were mapped to the reference genome using Bowtie2 (Langmead and Salzberg 2012). Transcript expression levels for each gene were calculated using the RSEM software package for all samples (Li and Dewey 2011). Relative expression levels were measured in fragments per kilobase of transcript per million mapped reads (FPKM).

To compare biological replicates, Pearson's correlation between all samples based on gene expression level was calculated in R using the function *cor*. Hierarchical clustering analysis of the samples was also performed in R using the *hclust* function.

Differential expression analysis between the ovaries of the fish exposed to the high feeding regime and the fish exposed to the low feeding regime was performed using the DEseq2 software (Love et al. 2014). This is based on a negative binomial distribution. Only transcripts which had an expression fold change of above or equal to 2 were compared. A Bonferroni correction was used to adjust the p-value for large volumes of data analysis. The cut-off adjusted p-value was set at <0.05 to obtain significant differentially expressed transcripts between the two treatments.

4.2.7 Hierarchical Clustering, Gene Ontology Enrichment and Pathway Analyses

Hierarchical clustering analyses were performed using the data provided by BGI Tech Solutions. These analyses were performed using the FPKM for the differentially expressed transcripts with an adjusted p-value of <0.1 using the *heatmap3* package in R (Zhao et al. 2014).

Gene ontology enrichment and pathway analyses was performed by BGI Tech Solutions. To carry out a gene ontology analysis, genes were first classified according to the official classifications. Then a GO-functional enrichment was carried out using the *phyper* function in R. False discovery rates were calculated for each area and then any which had a false discovery rate (FDR) lower than 0.001 was defined as significantly enriched. Finally, a pathway analysis was carried out using KEGG annotation and *phyper* was again used to calculate any functional enrichment. Any pathways which had an FDR lower than 0.001 were again considered enriched (Eisen et al. 1998).

4.3 Results

4.3.1 Representative Sampling

When all the obtained biometric data for the female fish considered for this experiment were compared there was no clustering into the different feeding regimes. The samples chosen for transcriptome analysis were more closely related to each other, than the other fish present in the population (Figure 4.1). The average body weight for the entire population was 870 ± 84 µm, and all sample fish except A10F6 were heavier than average (Table 4.1).

4.3.2 Similarity Between Biological Replicates

When Pearson correlation and hierarchical clustering was performed on the entire population of transcripts for each sample, there was limited similarity found between biological replicates (Figure 4.2 B). This was also true when a multidimensional scaling plot was performed on the same data. Overall LF3 was not closely clustered with any of the other samples sent. HF1 and LF1 were more closely correlated with one another in terms of expression than the other samples in their respective treatment groups (Figure 4.2).

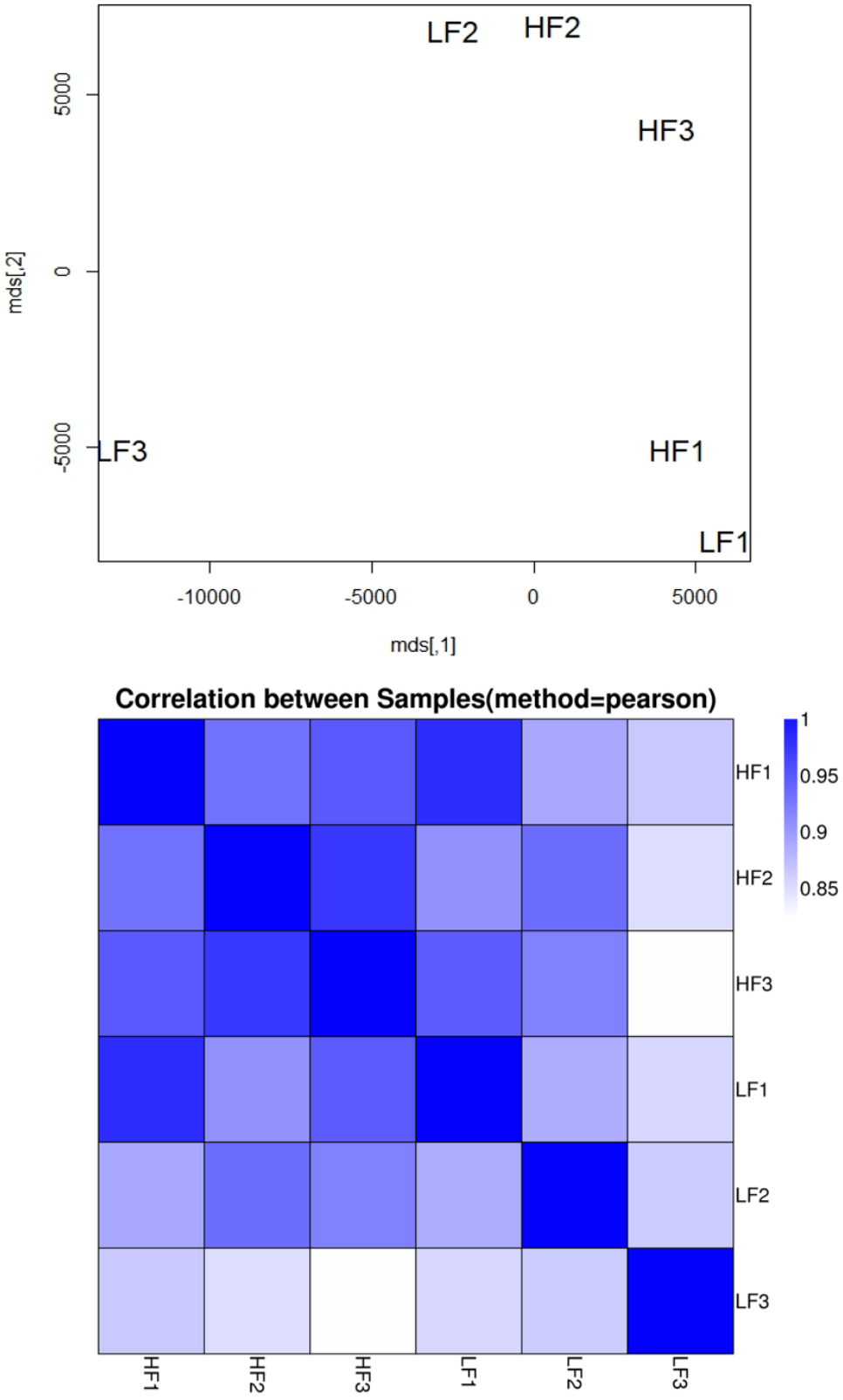

Figure 4.2: Comparison of biological replicates using A) a multidimensional scaling plot and B) a Pearson correlation between six female zebrafish (*Danio rerio*) when exposed to two different feeding regimes (high= 3% of bodyweight per day and low= 1.5% of bodyweight per day) using the fragments per kilobase of transcript per million mapped reads (FPKM) of all the genes sequenced in the transcriptome. Darker blue means a higher correlation between the samples (B).

4.3.3 Identification of Differentially Expressed Genes

Forty-one transcripts were found to be differentially expressed when ovary tissue from fish exposed to the low feeding regime was compared to fish exposed to a high feeding regime, with a FDR (p-value) of 0.05. When all transcripts annotated to the same gene were pooled, only 36 differentially expressed genes were found overall between the two treatments (Table 4.2). As there was high variation between biological replicates, an FDR of 0.1 was also considered, yielding a further 9 differentially expressed genes (Table 4.3).

4.3.4 Hierarchical Clustering and Gene Ontology Enrichment/Pathway Analysis

Among the genes that were differentially regulated between the feeding regimes, 29 genes were up-regulated in the ovaries of the fish fed less food compared to the fish on the higher feeding regime. 16 genes were down-regulated overall in the ovarian transcriptomes of the fish on the low feeding regime (Figure 4.3).

The biological processes that were found to be over-represented by gene ontology analysis included, cytoplasm ($p = 0.00064$), cytosol ($p = 0.005$) and regulation of protein stability ($p = 0.07$) (Figure 4.4). The molecular pathways that were found to be over-represented when the two treatments were compared using KEGGs pathway analysis were the peroxisome proliferator-activated receptor (PPAR) signalling pathway (Q-value = 0.0096), endocrine resistance (Q-value = 0.028) and the folate biosynthesis pathway (Q-value = 0.043) (Figure 4.5)

Table 4.2: Annotation of differentially expressed genes (n=36 within Q-value <0.05) between ovulated female zebrafish (*Danio rerio*) when exposed to different feeding regimes (high =3% of bodyweight daily and low= 1.5% of bodyweight daily)

