Guppy Gene Expression Dynamics: Effects of Sex, Reproductive Status, Feeding, and Temperature on Reproductive Function, Methylation, and Appetite

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Abstract

Introduction: This thesis examines the dynamics of gene expression in guppy fish *(Poecilia reticulata)* under various growth conditions, diets, and temperatures. Understanding the molecular processes underlying methylation, reproduction, and appetite regulation in these fish is the goal of this research. The process by which genetic information is transformed into useful products like proteins is known as gene expression. Guppy reproduction is highly impacted by elements like diet and temperature. This work investigates the effects of these factors on gene expression.

Methodology: Male and female guppies (*Poecilia reticulata*) were housed in 20L aquariums with filtered water at 25°C under a 12-hour light/dark cycle. Fish were acclimated for a week and fed daily with Fancy Guppy meal. For gene expression analysis, fish were divided into experimental groups: 1) fasting vs. feeding for 14 days, 2) temperature effects (25°C, 32°C, and 18°C). Tissues (brain, liver, intestine) were collected post-mortem for RNA extraction using the GeneJET RNA Purification Kit. Total RNA was reverse transcribed using the SensiFAST cDNA Synthesis Kit. Specific gene expression was analyzed via qPCR, and primer design was based on *Poecilia reticulata* sequences. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method, normalized to reference genes *GAPDH, EF-1α,* and *β-actin*. Statistical analyses included ANOVA and non-parametric tests with a significance level of $P<0.05$.

Results: A total of 45 guppies were examined, including 33 females and 12 males, with females classified into three reproductive stages. Analysis of brain gene expression showed no significant differences in *orexin (OX)* or *NPY* levels between groups, but *Kiss2* expression was higher in females with late-stage embryos, while *AVP* was elevated in males. Intestinal and liver genes expression showed no significant changes. In fasting experiments, females consumed more food than males, with brain orexin in fast male and hepatic *dnmt3b* expression in fed male being significantly higher. *Gnrh3* and *npvf* expression in fasted male and *IT* expression in fed female were higher. Temperature experiments revealed that food intake increased at higher temperatures, and *npvf* and *Orexin* levels were elevated at 32°C and 18°C in males. However, no significant changes were observed in epigenetic-related genes in response to temperature variations.

Conclusion: This study demonstrates how environmental elements like temperature and diet can have a substantial impact on guppy gene expression and phenotypic adaptation. The results of this study advance our knowledge of the molecular mechanisms underlying living things' flexibility and adaptation to environmental changes and may find use in aquaculture and conservation.

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Introduction

1.1 Introduction

Elucidation of the complex mechanisms underlying gene expression will provide insight into most biological processes, which range from development and reproduction to adaptation to the environment and behaviour. Gene expression in summary is the process by which information contained in genes is converted into functional products, such as proteins, used to carry out the biological processes defining an organism's phenotype. The dynamics of gene expression regulation are controlled by various factors: development stage, environmental stimuli, and genetic variability. The understanding of the changing pattern of gene expression related to these factors provides insight into the mechanism at play behind diverse physiological and behavioural traits (Uchiumi, 2022; Yamanaka et al., 2022).

Guppies are small freshwater fish that belong to the family Poeciliidae. They exhibit a very high rate of reproduction and are readily influenced by even slight environmental changes, such as those in temperature. Guppies are notorious for remarkable reproductive aptitude: high fecundity and early maturation. In guppies, reproduction is a complexly regulated process that involves intricate coordination of hormonal signalling, gamete production, and mating behaviours (Glavaschi et al., 2024; Sato et al., 2021; Yoshida et al., 2023; Zdanovich, 2023). The investigation into gene expression dynamics across the reproductive cycle can reveal molecular mechanisms involved in these processes and might highlight basic aspects of reproductive biology and evolutionary adaptation.

One of the major epigenetic modifications, DNA methylation, plays a decisive role in the regulation of the dynamics of gene activity during various stages of development, along with environmental ones. Methylation patterns can affect gene activity due to changes in chromatin structure and accessibility, which has consequences for different phenotypic traits. The dynamics of DNA methylation in guppies at disparate developmental stages and under varying environmental conditions may provide clues regarding the epigenetic mechanisms linked to phenotypic plasticity and adaptation associated with changing environments.

Feeding behaviour and dietary composition have recently been emphasized as some of the major factors influencing gene-expression profiles and metabolic pathways of organisms. Guppies exhibit highly diverse feeding behaviours in the wild, ranging from herbivory to omnivory, with diet playing an important role in growth, reproduction, and energy metabolism

(Herrera et al., 2022; Miranda et al., 2017; Raubenheimer et al., 2023; Zandonà et al., 2024). These dynamics of gene expression, in response to the various feeding regimes, can unravel the molecular mechanisms underlying nutrient sensing, metabolism, and energy allocation and hence offer valuable insights into the nutritional ecology of guppies and their adaptive strategies in different ecological contexts.

One of the most important abiotic environmental factors affecting physiological processes is temperature, which influences metabolism, development, and reproduction. Guppies exhibit phenotypic plasticity against temperature variation; thermal cues influence growth rates, timing of reproduction, and survival (Benavente et al., 2022; Rodrigues et al., 2022). Therefore, such studies on gene expression dynamics in guppies raised under different temperature regimes will help in illustrating the underlying molecular mechanisms of thermal adaptation and plasticity, thus providing insight into the evolutionary responses of organisms to shifting climatic conditions.

1.2 Endocrine Regulation of Feeding in Fish

1.2.1 Overview

Appropriate feeding is required for optimal growth and reproduction. In fish, adequate food intake results in improved food conversion efficiency, body composition and growth, as well as reduced feed waste, which is important in aquaculture not only to reduce feed supply costs but to reduce body build-up of nitrogenous wastes in the water, which could otherwise be harmful to fish (Arora and Pervez, 2024).