No.	Description	Name	Gene ID	Q-value	Function according to NCBI
1	Uncharacterized protein LOC563255	zgc:171517	563255	1.92E-55	uncharacterised protein coding
2	Uncharacterised LOC795984	LOC795984	795984	3.61E-27	Function unknown
3	ubiquitin B	*ubb*	550134	1.22E-22	molecular function
4	ubiquitin carboxyl-terminal hydrolase L5	*uchl5*	406357	2.73E-17	cysteine-type peptidase activity, hydrolase activity, thiol-dependent ubiquitin-specific protease activity
5	polyhomeotic homolog 2a	*phc2a*	171465	1.25E-14	DNA binding, metal ion binding, zinc ion binding
6	RIO kinase 2	*riok2*	407084	1.30E-13	ATP binding, protein serine/threonine kinase activity, transferase activity, protein phosphorylation
7	PREDICTED: beta-1,3-galactosyltransferase 2 isoform X1	*b3galt2*	404633	5.58E-13	galactosyltranferase activity, protein glycosylation
8	mitogen-activated protein kinase 14a	*mapk14a*	65237	1.06E-12	ATP binding, MAP kinase activity, protein serine/threonine kinase activity, transferase activity, p38MAPK cascade
9	tumor protein p53	*tp53*	30590	7.96E-08	DNA binding transcription factor activity, chromatin binding, damaged DNA binding, metal ion binding, p53 binding
10	L-rhamnose-binding lectin CSL2-like	LOC110438301	110438301	4.11E-07	Function unknown
11	epithelial cell adhesion molecule precursor	*epcam*	62955155	1.01E-06	cell-cell adhesion, liver development, morphogenesis of an epithelium
12	si:ch211-226h8.11	LOC561329	561329	8.23E-06	Function unknown
13	poly A binding protein, cytoplasmic 1 b	*pabpc1b*	393856	1.42E-05	RNA binding, nucleic acid binding
14	abnormal spindle microtubule assembly	*aspm*	554173	2.43E-05	mitotic cell cycle, spindle organisation
15	chromatin modifying protein 1B	*chmp1b*	336426	2.97E-05	molecular function, protein transport, vacuolar transport
16	myocyte enhancer factor 2cb	*mef2cb*	798771	3.16E-05	protein dimerization activity, positive regulation of transcription from RNA olymerase II promoter
17	roundabout homolog 1-like	LOC101882342	101882342	3.96E-05	Function unknown
18	putative ribonuclease	LOC110438491	110438491	1.00E-04	Function unknown
19	death associated protein 1b	*dap1b*	58094	1.73E-04	apoptopic signalling pathway, negative regulation of autophagy, cellular response to amino acid starvation
20	prostaglandin reductase 1	*ptgr1*	494108	6.54E-04	oxidoreductase activity
21	zgc:171474	zgc:171474	100137113	9.14E-04	molecular function
22	PREDICTED: pericentriolar material 1 protein isoform X4	*pcm1*	321709	2.01E-03	centrosome cycle, cilium assembly
23	member RAS oncogene family b	*rab1bb*	415219	2.33E-03	GTP binding, GTPase activity, ER to Golgi vesicle-mediated transport
24	zgc:172323	zgc:172323	564165	4.00E-03	Structural molecule activity
25	SRSF protein kinase 1a	*srpk1a*	323679	4.29E-03	ATP binding, protein serine/threonine kinase activity, transferase activity, intracellular signal transduction,
26	zgc:92606	zgc:92606	100000242	1.14E-02	autophagosome assembly
27	gem (nuclear organelle) associated protein 8	*gemin8*	100000842	1.52E-02	molecular function, spliceosomal snRNP assembly
28	DnaJ (Hsp40) homolog, subfamily C, member 28	*dnajc28*	550341	1.81E-02	Golgi organization, golgi vesicle prefusion complex stabilization
29	zgc:110183	zgc:110183	550534	1.81E-02	molecular function
30	zgc:173816	zgc:173816	100126140	1.85E-02	metal ion binding, nucleic acid binding
31	si:ch73-299h12.8	si:ch73-299h12.8	101883754	1.85E-02	metal ion binding, nucleic acid binding
32	wu:fc33b09	wu:fc33b09	324574	2.38E-02	Function unknown
33	glutaryl-CoA dehydrogenase a	*gcdha*	393860	2.84E-02	acyl-CoA dehydrogenase activity, flavin adenine dinucleotide binding, oxidoreductase activity
34	ubiquinol-cytochrome c reductase core protein 1	*uqcrc1*	393793	2.98E-02	catalytic activity, metalloendopeptidase activity, aerobic respiration
35	apolipoprotein O-like	*apool*	541537	3.94E-02	cristae formation, integral membrane component
36	transmembrane protein 74	*tmem74*	100331423	4.05E-02	Function unknown

Table 4.3: Annotation of genes which are tending to be differentially expressed (n=9 within Q-value <0.1) between ovulated female zebrafish (*Danio rerio*) when exposed to different feeding regimes (high =3% of bodyweight daily and low= 1.5% of bodyweight daily)

No.	Description	Name	Gene ID	Q-value	Function according to NCBI
1	dCTP pyrophosphatase 1	*dctpp1*	393744	5.00E-02	nucleoside-triphosphate diphosphatase activity
2	suppressor of cytokine signaling 1a	*socs1a*	445073	5.00E-02	protein kinase inhibitor activity, negative regulation of JAK-STAT cascade, negative regulation of insulin receptor signalling
3	SCO2 cytochrome c oxidase assembly protein	*sco2*	606683	5.00E-02	copper ion binding, copper ion transport, cellular copper ion homeostasis
4	potassium intermediate/small conductance calcium-activated channel	*kcnn1b*	570401	5.48E-02	calcium-activated potassium channel activity
5	SUMO1/sentrin/SMT3 specific peptidase 2	*senp2*	556400	5.60E-02	cysteine-type peptidase activity, protein desumoylation
6	si:dkey-229d11.5	si:dkey-229d11.5	555353	5.76E-02	Not available
7	zgc:101858	zgc:101858	449555	6.68E-02	oxidoreductase activity
8	mucin-5AC-like	LOC110439410	110439410	8.34E-02	Not available
9	estrogen receptor 2a	*esr2a*	317734	9.70E-02	estrogen receptor activity, intracellular estrogen receptor signalling pathway

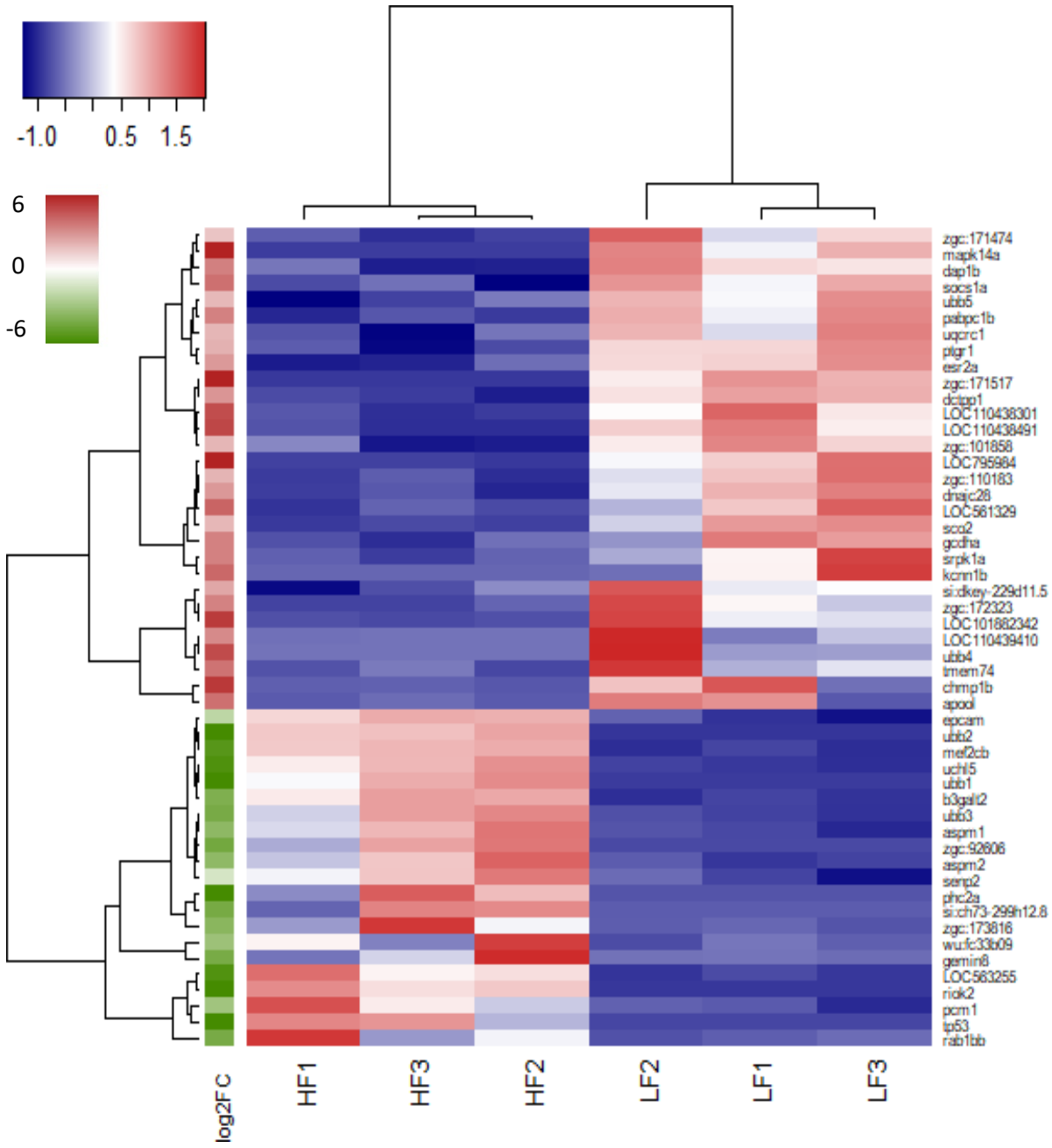

Figure 4.3: Heat-map of differentially (Q-value <0.05) and tending to be differentially (Q-value <0.1) ovarian genes in zebrafish (*Danio rerio*) when exposed to different feeding regimes (high= 3% of bodyweight per day and low= 1.5% of bodyweight per day). N=3 for each treatment. Expression levels were measured in fragments per kilobase of transcript per million mapped reads (FPKM) from normalised values (blue=lower expression and red= higher expression). The colour bar on the left represents the log2 fold changes in gene expression in the low females compared to the high females when the treatments are compared overall (red= higher expression, green=lower expression).