Similar to other vertebrates, in fish, hormones originating from the brain and peripheral organs govern feeding behaviour, food intake, and energy balance (Gorissen et al., 2006; Volkoff et al., 2005). These include factors that stimulate appetite, known as orexigenic factors, and those that decrease appetite, known as anorexigenic factors. Orexigenic factors include neuropeptide Y (NPY)[1] and orexin (OX)[2], produced mainly by the brain, and ghrelin, from the stomach and

[1] One of the brain's most powerful orexigenic peptides is neuropeptide Y (NPY). It increases appetite and favours foods rich in carbohydrates. Increasing meal size delays satiety, increases motivation to eat, and reduces consumption latency.

[2] Orexin (OX), also called hypocretin, is a neuropeptide that regulates arousal, alertness and appetite. It is produced by neurons in the hypothalamus and plays a crucial role in maintaining alertness and preventing narcolepsy.

brain. Anorexigenic factors include melanin-concentrating hormone (MCH) [3]and cocaine-and amphetamine-regulated transcript (CART)[4], which are mostly produced in the brain; leptin, which is principally synthesized in the liver; and cholecystokinin (CCK), which is produced in the gut (Volkoff, 2016). Other peripheral regulators include insulin from the pancreas, peptide YY (PYY) and glucagon-like peptides (GLP) from the intestine, adiponectin from adipose tissue, cortisol from the interrenal glands, and thyroid hormones. Brain-feeding centers control food intake in fish, based on energy reserves and metabolic demands. These centers are made up of neurons that produce orexigenic and anorexigenic hormones. Peripheral hormones convey information about feeding status and energy stores to the brain, either carried by the blood or by binding to receptors on the vagus nerve. The information is then integrated by central feeding centers, which regulate feeding accordingly (Fig.1-1) (Rønnestad et al., 2017; Volkoff, 2016).

Variations in gene expression and hormone levels significantly influence feeding behavior and appetite in fishes, particularly in response to environmental changes and fasting states. These variations can directly involve genes encoding hormones like neuropeptide Y (npy) and ghrelin, or indirectly through regulatory genes that modulate their expression and receptor sensitivity. Endocrine signals from the brain and gastrointestinal tract regulate food intake, with hormones acting as either orexigenic (stimulating appetite) or anorexigenic (suppressing appetite) agents. Key hormones include apelin, orexin, and cholecystokinin (CCK), which are influenced by factors such as nutritional status and environmental conditions. Fasting alters the expression of appetite-regulating peptides; for instance, orexin levels increase while CCK and leptin levels may decrease in certain species. These changes reflect the adaptive mechanisms of fishes to maintain energy homeostasis during periods of food scarcity. Environmental factors like temperature and food availability can modify the expression of feeding-regulating hormones, thereby affecting feeding behavior and energy storage (Assan et al., 2021; Butt et al., 2019).

[3] Melanin-concentrating hormone (MCH), derived from teleost fish pituitary gland, controls skin pigmentation, regulates feeding behaviour, mood, sleep-wake cycle, and energy balance.

[4] The cocaine-amphetamine regulated transcript, known as CART, is a neuropeptide protein encoded by the CARTPT gene in humans. CART appears to play a role in reward, feeding, and stress and it has the functional properties of an endogenous psychostimulant.

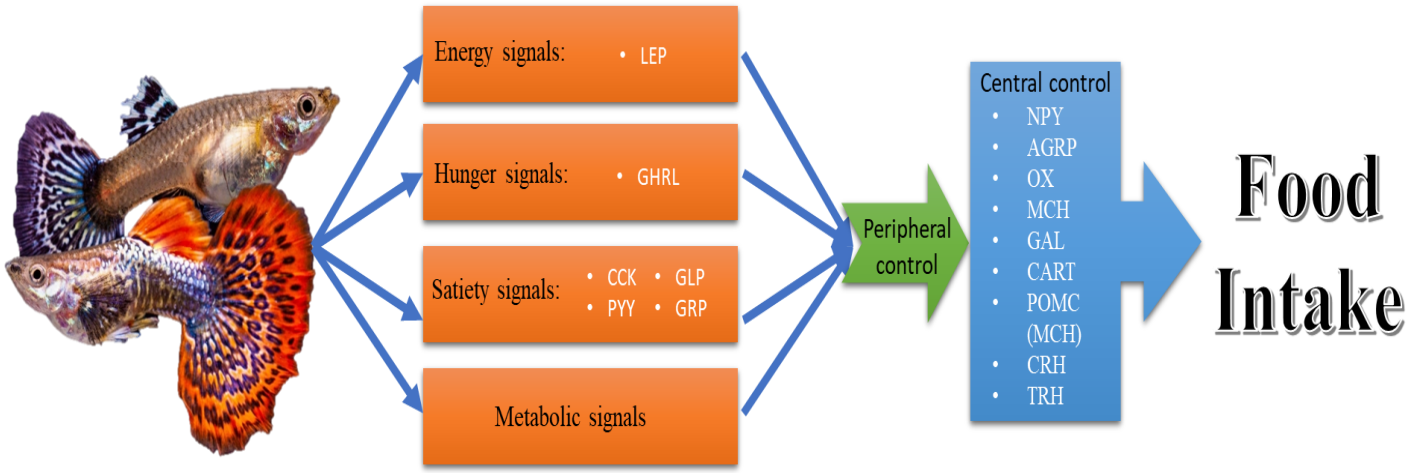

Figure 1-1 **Key organs and signalling pathways implicated in the regulation of appetite in fish are delineated. Presented are select central and peripheral endocrine factors investigated thus far. Energy Signals: - LEP (Leptin): An appetite-inhibiting hormone made by adipose (fat) tissue that aids in controlling energy balance. Hunger Indications: Ghrelin (GHRL) is a hormone that enhances food intake and stimulates appetite. It is mostly produced in the stomach. Satiety Signals: 1. Cholecystokinin (CCK): A hormone secreted by the small intestine that promotes digestion and fullness, helping regulate food intake. 2. PYY (Peptide YY): A hormone that decreases appetite secreted by the small intestine in reaction to food. 3. Glucagon-like Peptide (GLP): A hormone that controls satiety and glucose metabolism. 4. GRP (Gastrin-Releasing Peptide): A hormone that controls satiety and the digestive process by inducing gastrin release. Central Control: 1. NPY (Neuropeptide Y): A brain-produced peptide that increases appetite and decreases energy usage. 2. Agouti-Related Peptide (AGRP): A neuropeptide that lowers metabolism and promotes appetite. 3. OX (Orexin): An appetite, wakefulness, and arousal-regulating neuropeptide. 4. Melanin-concentrating hormone (MCH) is a neuropeptide that controls energy balance and hunger. 5. The neuropeptide GAL (Galanin) affects body weight, energy homeostasis, and feeding behaviour. 6. Cocaine- and amphetamine-Regulated Transcript (CART): A neuropeptide that controls reward circuits and hunger. 7. Proopiomelanocortin, or POMC: A precursor polypeptide that has several derivatives and functions in appetite regulation and energy balance. 8. Corticotropin-releasing hormone (CRH): A hormone that affects hunger and is involved in the stress response. 9. Thyrotropin-releasing hormone (TRH): A hormone that controls the thyroid gland and affects hunger and metabolic rate [This figure was created by the author using PowerPoint].**

Growth hormone (GH) is the main regulator of growth because it causes the liver to produce insulin-like growth factor-I (IGF-I). The physiological effects of GH usually occur indirectly through IGF-I's actions. Growth hormone-releasing hormone (GHRH)/pituitary adenylate cyclase-activating polypeptide (PACAP), ghrelin, and somatostatin (SS) are other hormones that are integrally involved in controlling pituitary GH secretion. Treatments with GH or IGF-I normally promote growth in fish, whereas SS decreases growth. However, there have been reported outliers (Chang and Wong, 2009; Degger et al., 2000; McLean et al., 1997; Very et al., 2001). For example, the gigantic danio, *Danio aequipinnatus*, responds to growth hormone (GH) treatment with increased growth, whereas the zebrafish, *Danio rerio*, does not (Biga and Meyer, 2009). Similar to hormones associated with appetite, growth is regulated by a variety of factors, and changes in the expression of a particular growth-related hormone gene or in its protein levels may not always indicate changes in the growth of the body. Fish growth endocrine regulators and hunger are anticipated to interact robustly. Interestingly, NPY not only increases feeding, but increases GH secretion; treatment with bombesin not only reduces feeding but increases GH production while simultaneously lowering the expression of the *SS gene* (Canosa et al., 2005; Himick and Peter, 1994a; Mazumdar et al., 2006). By contrast, feeding behaviour is increased when fish receive GH intraperitoneally, and IGF-I and pituitary GH mRNA levels are altered when fish are starved (Dalmolin et al., 2015; Delgadin et al., 2015; Gabillard et al., 2006; Tian Juan et al., 2012). Consequently, growth hormone (GH) and its related factors play a key role not only in regulating growth but also in interacting with feeding behavior and complex hormonal pathways in fish.

Fish exhibit diversity in both their habitats and feeding behaviors. For example, fish can be marine and freshwater fish, cold-water species (e.g., winter flounder, cod, and salmonids), or warm-water species. In addition, different anatomical and physiological characteristics are correlated with variations in fish feeding patterns. For example, whereas carnivorous species such as salmonids, cod, and seabass have well-developed teeth and stomachs, and short intestines, omnivorous/herbivorous species such as carp, catfish, and tilapia usually have long intestines and often lack true stomachs (Jiao et al., 2023). Environmental factors (e.g. temperature, pH, salinity) might affect feeding and the expression of appetite regulators (Volkoff, 2024). Finally, fish developmental stage, sex or reproductive stage (Volkoff, 2016) can influence feeding, both with regards to quantity and composition of the food.

As such, the optimization of food intake in aquaculture environments frequently depends on empirical methods, which include dietary manipulations such as changing the type of food, the quantity of the ration, the frequency, and the timing of feedings (Juell and Lekang, 2001; Petursdottir, 2002). One can alter environmental conditions, such as the duration of the light-dark cycle in indoor aquaculture facilities. It is possible to improve the size and quality of farmed fish by gaining a thorough grasp of the endocrine processes that control feeding and growth in fish (Biswas et al., 2006; Ergün et al., 2003). A deep understanding of the complex interactions between growth hormones, feeding behavior, and environmental factors, coupled with the use of knowledge-based approaches to nutritional management, can help optimize growth, improve quality, and increase aquaculture yields.

1.2.2 Description of Specific Feeding-Regulating Factors

1.2.2.1 NPY

In both mammals (Kuo et al., 2007; Robinson and Thiele, 2017) and fish (Shiozaki et al., 2020; Volkoff, 2016; Volkoff et al., 2005), NPY expressed in the central nervous system and the digestive tract is essential for controlling a number of physiological processes, such as stress response, appetite, circadian rhythms, and cardiovascular function. In all species examined, NPY appears to act as an orexigenic factor (Decressac and Barker, 2012). In fish, NPY has been cloned in a variety of taxa, including elasmobranchs and holocephalans (e.g., elephant fish, Chimaeriformes (Larsson et al., 2009)), Pleuronectiformes (e.g., olive flounder, *Paralichthys olivaceus* (Wang et al., 2015)), Salmoniformes *(Pereira et al., 2015),* Tetraodontiformes, Gonorynchiformes (e.g., milkfish *Chanos chanos* (Lin et al., 2017)), Perciformes (e.g., mandarin fish *Siniperca chuatsi* (Sun et al., 2015)), Siluriformes (e.g., channel catfish, *Ictalurus punctatus* (Schroeter et al., 2015)), and Tetraodontiformes (Xu et al., 2016). The broad role of NPY in regulating physiological and behavioral processes, along with its presence in various fish species, indicates the critical importance of this factor in maintaining energy balance and other vital functions in aquatic organisms.