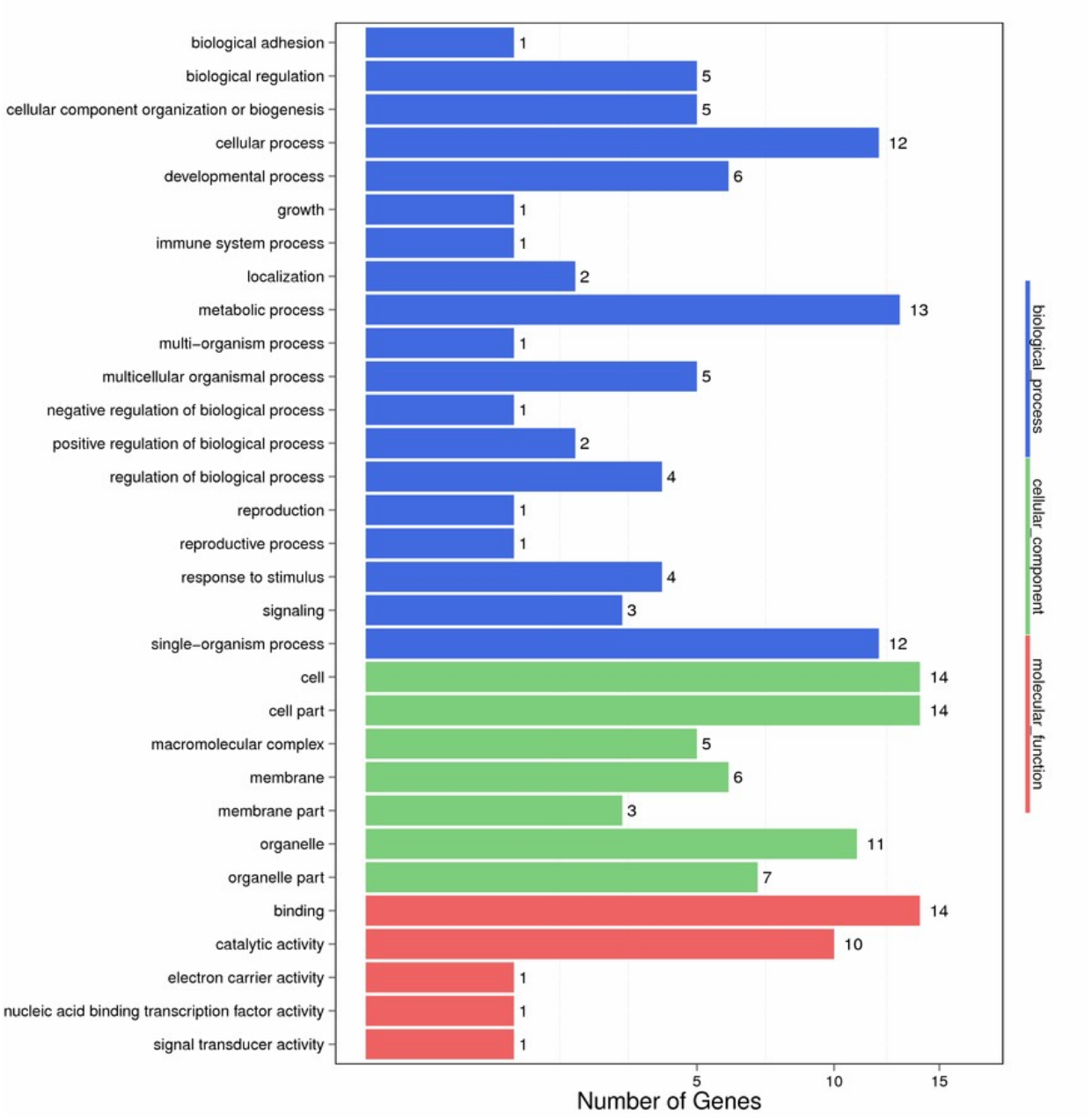

Figure 4.4: Gene ontology analysis of the genes which were differentially expressed in the ovaries of zebrafish, *Danio rerio*, when exposed to different feeding treatments, high (3% of bodyweight daily) and low (1.5% of bodyweight daily). Numbers beside the bars represent the number of differentially expressed genes in each pathway, genes have been accredited to multiple pathways (overall n= 45). The colours of the bars represent the broad gene ontology category each biological process fits into.

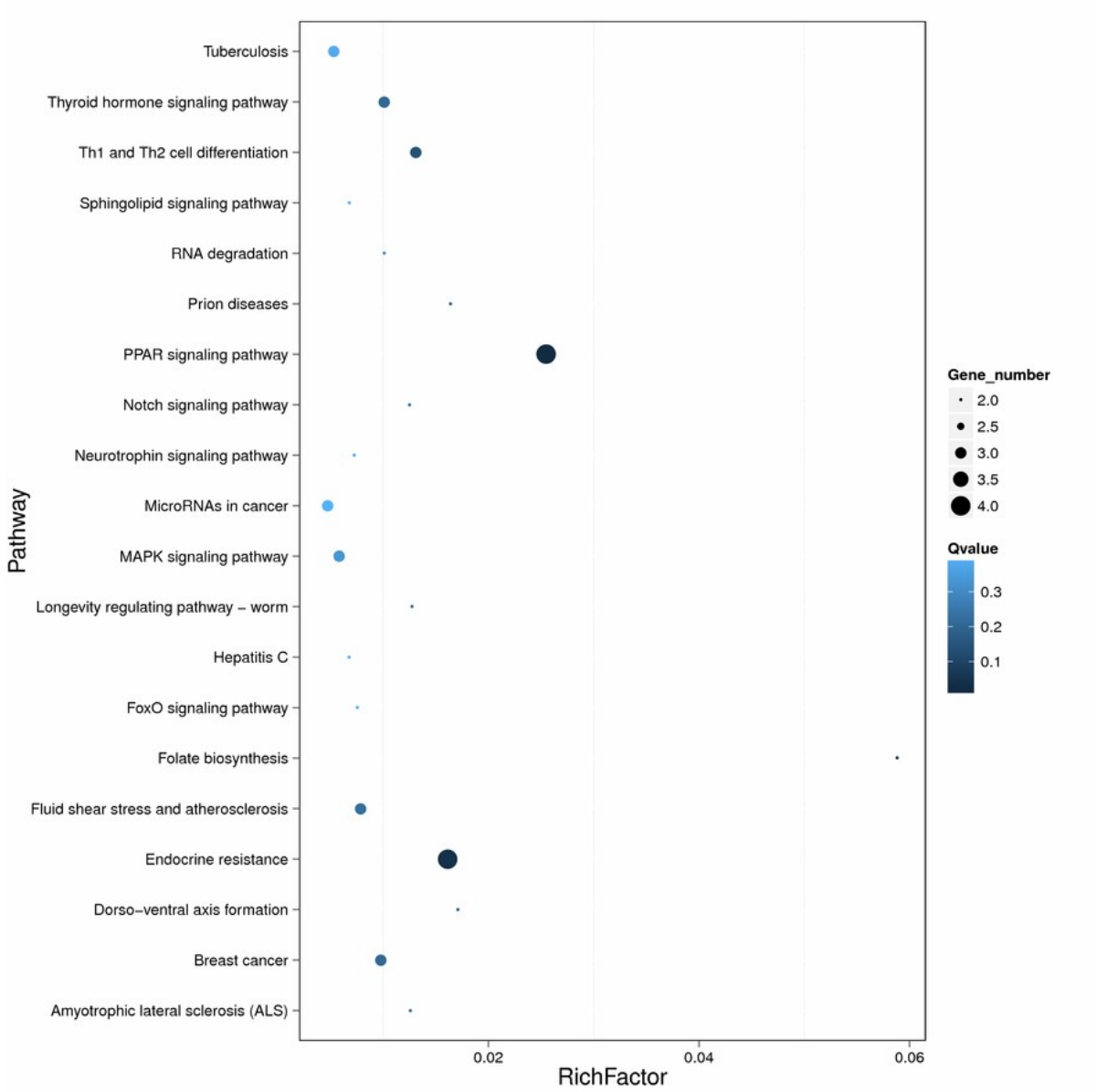

Figure 4.5: Kegg pathway analysis of the genes which were found to be differentially expressed in the ovaries of zebrafish, *Danio rerio*, when exposed to two nutritional environments, a high feeding group (3% of bodyweight per day) and a low feeding group (1.5% of bodyweight per day). The y-axis represents the enrichment factor, while the x-axis shows the pathways found in the analysis. Larger sized circles represent a higher number of genes that were differentially expressed in that pathway and the darker colour represents the more over-represented pathways. Q-value is a corrected p-value taking into account the large number of genes present in this analysis.

4.3.5 Potential Future qPCR Targets

The estrogen receptor beta (*esr2a*) was found to be differentially expressed with an FDR of 0.09 (Figure 4.6, A). To evaluate other candidate genes for future research, the FPKM of genes related to the estrogen/steroid hormone pathway were analysed between feeding regimes. The ones that showed a tendency to be different between the feeding regimes are shown below. The estrogen receptor had a trend for higher expression in the low feeding regime, when compared to the females on the high feeding regime. The mRNA levels of zona pellucida proteins (*zp3* and *zp2.3*) and the cathepsins (*ctsba* and *ctsla*) followed a similar trend (Figure 4.6, C, D, E and F). Aromatase (*cyp19a1a*), interestingly was not found to be expressed at all in the ovarian transcriptome of the fish on the low feeding regime, with only low expression found in the high fed females (Figure 4.6, B). Activin (*acvr2aa*) was found to have a trend for higher transcript abundance in the fish subjected to the high feeding regime compared to those in the low regime (Figure 4.6, G).

Table 4.4: Potential endocrine and estrogen-related genes for future qPCR research from the RNA-seq results comparing female zebrafish, *Danio rerio*, ovaries when exposed to different nutritional environments, high (fed 3% bodyweight daily) and low (fed 1.5% bodyweight daily). All p-values are before an FDR was applied.

Gene ID	Description	Gene Name	Function	p-value
317734	estrogen receptor 2a	*esr2a*	Estrogen receptor activity, RNA polymerase II transcription factor	0.0002
114439	zona pellucida glycoprotein 2, tandem duplicate 3	*zp2.3*	Extracellular matrix	0.001
30594	zona pellucida glycoprotein 3	*zp3*	Extracellular matrix	0.025
321453	cathepsin La	*ctsla*	Proteolysis involved in cellular protein catabolic process	0.046
664700	zona pellucida glycoprotein 2, tandem duplicate 1	*zp2.1*	Extracellular matrix	0.070
406645	cathepsin Ba	*ctsba*	Proteolysis involved in cellular protein catabolic process	0.075
30390	cytochrome P450, family 19, subfamily A, polypeptide 1a, aromatase	*cyp19a1a*	Regulation of estrogen biosynthetic process	0.075
1E+08	activin A receptor type 2Ab	*acvr2ab*	transmembrane receptor protein serine/threonine kinase signalling pathway	0.082
558677	zona pellucida glycoprotein 3a, tandem duplicate 2	*zp3a.2*	Extracellular matrix	0.098
553359	activin A receptor type 2Aa	*acvr2aa*	transmembrane receptor protein serine/threonine kinase signalling pathway	0.100
64815	hydroxysteroid (17-beta) dehydrogenase 8	*hsd17b8*	Steroidogenesis	0.100

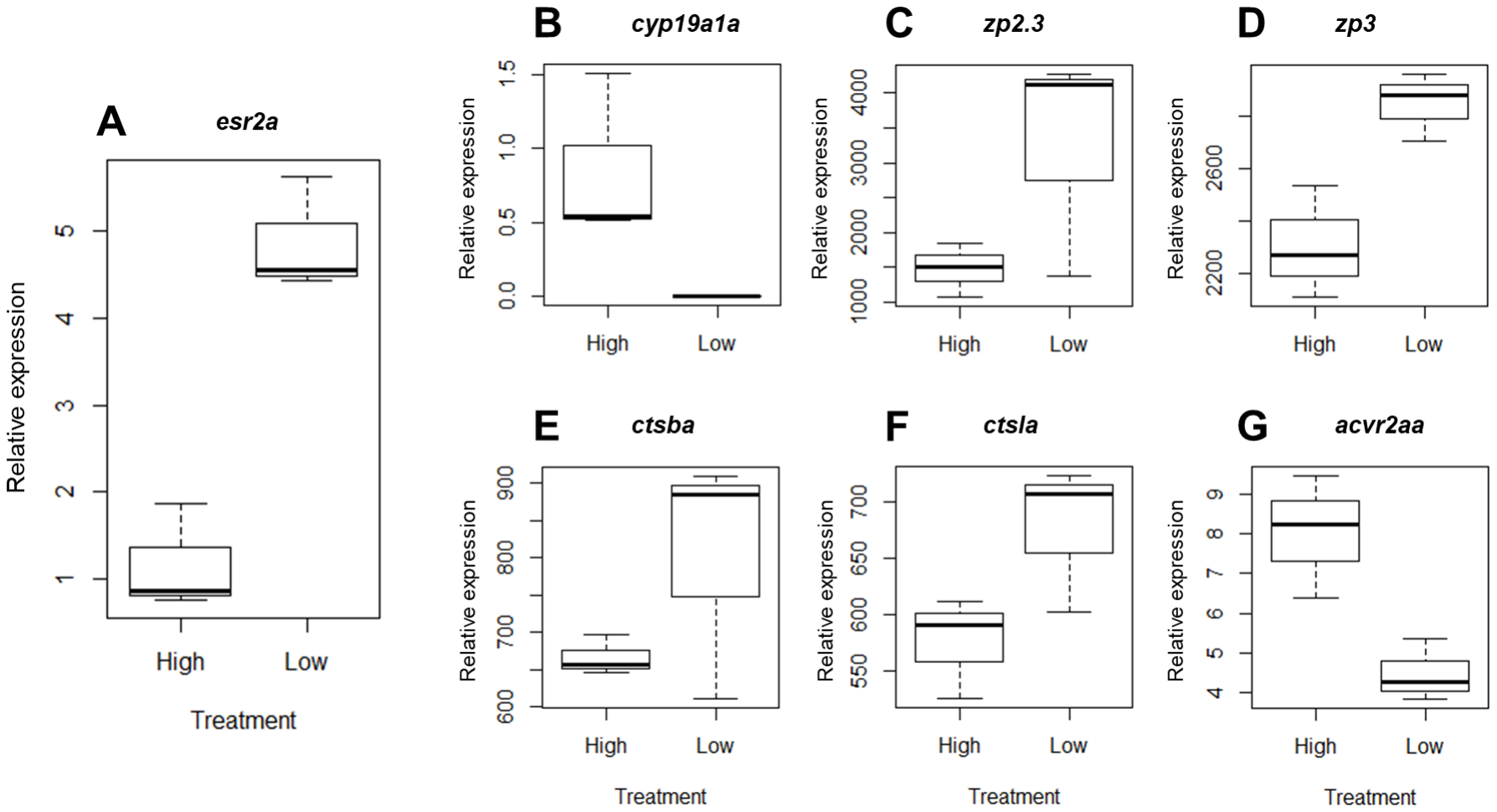

Figure 4.6: Relative transcript abundance of potential future qPCR targets related to the estrogen pathway, n = 3 for each treatment in all figures. For detailed gene information refer to table 4.4.

4.4 Discussion

Understanding the molecular mechanisms determining oocyte size and fecundity in different nutritional environments will have wide-reaching implications across many fields. Studies which investigate the response of an organism at the transcriptome level, offer chances to understand potential underlying genetic causes of adaptation and phenotypic response (Smith et al. 2013).There have been very few studies that investigate molecular mediators for the trade-off between offspring size and number (Forbes et al. 2010, Green and Extavour 2014). This study looked at using RNA-Seq Illumina sequencing technology to compare ovaries from zebrafish, *Danio rerio*, after they were exposed to different levels of resources (food) in the environment. DEG identified through this process were used to identify target genes for future research.

As a model species with a fully sequenced genome was used, all gene transcripts were annotated and both gene ontology and pathway analysis were performed. Overall, 36 differentially expressed genes were identified when the ovarian transcriptomes of the fish subjected to a low feeding regime (LF), were compared with the transcriptomes of the fish kept on a high feeding regime (HF). There were both up and down-regulated genes, with a higher proportion of transcripts up-regulated in the low feeding group (29 genes) when compared to the high feeding group (16 genes up-regulated). These were split between a range of different biological processes and pathways, a few of which were considered over-represented.

Overall there was a large level of heterogeneity in biometrics seen within the different feeding regimes. Due to complexities in the ovarian states of the females at the end of the experimental period, some spawned and some ovulated, choosing samples representative of the entire population was difficult. As seen in the multidimensional scaling plot, the samples chosen for transcriptome were more closely clustered towards each other, regardless of feeding regime, than the other fish. This means the molecular results obtained in this chapter may not be truly representative of the effects of the different feeding regimes. In future studies, a design with a single endpoint may provide clearer responses.

As there was high variation between the biological replicates, limited clustering shown within treatment groups and large variation in the expression levels of most genes within treatment groups, a two-pronged approach was used in the analysis. Initially both the gene ontology and pathway analysis were considered for candidate genes. Due to the lack of differentially expressed genes overall, the cut-off for the FDR was extended to 0.1. This meant that a nuclear estrogen receptor, *esr2a*, was considered differentially expressed. The estrogen receptor provided the first glimpse into possible endocrine mediators of phenotypic plasticity in follicle size and fecundity.

4.4.1 Gene Ontology and Pathway Analysis

In the whole analysis there were only 36 genes that were differentially expressed, (FDR = 0.05) or 45 if the FDR was extended to <0.1, out of over 24,000 genes that had sufficient quality and expression levels to be analysed. This low number of differentially expressed genes may be due to the lack of similarity seen between biological replicates and the low power of this experiment in general. Due to budget constraints, only 3 samples representative of each feeding regime were sequenced. Limited biological replicates result in low statistical power to detect lower fold changes in expression between treatments. The effects of intra-treatment variation also play a much larger role when fewer replicates are sampled (Todd et al. 2016).

There were five biological processes and pathways that were considered over-represented, when the ovaries from the low fed and the high fed fish were compared. In general, these only had two or three differentially expressed genes mapped to the specific pathway. One over-represented pathway was the peroxisome proliferator-activated receptor signalling pathway (PPAR). This pathway is involved in intra and extra-cellular lipid metabolism and is up-regulated in organisms undergoing fasting or food-limited periods (de Lange et al. 2007, Fuentes et al. 2013). As the current study is looking at differences in between fish exposed to different feeding regimes, this pathway could be up-regulated in LF females, indicating they are under food-limited conditions (de Lange et al. 2007). However, the PPAR pathway only had 4 transcripts of ubiquitin B

mapped to it, which were both up and down-regulated (Appendix 3 – Figure A.4). Due to the overall lack of genes that were mapping to each pathway in the analysis and the large numbers of pathways represented overall, it is difficult to identify any treatment effect here.

4.4.2 Potential Sources of Error or Bias

The statistical power to detect true expression differences between treatments or populations of data is reflective of the ability to distinguish the treatment effects from background noise (Busby et al. 2011, Todd et al. 2016). There are three main sources of noise or false readings in any RNA-seq analysis, measurement error (or Poisson counting error), technical variation and biological variation (Busby et al. 2011, Hansen et al. 2011, Todd et al. 2016). The extent of these errors and how they are managed can significantly impact the accuracy of assigning differentially expressed genes and the reliability of the conclusions that can be drawn (Busby et al. 2011, Todd et al. 2016).

Poisson counting error and biological variation may be playing a role in the accuracy of this analysis. Poisson counting error, is the inherent uncertainty that is present in any count-based measurement like an RNA-seq (Busby et al. 2011). The shotgun sequencing method used in RNA-seq, only measures a small fraction of the entire RNA population in a sample, which is why sampling depth is also important. Poisson error is disproportionately large for low count data and can be a large part of the variation seen in expression counts that are measured below 10 (Busby et al. 2013). Filtering any genes with expression counts below 10 can drastically reduce the effect of Poisson error (Busby et al. 2013, Todd et al. 2016). In this analysis 19 out of the 45 differentially expressed genes measured had expression levels (FPKM) that were below 10. Due to Poisson error it is unlikely that these accurately reflect the true expression level of the genes shown and that the treatment effect seen in these genes could be created by the bias caused by this uncertainty.

There was a high variation seen between biological replicates when the entire transcriptomes were compared between all 6 fish regardless of feeding regime. This may

be due to the zebrafish being obtained as a wildtype from a pet supplier, rather than a purpose bred laboratory strain. Wild populations inherently have higher variation between individuals (Todd et al. 2016).

Another opportunity to introduce bias into the analysis, is the random variation that inherently occurs in gene expression between samples (Hansen et al. 2011). Gene expression is considered stochastic or random and is known to vary considerably between units of the same population of samples (Elowitz et al. 2002). At any given time, it is highly unlikely that biological replicates will be at the exact same level of development. For the samples from my experiment, there was high variation in mRNA transcript abundance between biological replicates. For some of the genes that were considered differentially expressed between treatments, there were 10, 20 and even 50-fold changes in transcript abundance within the same biological treatment group. Increasing the number of biological replicates in future experiments could reduce the overall effects of biological variation and produce more robust datasets (Todd et al. 2016).