1.2.2.2 Orexin

Orexins (or hypocretins) consist of two peptides orexin A and B, originating from the single gene *preproOX*. These neuropeptides play a critical role in regulating various physiological functions such as appetite, sleep-wake cycles, and reproduction. In mammals,

orexins bind to two types of G protein-coupled receptors called *orexin-1 (OXR1)* and *orexin-2 (OXR2)* (Bouâouda and Jha, 2023; Nakamachi, 2016; Wang et al., 2018). *PreproOX* cDNAs have been identified in several fish species, such as Atlantic cod (*Gadus morhua* (Xu and Volkoff, 2007)), zebrafish, pufferfish (*Tetraodontidae*) (Alvarez and Sutcliffe, 2002), and goldfish (*Carassius auratus* (Volkoff et al., 1999)). These species typically have one *OX* receptor gene, the *OXR2* (Wong et al., 2011). In a number of species, orexin injections increase appetite and motor activity and fasting increase orexin mRNA expression (Volkoff, 2016). In guppies, *orexin* expression is correlated with stressors, suggesting that it plays a modulatory role in behaviours connected to stress (Killen, 2020). Sleep, feeding, and stress response vary among guppy populations due to genetic polymorphisms in the orexin gene (Buckley et al., 2010). Therefore, orexins play a vital role in maintaining physiological and behavioral balance in fish, as key regulators of behaviors related to feeding, sleep, and stress response.

1.2.2.3 CCK

In both fish (Blanco et al., 2021; Volkoff, 2016) and mammals (Moran and Kinzig, 2004; Rehfeld, 2017), CCK is a member of the gastrin/cholecystokinin family produced by the *proCCK* gene regulating stomach acid production, stomach emptying midgut motility and food intake. *ProCCK* is processed into several peptides, including peptides such as cholecystokinin-8, -12, and -33, CCK-8 being one of the most biologically active forms (Moran and Kinzig, 2004). In fish, CCK has been shown to increase gut motility [e.g., Ballan wrasse (*Labrus bergylta*) (Le et al., 2019)], and promote gallbladder emptying (Le et al., 2019). In most fish studied to date, CCK acts as a satiety factor to decrease food intake. Injections of CCK decrease feeding in several fish species including goldfish (Himick and Peter, 1994b), CCK mRNA expression levels increase postprandially [e.g., goldfish (Peyon et al., 1999)] and fasting decreases intestinal CCK mNA lavels [e.g., Characiformes (Volkoff et al., 2017), Salmoniformes (Valen et al., 2011), Gadiformes, Perciformes, Pleuronectiformes (MacDonald and Volkoff, 2009a), and other Cypriniformes (Peterson et al., 2012).] [reviewed by (Blanco et al., 2021; Rønnestad et al., 2017)]. This evidence indicates that CCK, as a key factor in regulating digestion and reducing appetite, plays a fundamental role in maintaining nutritional and physiological balance in fish.

1.2.2.4 PYY

PYY belongs to the NPY family of peptides, which include pancreatic polypeptide (PP), and NPY. While PP is only expressed in the pancreas, tetrapods exhibit the presence of both NPY and PYY mRNA in brain and intestine. PYY is mostly produced by L-cells in the gastrointestinal tract. In mammals, PYY acts as a satiety factor and decreases gastrintestinal motility (Troke et al., 2013). Fish have two forms of PYY, PYYa (the homologue of PYY in mammals) and Pyyb (formerly called PY). In fish, injection of PYYa have an anorexigenic effect in several species [eg., goldfish (Gonzalez and Unniappan, 2010)., and mRNA PYYA expression increases postprandial [e.g., brain of grass carp (Chen et al., 2013)] and decrease following fasting [e.g., goldfish (Gonzalez et al., 2010); Ya fish (Yuan et al., 2014)] [reviewed by (Blanco et al., 2021), (Volkoff, 2016)], suggesting a role as a satiety factor. However, fasting did not impact the expression of PYY mRNA in either the brain or gut of Atlantic salmon (Salmoniforme) (Schroeter et al., 2015), cavefish *Astyanax mexicanus* (Characiforme) (Wall and Volkoff, 2013) or red-bellied piranha (Volkoff, 2014) and increases PYY gut expression in Japanese grenadier anchovies *Coilia nasus* (Clupeiformes) (Yang et al., 2016) and yellowtail (Perciformes) (Murashita et al., 2006, 2007). Existing evidence suggests that PYY plays an important role as a satiety factor in regulating feeding behavior and gastrointestinal function in fish. However, the response of PYY to conditions such as starvation and feeding may vary depending on different species and physiological differences. This diversity reflects the complexity of the regulatory mechanisms involved in energy balance and nutrition in fish.

1.3 Effects of Feeding and Nutritional Status on Food Intake and Appetite Regulators

Food intake, circulating metabolite levels, and duration of food deprivation have all been shown to affect feeding behaviour and the expression of appetite-regulating peptides. Changes in particular levels of circulating metabolites might affect the amount of food consumed. For example, food intake is reduced in trout-fed diets rich in carbohydrates and in carp that received intraperitoneal injections of essential amino acids (Kuz'mina, 2005). The expression of appetite regulators can be affected by food composition. For example, in goldfish, diets consisting of different macronutrient modulate the expression of *NPY* (Narnaware and Peter, 2002). Ingesting

food alters the gene expression of appetite regulators, usually with an increase in the expression levels of orexigenic factors either before or during a meal. In most cases, food deprivation causes the expression of orexigenic factors to increase while that of anorexigenic factors to decrease (Volkoff, 2016). Interestingly, fish have the ability to modify their food consumption in accordance with their energy needs, consuming more food when given low-energy diets (Boujard and Médale, 1994; Geurden et al., 2006; Paspatis and Boujard, 1996; Yamamoto et al., 2000) .Feeding behaviour and expression of appetite-regulating peptides in fish depend on food composition, metabolite levels, and starvation duration, and this adaptability plays an important role in maintaining their energy balance and physiological needs.

1.4 Effects of Temperature Changes on Appetite in Fish

One of the most important environmental variables affecting fish physiology is temperature. Fish rely on behavioural adaptations to maintain their internal temperature because most of them are ectotherms, meaning that their body temperature reflects that of their aquatic environment (Johansen et al., 2021). Any change in temperature can have a substantial impact on metabolic rate and raise energetic demands since it directly affects the rates of biological reactions.