In this experiment, it could be argued that due to both the high level of low count expression data (Poisson counting error or measurement error) and biological variation, it is likely that the majority of the differentially expressed genes may be a result of introduced bias. Even though a false detection rate (Bonferroni correction) was used to correct for false positives introduced by the sheer size of the dataset, it may be wise to also carefully consider both Poisson counting error and biological variation before considering a gene to have a significant treatment effect.

4.4.3 Potential Candidate Genes for Future Exploration

The link between nutritional state or body condition and reproductive traits such as fecundity has been well established in a variety of fish species (Duan 1997, Campbell et al. 2006). It is reasonable to infer that an endocrine signal may be playing a major role in the signalling of the changing nutritional state of the organism and potentially

mediating the phenotypic changes of reduced fecundity and larger egg size in food-limited individuals.

Estrogens are the major female sex hormones and have many different functions in both reproductive, and non-reproductive tissues (Mueller and Korach 2001, Hewitt et al. 2005, Lu et al. 2017). Nuclear estrogen receptors, such as the estrogen beta receptor (*esr2a*) bind to estrogen and the estrogen/receptor complex binds to transcriptional machinery to cause changes in expression of different genes (Couse and Korach 1999, Klinge 2000, Hewitt and Korach 2002). In zebrafish, *esr2a* has been shown to be the primary nuclear estrogen receptor present in the ovaries and the only receptor expressed in the oocytes themselves (Lu et al. 2017). In the ovaries of the fish that were kept on a low feeding regime, there was a higher abundance of the *esr2a* transcripts. This could provide a first glimpse into the molecular mechanisms regulating the trade-off between egg size and fecundity. Using this receptor as a starting point, related genes that may not have been considered differentially expressed in this analysis were considered. Potential candidate genes were chosen for future research, where larger sample sizes should be used.

Multiple studies have found that exposure to higher levels of estrogen (majority looking at environmental toxins) will cause an increase in the production of zona pellucida proteins in fish (Arukwe et al. 2000, Thomas-Jones et al. 2003, Santos et al. 2007). In zebrafish, the zona pellucida proteins (Zp2 and Zp3) are secreted by the developing oocytes during the primary growth phase, and are deposited as an extracellular matrix, forming a layer between the oocyte and surrounding follicle cells (Mold et al. 2009). Recently, a study looking at the specific effects of the nuclear estrogen receptors in zebrafish, demonstrated that when *esr2a* was knocked out, the levels of zona pellucida proteins present in the oocytes were significantly reduced. Earlier hatching also occurred due to misshapen chorion envelopes (Lu et al. 2017). In the current experiment, there were trends in *zp3* and *zp2.3* which mirrored the trends found in the expression of *esr2a* between the two treatment groups. Changes in these proteins could create different hatching times between treatments and convey selective advantages to the offspring.

Cathepsins belong to the endosomal/lysosomal protease family and are widely distributed in many tissue types (Roberts 2005, Tingaud-Sequeira et al. 2011). Certain cathepsins, including cathepsin D, L and B, have been implicated in the metabolism of yolk proteins in the developing oocytes (Matsubara and Sawano 1995, Tingaud-Sequeira et al. 2011, Palomino et al. 2017). Cathepsin D (ctsd) cleaves vitellogenin molecules into yolk proteins inside the oocyte (lipovitellins and phosvitins), during vitellogenesis (Matsubara and Sawano 1995, Hiramatsu et al. 2002). Cathepsin B (ctsb) and L (ctsl) have been implicated in the hydrolysis of yolk proteins that occurs during oocyte maturation (Matsubara et al. 2003, Tingaud-Sequeira et al. 2011, Palomino et al. 2017). This processing at the maturation stage produces a free fatty acid pool that is essential for hydration of the oocyte and subsequent absorption as an energy source by the embryo (Ohkubo and Matsubara 2002, Palomino et al. 2017). Cathepsin La (ctsla) has been shown to be expressed in developing embryos in zebrafish and it has been suggested that this is the main cathepsin involved in embryonic yolk processing to allow subsequent absorption (Tingaud-Sequeira and Cerda 2007). There was a general increase in both *ctsba* and *ctsla* mRNA transcript expression in the ovaries of the food-limited females which could suggest larger quantities of yolk present. This would support the expected phenotype of larger eggs being produced in food-limited mothers (Forbes et al. 2010).

4.4.4 Conclusions

Overall, due to high levels of biological variation and other introductions of bias, the 36 differentially expressed genes identified by RNA-seq are hard to reconcile with changes in feeding regime. A future study with larger numbers of biological replicates may provide further insight into patterns in gene expression in the ovary when exposed to different nutritional environments. Through exploration of the pathways related to the differentially expressed estrogen beta receptor, candidate genes for future analyses were identified.

Chapter Five

General Discussion

The amount of resources available to any given organism is directly determined by the environment in which they reside. Any resources obtained by a mother are finite and must be divided between competing life history traits (Auer 2010, Burton et al. 2010, Jørgensen et al. 2011). This means maternal provisioning of one component of reproduction will reduce the resources available to another (Auer 2010). One classically studied trade-off between competing traits, is the relationship between the size and the number of offspring produced (Smith and Fretwell 1974, Reznick et al. 2002, Jørgensen et al. 2011, Riesch et al. 2012). This relationship is directly dependent on both the resources available in the local environment and the amount of physical space the mother has available for reproduction (Uller and Olsson 2005, Bauerfeind and Fischer 2008, Auer 2010, Jørgensen et al. 2011). If external cues are reliable, a mother can adjust the phenotype of her offspring to maximise her fitness in any given environment (Pigliucci et al. 2006, Kuijper and Johnstone 2013). Many studies have demonstrated that in resource-limited environments, mothers can increase the investment in each individual offspring, but reduce the number of offspring produced. Conversely, it has been shown that in the same species, in environments which are resource abundant, large clutches of comparatively small offspring are produced (Reznick and Yang 1993, Bashey 2003, Fischer et al. 2003, Bashey 2006, Hassall et al. 2006, Forbes et al. 2010, Riesch et al. 2010, Riesch et al. 2012). These maternal effects (phenotypic plasticity in offspring) are directly related to both offspring and maternal fitness (Jørgensen et al. 2011, Kuijper and Johnstone 2013). Although adaptive plasticity in offspring size and number has been clearly demonstrated in a range of species, there are few studies that evaluate the mechanisms that allow this variation to occur.

In invertebrates, such as *Drosophila*, the fecundity of the female has been directly linked to the metabolic hormone, IGF-1 (Green and Extavour 2012). In locusts, offspring size and number have been demonstrated to be mediated by the upstream regulation of vitellogenin (Chen et al. 2015). In vertebrates, both gonadotropins and upstream regulation of vitellogenin expression have been shown to directly influence both fecundity and offspring size, although studies have focused mainly on mammals, birds and lizards (Sinervo and Licht 1991, Sinervo 1999, Williams 2001, Oksanen et al. 2002).

This thesis aimed to elucidate mechanisms allowing for adaptive plasticity in offspring phenotype when exposed to different resource availability in the environment. Zebrafish, *Danio rerio*, have previously demonstrated adaptive plasticity in both offspring size and number (Forbes et al. 2010), and as a widely established model organism (Ribas and Piferrer 2014) were deemed appropriate to investigate molecular mediators of offspring size and offspring number. Female zebrafish were exposed to either a low feeding regime (1.5% of body weight) or a high feeding regime (3% of body weight) and both phenotypic changes in fecundity, offspring size and gene expression of candidate genes in the ovary were evaluated. In a second experiment to evaluate the sensitivity of the plasticity response, female zebrafish were exposed to first one feeding regime and then swapped to the other feeding regime. Phenotypic changes in fecundity and offspring size were evaluated.

Mothers exposed to harsh or limiting environments can increase offspring survival by manipulating offspring phenotypes (Winemiller and Rose 1993, Jorgensen et al. 2014, Paul et al. 2015). Larger offspring have a selective advantage in limited environments, increasing maternal fitness through increased survival when compared to smaller, less developed offspring (Riesch et al. 2010, Murphy et al. 2014). Although these maternal effects in zebrafish have previously been demonstrated (Forbes et al. 2010), this adaptive plasticity was not recorded in my experiments, as females in both food-abundant and food-limited environments produced similar sized offspring.

When fish were analysed regardless of feeding regime, there was a positive relationship between fecundity (or reproductive investment) and offspring size. A positive relationship between maternal body size and offspring size was also found. The larger females that were obtaining the most food, were producing both larger offspring, and more of them. There was clear heterogeneity in maternal condition or quality within both feeding regimes. Each tank had larger dominant females present, and these fish were likely to have limited access to the food for the subordinate females (Filby et al. 2010). This meant that the effects of environmental resource availability and any phenotypic plasticity present was obscured between the feeding regimes.

When female condition was not corrected for, there was no trade-off between offspring size and number occurring in the female zebrafish overall. When offspring size was

standardised over female body weight, a negative relationship was found between this value and reproductive investment. This indicates that at an individual level the trade-off may still be present (Lim et al. 2014). Individuals vary in the way they obtain resources. When there is heterogeneity in both resource allocation and resource availability in a population, correlations between competing traits can appear positive (Reznick et al. 2000, Lim et al. 2014). This highlights the importance of controlling for individual quality when looking at phenotypically expressed trade-offs (Lim et al. 2014).