A thermal performance curve is frequently used to illustrate the temperature sensitivity of fish. When temperatures rise, performance usually increases until it reaches an ideal temperature, after which it decreases (Haesemeyer, 2020; Johansen et al., 2021; Volkoff and Rønnestad, 2020). Similar to a bell-shaped model, this pattern applies to food intake (Volkoff, 2024; Volkoff and Rønnestad, 2020). Changes in temperature affect the way hormones that control appetite are expressed, albeit the effects vary depending on the species. Higher temperatures, for example, increase the amount of food that goldfish (*Carassius auratus*) eat; this effect is probably caused by increased *orexin* levels and lower *CART* levels (Nadermann et al., 2019). On the other hand, rising temperatures in clownfish (*Amphiprion ocellaris*) are linked to lower levels of the anorexigenic peptide proopiomelanocortin (POMC)[5] expression (Pham et al., 2022). Rising

[5] Proopiomelanocortin (POMC), a multifunctional precursor protein, is the source of various physiologically active hormones such as ACTH, MSH (α-, β- and γ-MSH) and the endogenous opioid β-endorphin (β-ED). Numerous physiological functions such as energy balance, adrenal function, sexual behavior, thermoregulation, nociception, exocrine gland activity, immune system function and pigmentation are significantly influenced by these peptides.

temperatures increase food intake in black tetra (*Gymnocorymbus ternetzi*) but decrease brain *orexin* expression, brain *CART*, and intestinal *CCK* expressions (Kuhn et al., 2023). Changes in ghrelin and leptin plasma concentrations in Atlantic salmon have been associated with temperature-induced feeding variations (Kullgren et al., 2013; Vikeså et al., 2017). These indicate that temperature changes have a significant impact on feeding behavior and the expression of appetite-regulating hormones in fish.

Decreases in temperature affect feeding. For instance, in Atlantic cod, reduced temperatures suppress food intake while increasing CART transcript expression (Kehoe and Volkoff, 2008), and in black tetra, low temperatures inhibit food intake and decrease CART brain expression (Kuhn et al., 2023). Temperature-induced changes in eating behaviour are probably influenced by the hypothalamic-pituitary-interrenal axis because environmental stress has been shown to increase the release of cortisol and corticotropin-releasing factor (CRF) (Volkoff, 2020). Studies on fish species such as rainbow trout (Madison et al., 2015), Atlantic salmon (Folkedal et al., 2012), and carp (Jaxion-Harm and Ladich, 2014) have demonstrated that both cortisol and CRF suppress feeding in fish.

Changes in food intake in response to temperature variations may result from changes in fish cognitive abilities, which could hinder their ability to forage. Excessive temperatures, namely 18°C and 34°C compared to the typical 26°C, affect energy metabolism and cause the downregulation of proteins related to neurotransmitter release and synaptic function, according to studies conducted on wild-type zebrafish (Toni et al., 2019). Additionally, temperature variations have been demonstrated to influence locomotor activity in fish. Reductions in locomotion occur both below and above the optimal temperature range for fish performance, potentially impairing their capacity to capture prey and locate food resources (Volkoff and Rønnestad, 2020). As a consequence, there is a reduction in interest in unfamiliar surroundings and a deterioration in cognitive abilities noted in behavioural evaluations.

1.5 Endocrine Regulation of Fish Reproduction:

While POMC and its derived peptides were originally described as stress-induced neurohormones within the traditional hypothalamic-pituitary-adrenal (HPA) stress axis, it is now known that they can also be produced autonomously in other peripheral tissues, such as the skin.

1.5.1 The Hypothalamic-Pituitary-Gonad Axis (HPG) and the Regulation of Reproduction in Fish

The pituitary gland secretes gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which act on gonadal receptors (FSH-R and LH-R), which in turn triggers the release of gonadal sex hormones, hence promoting gametogenesis and regulating sexual behaviour.

Estrogens and androgens influence sexual characteristics and reproductive processes. Female fish ovaries secrete estrogens and progesterone. The main estrogen, estradiol 17 beta (E2), promotes vitellogenesis and oogonial proliferation. Progestogens include 17-alpha and 20-beta dihydroxy-4-pregnen-3-one (DHP) and are essential for the initiation of follicular maturation and ovulation (Marques et al., 2022; Perrett and McArdle, 2013). On the other hand, male testis produce androgens, specifically 11-ketotestosterone (11-KT), which controls spermatogenesis and spermiogenesis (Cavaco et al., 2001). The major endocrine factor regulating the release of pituitary gonadotropins is gonadotropin-releasing hormone (GnRH), a hormone released by the hypothalamus.