Oogenesis is heavily controlled by the pituitary through the production of the gonadotropins, luteinising hormone and follicle stimulating hormone (Fsh). In many species, recruitment into the secondary growth phase and vitellogenesis is regulated by Fsh. For example, super ovulation in mammals is induced by exposure to gonadotropins (Fowler and Edwards 1957). When bank voles, *Clethrionomys glareolus*, were exposed to human menstrual urine (which contains both luteinising hormone and FSH), the number of pups produced per litter increased, and on average these were smaller (Oksanen et al. 2002). Moreover, in the side-blotched lizards, *Uta stansburiana*, when individuals were exposed to ovine FSH, clutch size increased and concomitantly egg size decreased (Sinervo and Licht 1991, Sinervo 1999). They suggested that the number of eggs present in the clutch was under the control of circulating levels of gonadotropins, specifically FSH but that the egg size was set by the number of follicles undergoing vitellogenesis (Sinervo and Licht 1991). In teleosts, when rainbow trout (*Oncorhyncus mykiss*) were subjected to a unilateral ovarioectomy, levels of Fsh in the plasma increased concomitantly with recruitment of a new cohort of oocytes into the secondary growth phase in the remaining ovary (Tyler et al. 1997). Recently, gene knockout studies looking at the specific effects of gonadotropins and the gonadotropin receptors in zebrafish have demonstrated that Fshr is essential for oocytes to develop past the primary growth stage (previtellogenesis-vitellogenesis transition) (Chu et al. 2015, Zhang et al. 2015, Li and Cheng 2018). In the current study, *fshr* mRNA levels tended to increase in fish in a food-abundant environment. This was also accompanied by a tendency for a higher fecundity in these fish when compared to food-limited females. As activation of Fshr is essential for the recruitment of oocytes into vitellogenesis (Chu et al. 2015, Li and Cheng 2018) changes in the level of this protein could be a mechanism

allowing for larger clutches overall. In mammals, the "fecundity gene" increases the number of follicles brought through to ovulation through preventing the down-regulation of *fshr* expression and preventing the loss of follicles to atresia (Moore et al. 2004, Moore and Shimasaki 2005). Limitation of space occurs in the mother (Uller and Olsson 2005), thus this increase in the number of follicles would decrease the overall size of the eggs produced. Future studies looking at the expression of the *fshb* in the pituitary or the plasma levels of Fsh in females exposed to different feeding regimes could illuminate upstream regulation of this pathway.

Vitellogenin is a key component of yolk, and therefore levels of vitellogenin play a direct role in the provisioning of the future larvae (Tyler et al. 1990, Sun et al. 2015). Vitellogenesis is often described as the major growth stage of oogenesis and in some species, yolk can account for up to 80 % of the final oocyte size (Tyler et al. 1991). In species where plasticity in offspring size is seen between populations, the larger eggs are often shown to have increased levels of lipoprotein when compared to the smaller ones (Guisande and Gliwicz 1992, Hassall et al. 2006, Sun et al. 2015). This is hypothesised to enhance initial survival of these offspring, as more yolk means more initial resources for the offspring (Sun et al. 2015). This can lead to both a larger initial size and faster development (Levitan 2000). In harsh environments, larger offspring have a selective advantage over smaller ones.

The production of vitellogenin and the uptake into the oocyte is mediated in vertebrates by circulating estrogens (Williams 2001). In zebra finches, *Taeniopygia guttata*, if females are treated with an estrogen receptor blocker, they show increased fecundity but smaller eggs overall. This decreased egg size, an 8 % decrease in egg mass, was accompanied by a 50 % decrease in plasma levels of vitellogenin (Williams 2001). In my study, transcriptome results showed a trend for an increase in the primary ovarian estrogen receptor, *esr2a*, in the females that were food-limited, when compared to females that had an abundance of food. When target genes were evaluated in ovary samples from females in phase one of experiment two, *lrp8* was also found to have a tendency for an increase in food limited females. In teleosts, *lrp8*, has been designated as the main vitellogenin receptor (Prat et al. 1998, Johnson 2009, Hiramatsu et al. 2013).

This could suggest that, as in birds, in teleosts the regulation of the vitellogenin uptake into the oocytes can mediate the size and number of offspring produced by a mother.

It is likely that the Fsh pathway and regulation of vitellogenin uptake play key roles in determining size and number of oocytes present in the ovary. However, based on the current study it is impossible to definitively state that these are involved in adaptive plasticity of offspring size. To clearly evaluate the effects of changes in the expression of these genes on both fecundity and offspring size, further research is required with females which clearly exhibit phenotypic plasticity in these traits.

For future research it would be wise to carefully consider the state of the target tissue at the end of the study. All of my experiments concluded with three different states of ovarian tissue, spawned, ovulated and unovulated ovaries. In the previous literature, studies looking at plasticity in offspring size have looked at spawned offspring or eggs (Forbes et al. 2010, Vrtílek and Reichard 2015). Due to low spawning rates across both feeding regimes this was not possible in these experiments. As zebrafish are asynchronous spawners (Selman et al. 1993), there are multiple clutches present in the ovary, and sampling across the top 20% of follicle size is not guaranteed to only measure follicles from a single clutch.

A consistent lack of spawning was seen across all experiments, with low incidence of spawning occurring in each feeding regime but not enough to allow for statistical analysis. Previous studies have shown that reproduction is cued by food availability (Lawrence 2006), which could explain the lower incidence of spawning that occurred in the low feeding regimes of the experiments in this thesis. In Forbes (2010), only three fish in the lower feeding regime spawned compared to eleven in the higher feeding treatment, showing a similar trend. Dominant females can inhibit the spawning of subordinate females through pheromones in the water (Gerlach 2006), as there were clearly larger dominant females in each tank and this could have produced a lower reproductive output in the experiment. In future studies, larger numbers of fish present in each tank could help diffuse aggression and dominance behaviour increasing reproductive output.

There was a lack of specificity in targeting the underlying mechanisms causing changes in offspring size, due to the treatment directly relating to body condition (Bernardo 1996). The molecular mechanisms underlying maternal effects are hard to discern due to unavoidable effects of the treatment on parental condition (Lim et al. 2014). Manipulating the environment rather than the organism allowed heterogeneity in resource acquisition, potentially through dominance behaviour (Filby et al. 2010), to blur the effects of the feeding regimes. In these fish, maternal size was found to be directly correlated with follicle diameter, so the larger dominant fish had larger follicles regardless of feeding regime. Increasing the stocking densities in individual tanks can mitigate dominance behaviour in this species (Filby et al. 2010). As the fish grew the strength of the negative relationship between offspring size and offspring number decreased. This meant there was lower pressure to show adaptive plasticity as the constraints of space limitation are removed (Uller and Olsson 2005) and the large fish with abundant resources could produce larger numbers of large offspring. In future studies, higher stocking levels and choosing younger fish that have higher energy constraints and less space for offspring could remove some of the heterogeneity seen in the current study. Other species who do not have this relationship may also be more appropriate to study changes in resource allocation between different environments (Lim et al. 2014).

Based on the experiments carried out for this thesis, the study of phenotypic plasticity responses under different resource levels in the environment is complex due to the effects of maternal condition. Previous literature, and to an extent my study, have shown that fecundity will increase in food abundant conditions. This is likely to be mediated through increases in the expression of the Fshr in teleosts and potentially pituitary Fsh as well, stimulating more oocytes to enter vitellogenesis. Future exploration of increases in the estrogen receptor and vitellogenin receptor in food-limited ovaries may illuminate the mechanisms that allow for increased provisioning of individual offspring.

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Appendix

Appendix 1 – Collagenase Digestion Trials

Methods:

Five female zebrafish were euthanised in 1 ml of benzocaine per 200 ml of water.

Ovary tissue was removed and separated evenly between 6 different falcon tubes.

These falcon tubes contained zebrafish ringer and varying concentrations of collagenase, 0.5, 0.75 and 1 mg/ml.

The zebrafish ringer contained:

116mM NaCl

2.9 mM KCl

1.8 mM $CaCL_2.6H_2O$

5 mM HEPES

1 g/l glucose

One falcon tube at each collagenase concentration was placed on a shaker at a gentle speed at room temperature. The other three falcon tubes were placed upright in a rack on the bench beside the shaker.

They were incubated for an hour.

Results:

All falcon tubes even at the lowest collagenase concentrations contained yolk globules floating in the medium from digestion of the largest mature follicles. No concentration was found to be suitable for use in the experimental analyses (Figure A1).