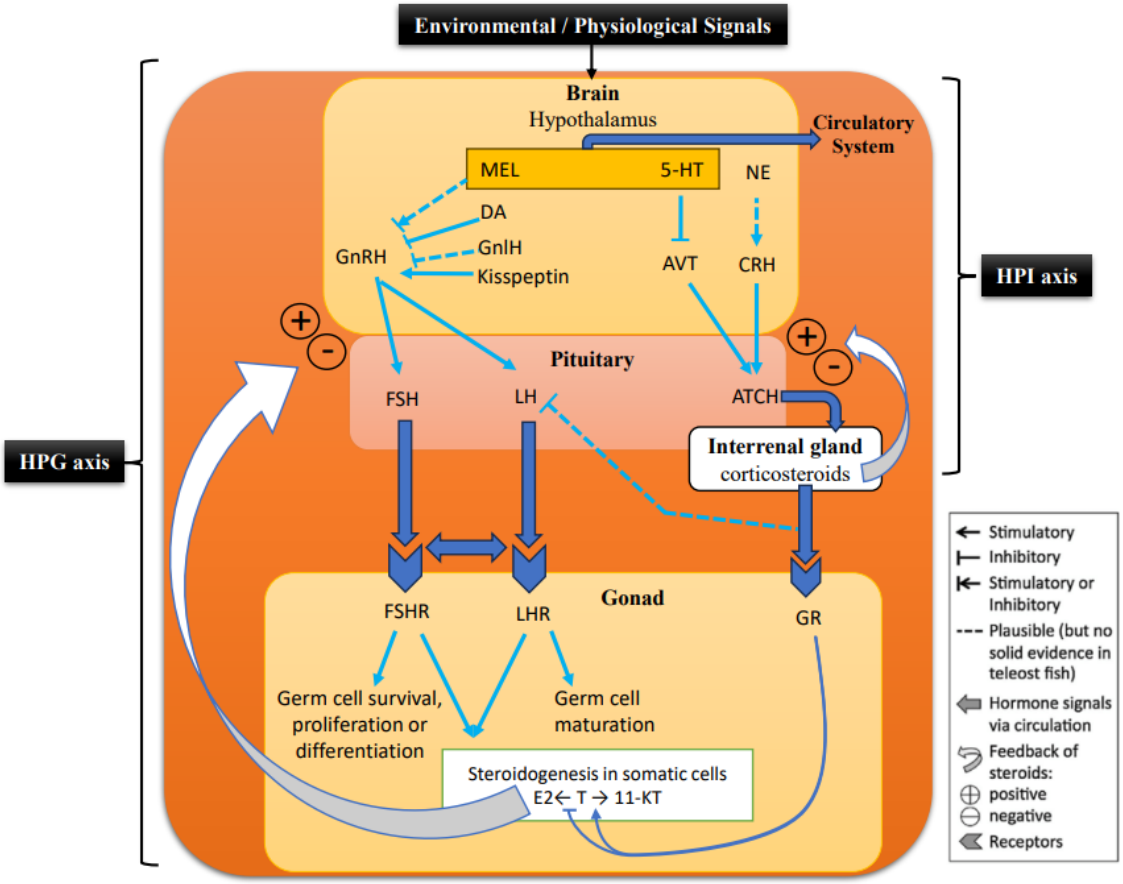

Figure 1-2 Diagram illustrating the Hypothalamic-Pituitary-Gonadal (HPG) and Hypothalamic-Pituitary-Interrenal (HPI) axes, as well as the neuroendocrine mechanisms governing steroidogenesis in fish [This figure has been reconstructed from (Liu et al., 2016) using PowerPoint. All rights belong to the original source.].

Fish can display up to three different GnRH forms (Carolsfeld et al., 2000; Santhakumar, 2023). Gonadotropin release is regulated by GnRH1, which is regarded as the hypothalamic variant (Zohar et al., 2010). It is thought that GnRH2, which is mainly located in the midbrain, has neuromodulatory properties. GnRH3 is found in the olfactory bulb and is linked to reproductive behaviour in teleosts that have three GnRH forms (Okubo and Nagahama, 2008; Santhakumar, 2023). GnRH synthesis and release are regulated by RFamide peptides, which have an Arg-Phe-NH2 pattern at the C-terminus. Important roles in this process are played by kisspeptin, which is produced by the KISS1/KISS1 gene, and gonadotropin-inhibitory hormone (GnIH, called LPXRFa in fish), which is encoded by the NPVF (Neuropeptide VF) gene.

Kisspeptin is synthesized by the *KISS1* gene and functions as a regulator of the hypothalamic-pituitary-gonadal (HPG) axis. Kisspeptin is a critical regulator of GnRH production and has a role in the onset of sexual maturation (Beltramo et al., 2020; Tena-Sempere, 2006). Kisspeptin forms synaptic connections with GnRH neurons expressing the kisspeptin receptor to prevent the release of luteinizing hormone (LH) in various animal species, including humans, rats, sheep, and primates (McCartney and Marshall, 2014). Studies conducted on teleosts and mammals indicate that some kisspeptin neurons are sensitive to steroids, which may suggest that the regulatory elements of the kisspeptin gene are estrogen-responsive (Kanda and Oka, 2021; Shahjahan et al., 2010b). Two *kisspeptin* genes, *kiss1,* and *kiss2*, have been found in teleost fish (Somoza et al., 2020). The expression of the kiss1 receptor (Kiss1R, also called GPR54) in GnRH neurons in cichlid fish (e.g., *O. niloticus* and *Astatotilapia burtoni* (Ohga et al., 2018)) indicates that kiss1 may regulate GnRH production in this species.

The regulation of GnRH secretion, which is crucial for healthy reproductive development, is controlled by the kisspeptin system. It functions as a "gatekeeper" of puberty and aids in integrating the impact of metabolic cues and sex hormones on GnRH secretion (Ogawa and Parhar, 2014). Kisspeptin, originally known as metastin because of its capacity to prevent the growth of breast and melanomas, has been discovered to have a significant impact on LH release in a variety of animals, including rodents, sheep, monkeys, and humans (Belchetz et al., 1978; Marshall and Kelch, 1986). Kisspeptin fibers make synaptic connections with GnRH neurons and the majority of GnRH neurons have been found to express the kisspeptin receptor (Roseweir and Millar, 2009; Tena-Sempere, 2006). The kisspeptin system, as a gatekeeper of puberty, plays a fundamental role in regulating GnRH secretion and integrates the influence of metabolic signals and sex hormones on this process. The direct interaction of kisspeptin with GnRH neurons and the expression of kisspeptin receptors on these neurons emphasize its importance in healthy reproductive development.

GnIH is a new hypothalamic peptide encoded by the *NPVF* gene that was originally identified from the brain of a Japanese quail. In birds, GnIH has been shown to act directly on gonadotropes to inhibit the release of LH and FSH, as well as on hypothalamic GnRH neurons to inhibit GnRH synthesis and release (Kumar, 2021; Tsutsui, 2016; Tsutsui et al., 2000). The *NPVF* gene produces the *NPVF* protein. In both mammalian and fish species, the NPVF gene

and the neuropeptide it produces play a variety of roles in the control of eating behaviour, energy homeostasis, stress response, and reproduction. Although there has not been as much research on *the NPVF* gene in fish species as there has been in mammals, new studies have shed light on its significance. These studies suggest that the *NPVF* gene may be very important in controlling eating behaviour and energy balance in fish species (Hu et al., 2016). For instance, the *NPVF* gene is predominantly expressed in mammalian-like brain areas involved in hunger control and energy regulation in zebrafish. It has been demonstrated that altering the NPVF signalling pathway in zebrafish affects feeding behaviour, control of body weight, and energy expenditure (Lee et al., 2020). The NPVF gene may also affect fish reproduction (Edgecombe, 2020): In several fish species, *NPVF* expression rises throughout the breeding season, suggesting that it regulates reproductive processes, similar to what has been shown in mammals.

The nonapeptides arginine vasotocin (AVT, the teleost from of mammalian arginine vasopressin, AVP) and isotocin (IT, the teleost form of mammalian oxytocin, OT) have been shown to be essential for regulating fish behaviour in both social and reproductive contexts. In teleosts, extensions of the AVT and IT neurons have been shown in a variety of brain areas, including the hindbrain, telencephalon, mesencephalon, diencephalon, and hypothalamus. In some species, AVT and IT neurons have been shown to modulate reproductive behaviour and gamete release (Mennigen et al., 2022). Stress and aggression in fish are controlled by arginine vasotocin (AVT) and corticotropin-releasing factor (CRF)[6]. New research suggests that AVT might play a role in socially mediated sexual differentiation (Balment et al., 2006; Sokołowska et al., 2020). Therefore, arginine vasotocin (AVT) and isotocin (IT), as the fish equivalents of vasopressin and oxytocin in mammals, play a vital role in regulating social and reproductive behaviors in fish.

For instance, IT-positive fibers have been found in the olfactory bulb of male zebrafish (*Danio rerio),* which are reactive to female sex pheromones (Altmieme et al., 2019; Yabuki et al., 2016). In plain fin midshipman (*Porichtys notatus*), in which courtship behaviour depends on vocalization, AVT and IT innervation has been detected in vocalization-related fore- and mid-brain regions as well as diencephalic regions along the ascending auditory pathway (Forlano and

[6] Corticotropin releasing factor (CRF), a neuropeptide, regulates the hypothalamic-pituitary-adrenal system and is found in extrahypothalamic areas of the brain and hypothalamus, deeply involved in stress responses.

Bass, 2011; Goodson and Bass, 2000; Goodson et al., 2003). AVT neurons are present in the medulla close to the pacemaker nucleus that regulates electric organ discharge (EOD) signals in the weakly electric gymnotiform bluntnose knife fish (*Brachyhypopomus audio*), which uses EOD signals for mating selection (Perrone et al., 2014; Silva et al., 2007). The hypothalamic paraventricular and supraoptic nuclei are the primary sites for OT gene production. These nuclei are where the processed peptide is released in response to events like parturition, nursing, or stress. OT has many physiological effects, most notably in causing mammals to contract their smooth muscles and go into labor (Lyu et al., 2021). IT controls various social behaviours in fish species, including decision-making, anxiety, protectiveness toward the father, social hierarchy, defense of the territory, care of the eggs, and courting behaviours. While studies show that IT is involved in the spawning response and ovulation induction in some fish species, the exact effect of IT on teleost ovulation is still poorly understood (Ramsey et al., 2019). Both OT and IT originate from the same precursor and have a structurally comparable makeup (Gimpl and Fahrenholz, 2001; Lyu et al., 2021). All of this neuroanatomical data points to the involvement of nonapeptides in regulating the emission and reception pathways of different sensory signals linked to different teleost fish reproductive behaviours.

1.5.2 Oocyte Growth and Maturation in Fish

In female fish, the oocyte grows throughout fish puberty but is stopped at the prophase of the first meiotic division. During this phase, the oocyte stores chorionic proteins (choriogenins), which are mostly produced by the liver in response to estrogen stimulation, and yolk precursors (vitellogenins). This concentration of yolks is what causes the oocyte diameter to rise. The regulation of vitellogenin incorporation into oocytes and the release of estradiol are both influenced by FSH (Arukwe and Goksøyr, 2003; Weber, 2023). In response to environmental, social, or pheromonal signals, the post-vitellogenic oocyte displays dormancy for a few months before beginning the final maturation phase, which denotes the restart of meiosis. GnRH is released at the start of this process, and it may be followed by circulating LH and a decrease in dopaminergic inhibition (Figure 1-2). The ovarian follicle begins to mature when LH binds to its receptors on granulosa cells[7]. Ovulation, or the discharge of eggs into the coelomic cavity or ovarian lumen, is made possible by the breach of the follicular membrane caused by subsequent

[7] Granulosa cells are found in ovaries. These cells produce estrogen, progesterone and other hormones.

oocyte growth by water absorption. At metaphase II, meiosis stops once more, delaying the second polar body's [8]extrusion and the second meiotic division's completion until fertilization (Yaron and Levavi-Sivan, 2011). Therefore, oocyte growth and maturation in female fish is regulated by sex hormones and environmental signals and is associated with meiosis arrest in metaphase II until fertilization.

1.6 The Effect of Temperature on Reproduction in Fish

Temperature plays a crucial role in regulating various physiological processes in fish, including reproduction. As ectothermic organisms, fish are highly sensitive to changes in environmental temperature, which can directly influence their reproductive physiology. In reproductively mature adults that reproduce seasonally, temperature (and photoperiod) cues control reproductive episodes (Volkoff and Rønnestad, 2020). This control varies among species. For instance, spring and early summer spawners require higher temperatures during spring to trigger maturation, while high temperatures in summer delay maturation and ovulation in autumn-spawning species (Pörtner et al., 2017; Whitney et al., 2016).