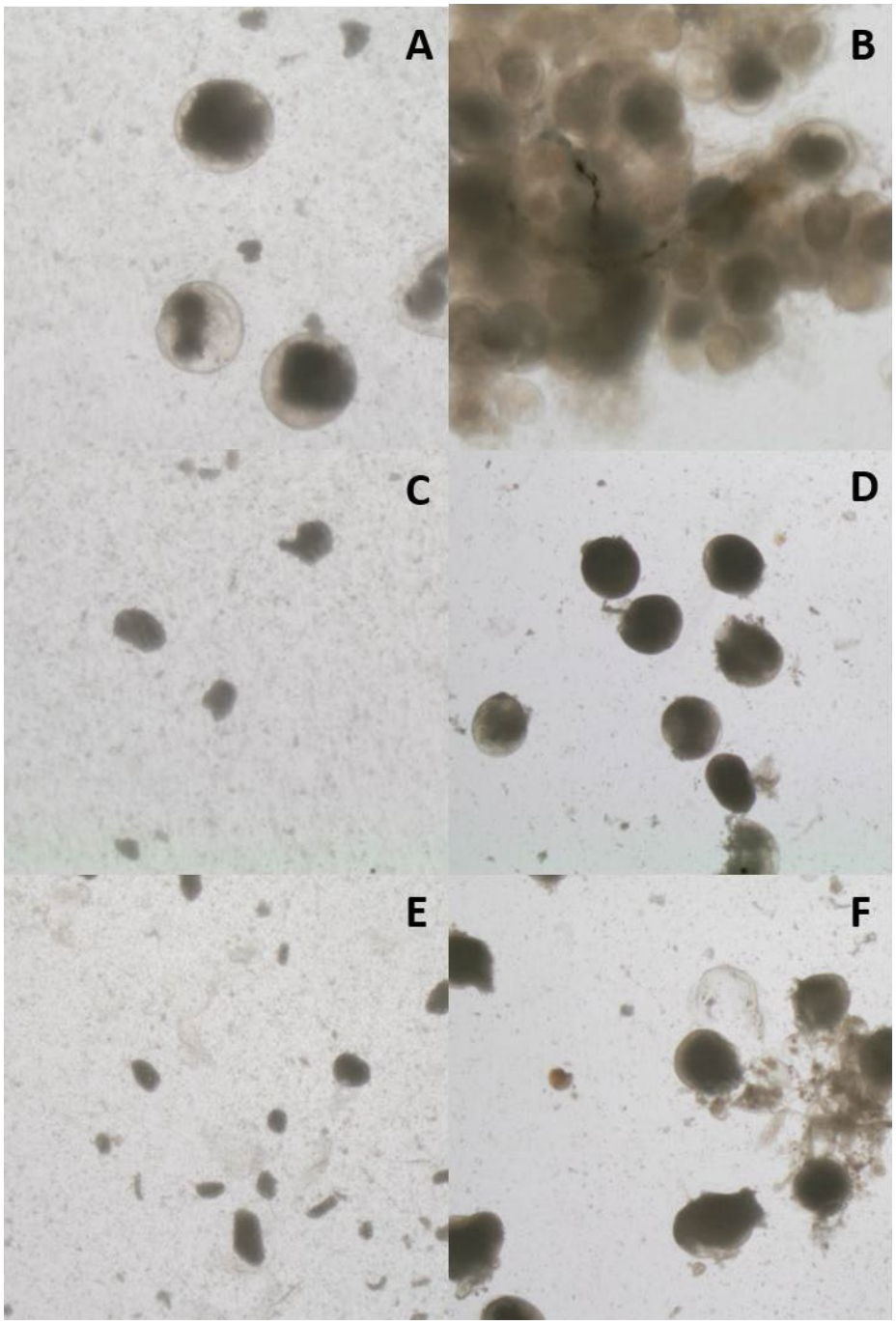

Figure A1: Collagenase digested ovarian tissue from female zebrafish, *Danio rerio*. Six different treatments were used; 0.5 mg/ml collagenase + shaker (A), 0.5 mg/ml collagenase (B), 0.75 mg/ml collagenase + shaker (C), 0.75 mg/ml collagenase (D), 1.0 mg/ml collagenase + shaker (E), 1.0 mg/ml collagenase (F).

Appendix 2 – Spawned Fish from Experiment 2a and 313

During the 2016 lab work component of ZOOL313 an experiment was set up to evaluate the effect of food availability in the environment on fecundity and egg size in zebrafish (*Danio rerio*). Fifty mixed sex females were divided evenly between 8 different tanks, these were randomly designated to two feeding regimes, 1.5% or 3% of body weight daily. After four weeks fish were placed in spawning tanks with a male overnight. Any eggs spawned were collected and counted. Yolk diameters were measured. Eggs were kept in petri dishes until hatched and initial hatchling size was measured.

Spawning was relatively unsuccessful, only 1 low feeding regime female spawned and 3 high feeding regime females.

In the Experiment 2a females, 4 fish also spawned. There were 3 females on the low feeding regime that spawned and 1 on the high feeding regime.

The yolk diameters were comparable between the experiments. To look for trends the experiments were combined. It appears that there is a strong trend for a reduction in yolk diameter and hatchling length in females that are in food-abundant environments when compared to food-limited females. (Figure A.2 and Figure A.3)

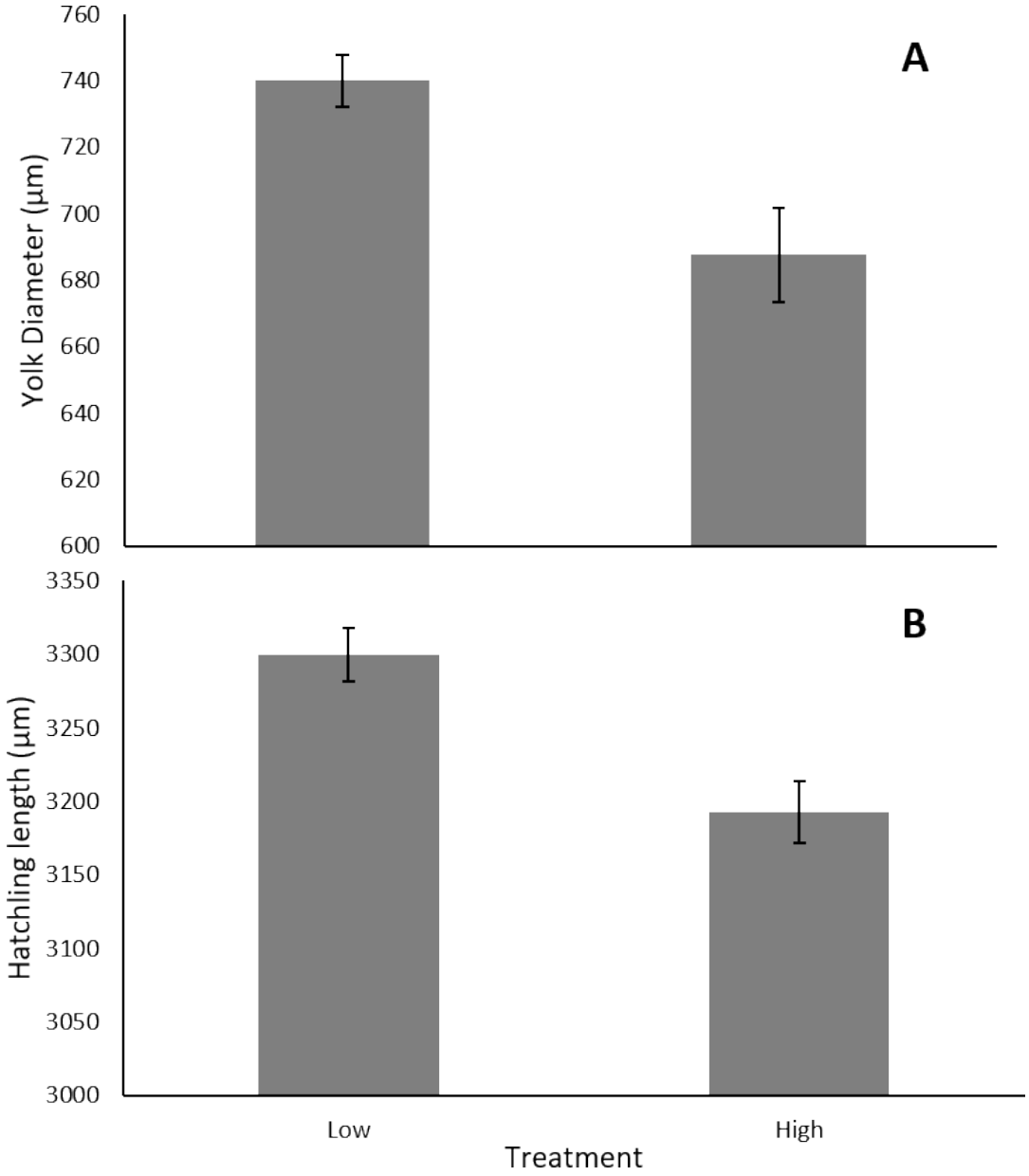

Figure A.2: Average yolk diameter (µm) and hatchling length (µm) in female zebrafish, *Danio rerio*, when they are fed 1.5% (Low) or 3% (High) of body weight daily. Bars represent the average value across all fish present ± 1 se, n (number of fish) = 4 for each feeding regime.

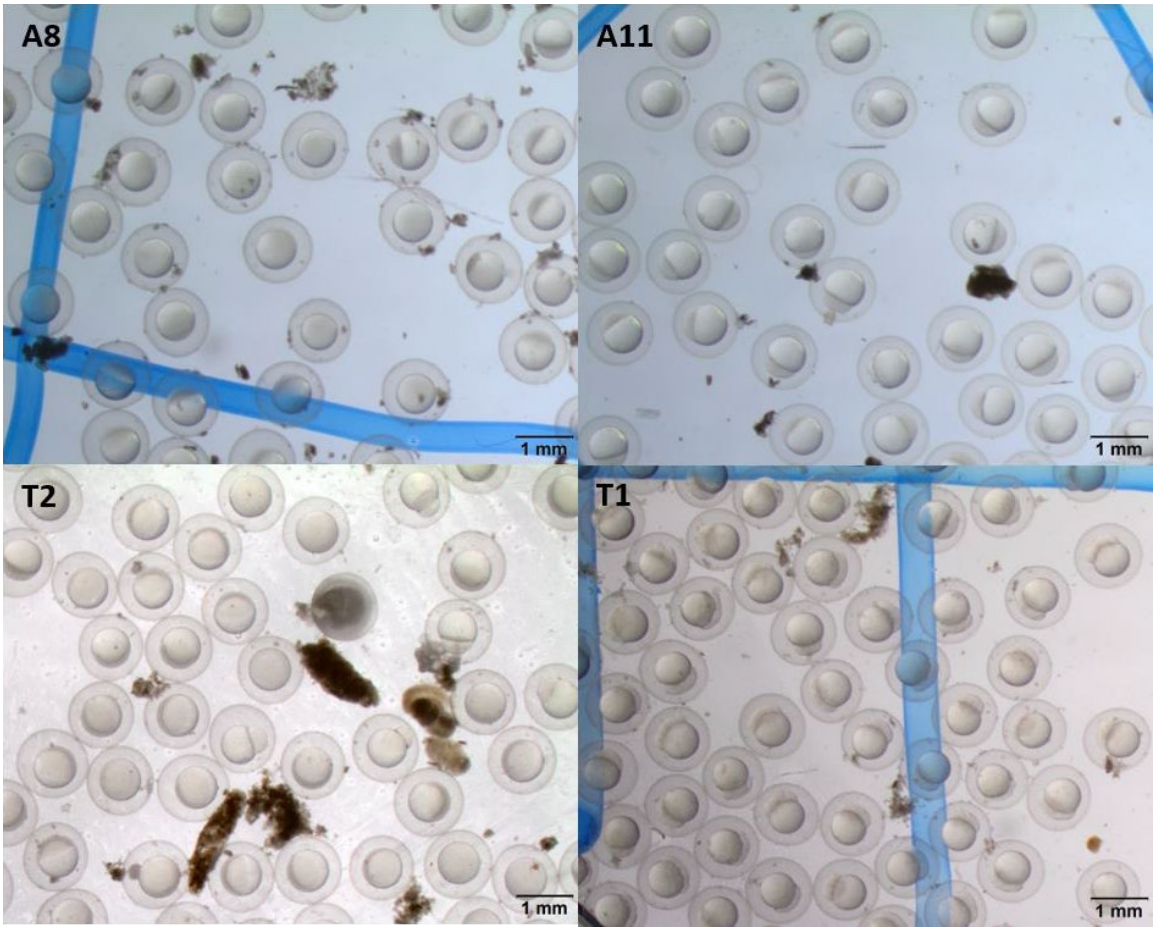

Figure A.3: Photos of eggs spawned when female zebrafish are exposed to different feeding regimes; 1.5% (A8, T2) or 3% (A11, T1) of body weight daily.

Appendix 3 – PPAR Enrichment from Gene Ontology Analysis

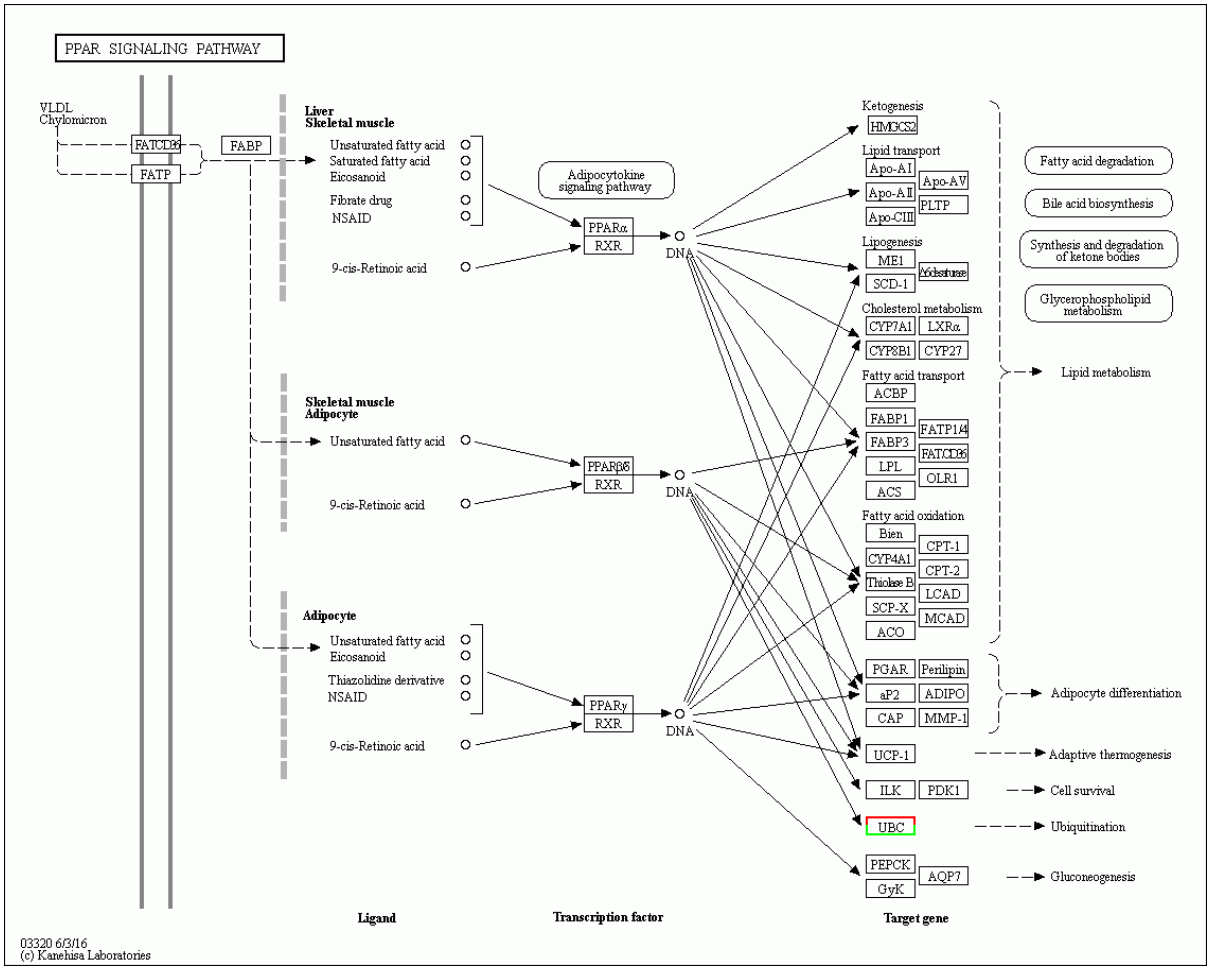

Figure A.4: PPAR pathway enrichment by differentially expressed genes identified in zebrafish, *Danio rerio*, ovarian tissue by RNA-Seq analysis

Appendix 4 – Average Body weight and GSI for Phase 2 of Experiment 2

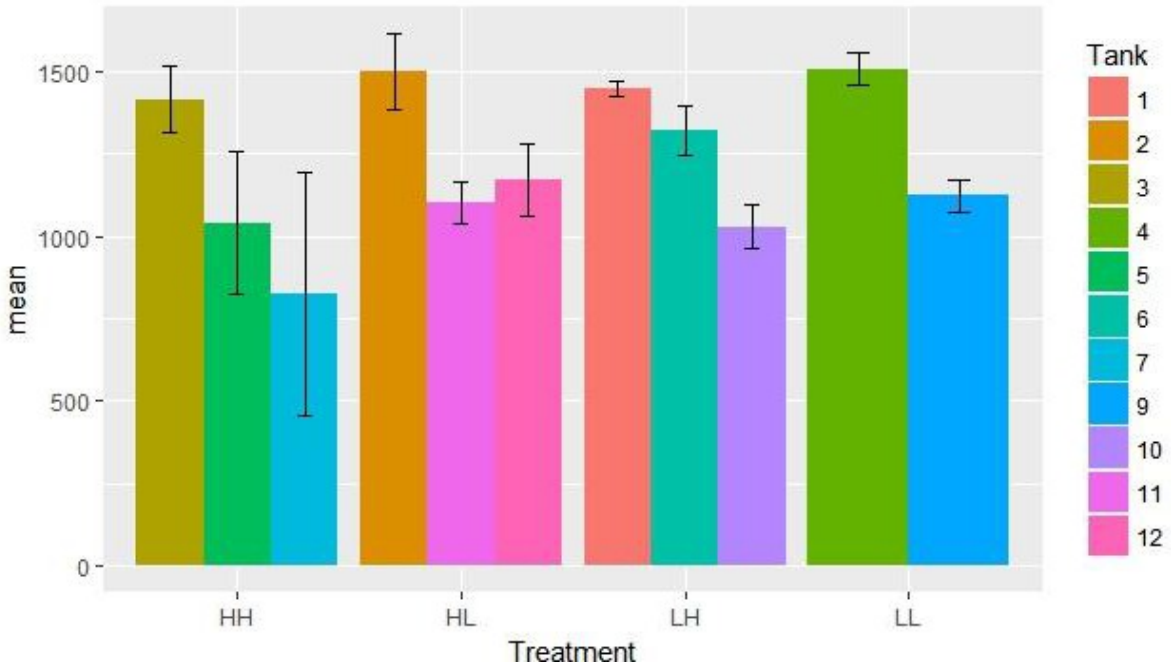

Figure A.5: The average body weight (mg) of female zebrafish, *Danio rerio*. There were four different treatments present all consisting of a combination of two different feeding regimes; Low: 1.5% of body weight or High: 3% of bodyweight, in four-week phases. Each bar represents the average body weight per tank ± 1 se**.**

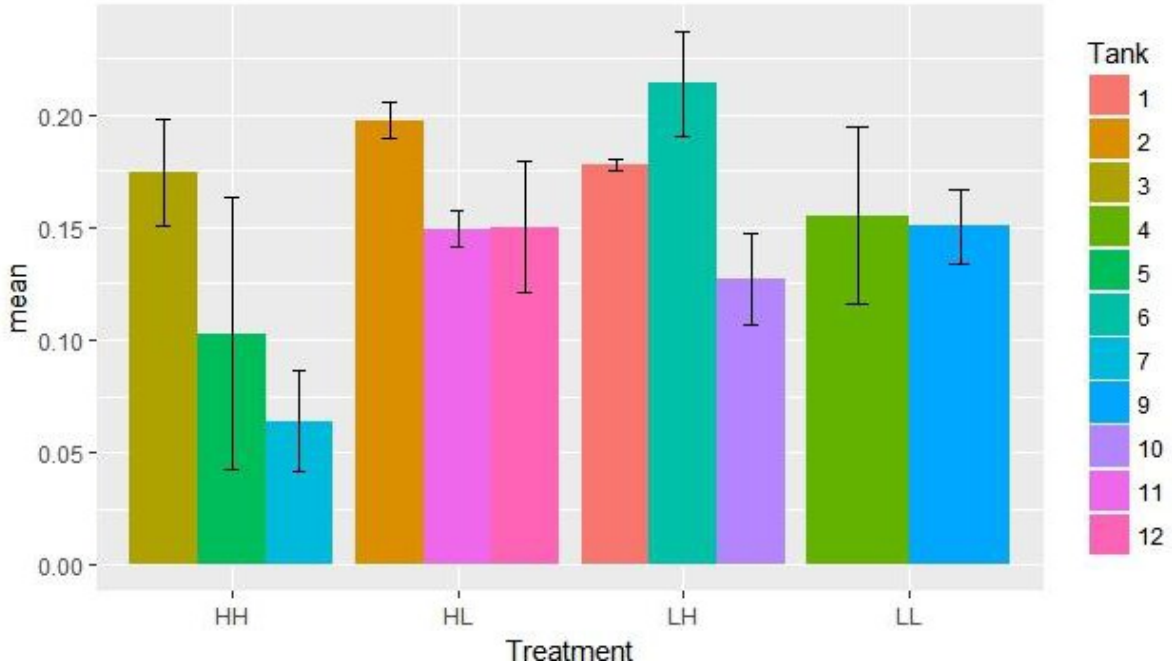

Figure A.6: The average GSI (%) of female zebrafish, *Danio rerio***.** There were four different treatments present all consisting of a combination of two different feeding regimes; Low: 1.5% of body weight or High: 3% of bodyweight, in four-week phases. Each bar represents the average GSI per tank ± 1 se**.**