Temperature affects many aspects of reproduction, including the development and maturation of gametes, the timing of ovulation and sperm release, spawning, embryogenesis and hatching, and the development and survival of larvae and juveniles (Brett, 1971; Kovacevic et al., 2019; van de Pol et al., 2017). Temperature exerts profound effects on the endocrine system, which plays a central role in regulating reproductive processes in fish. Changes in temperature can alter the synthesis, secretion, and activity of reproductive hormones, including gonadotropins, sex steroids, and growth factors and influence the expression of genes encoding hormone receptors, enzymes involved in hormone synthesis, and signalling pathways regulating hormone action. For instance, temperature-induced changes in thyroid hormone signalling have been implicated in the regulation of reproductive timing and fecundity in fish (Pankhurst and King, 2010; Pankhurst and Porter, 2003; Shimizu, 2003). Temperature acts as a key factor in regulating the reproductive processes of fish, influencing gamete maturation, ovulation,

[8] As the egg cell divides during meiosis, a polar body is created as a byproduct, helping to ensure that the resulting egg cell has the proper amount of cytoplasm and organelles necessary for fertilization and early development. After the first meiotic division (meiosis I), the first polar body is extruded, and after the second meiotic division (meiosis II), which ends with fertilization, the second polar body is extruded.

reproductive timing, and hormonal regulation, all of which affect offspring survival and reproductive success.

1.6.1 Gamete Development and Maturation

Temperature influences the timing and efficiency of gametogenesis, the process by which germ cells develop into mature gametes (Trudeau, 1997). Molecular studies have shown that temperature regulates the expression of genes involved in germ cell development, including those encoding transcription factors, signalling molecules, and cell cycle regulators (Weltzien et al., 2004). For example, in some fish species (Hu et al., 2011; Mylonas et al., 2010; Selman et al., 1993; Skinner et al., 2014), exposure to high temperatures accelerates germ cell proliferation and meiotic progression, leading to early maturation and spawning.

Temperature affects the timing and frequency of spawning events in fish, which are crucial determinants of reproductive success (Hofmann and Todgham, 2010). Temperature variations can affect the genes that control spawning behaviour by activating them differently (Zohar and Mylonas, 2001). These genes include those that make neuropeptides, neurotransmitter receptors, and ion channels (Munday et al., 2017). Furthermore, temperature can impact sperm and egg quality, affecting fertilization success and embryonic development (Schulz et al., 2010). Changes in temperature may alter the expression of genes related to sperm motility, egg maturation, and embryonic gene activation, ultimately shaping the viability and fitness of offspring (Pankhurst and Porter, 2003; Pankhurst, 1997b). There is relatively little information of the effects of temperature on the expressions of GnRH, gonadotropins, kisspeptin, GnIH and their receptors.

In the blue gourami (*Trichogaster trichopterus*), increases and decreases in water temperature suppress GnRH3 mRNA levels (David and Degani, 2011), in red seabream, high water temperatures inhibit brain GnRH1 and pituitary LHb (Okuzawa and Gen, 2013) and in pejerrey (*Odontesthes bonariensis*), higher temperatures lower mRNA levels of FSHB[9] and LHB[10]. Little is known about the effect on kisspeptin one study found that GnRH and kiss2

[9] FSHB (Follicle-Stimulating Hormone Beta Subunit) is the beta subunit of follicle-stimulating hormone (FSH), which is produced by the anterior pituitary gland

[10] LHB (Luteinizing Hormone Beta Subunit) is the beta subunit of luteinizing hormone (LH), produced by the anterior pituitary gland

mRNA levels are decreased at both high and low temperatures (Shahjahan et al., 2017). Therefore, more research is necessary to fully understand how temperature affects kisspeptin and the expression of its receptor gene.

The expression levels of genes related to GnRH, kisspeptin and GnIH and their receptors generally decrease in response to temperature changes in the brain, with some exceptions such as GnRH3 and GnIH in sheepshead minnows (*Cyprinodon variegatus*) and Kiss1 in zebrafish (Shahjahan et al., 2013a). In particular, preoptic neurons that produce GnRH1 or GnRH3 innervate the pituitary gland and stimulate the release of gonadotropins (GTH), which varies depending on the fish species and may have two or three GnRH isoforms in their brain. Both high and low temperature abnormalities often reduce gene expression of these GnRH isoforms. Notably, GnRH3 does not appear to play a hypophysiotropic role in sheepshead minnows, as evidenced by lower pituitary expression of FSHB and LHB and increased GnRH3 expression at high temperatures. Conversely, elevated GnIH levels in high temperature environments appear to inhibit GTH secretion in sheepshead minnows (Bock et al., 2021; Muñoz-Cueto et al., 2020). However, in *Takifugu alboplumbeus* (grass puffer), high temperatures inhibit GnIH expression, leading to increased GTH production. In zebrafish, Kiss1 neurons in the habenula are thought to regulate the serotonergic system, while Kiss2 neurons in the hypothalamic nuclei are thought to be involved in reproduction. Therefore, upregulation of Kiss1/Kissr1 expression at low temperatures is thought to be related to behavioural changes in zebrafish under such conditions (Ogawa and Parhar, 2018). These observations indicate that temperature changes affect the expression of genes related to GnRH, kisspeptin, and GnIH in the brain of fish and, depending on the species, can alter the regulation of reproduction, secretion of gonadotropins, and related behaviours.

1.7 Interface Between Nutrition and Reproduction

Interactions between nutrition and reproduction have long been known in mammals (Garcia-Garcia, 2012; Jazwiec and Sloboda, 2019; Schneider, 2004). Undernutrition has a major effect on the reproductive system, delaying the beginning of puberty, lengthening the interval among pregnancies, interfering with ovarian cycles, and raising the incidence of infertility. Animal reproductive performance is linked to poor nutrition, namely insufficient calories, protein, vitamins, and minerals. Since energy availability has a direct impact on an animal's

capacity for successful reproduction, energy balance is particularly important for reproductive function (Bindari et al., 2013; Pradhan and Nakagoshi, 2008). Animal metabolism is influenced by nutritional status, which has an impact on both the success of animal husbandry as a whole and reproductive efficiency (Puls, 1988; Randel, 1990). When the food supply is out of line with reproductive needs, the energy requirements of reproduction present difficulties and endanger the health of both mothers and offspring (Apter and Hermanson, 2002; Zhang, 2015). The interaction between nutrition and reproduction plays a critical role in reproductive success and general health, especially when imbalances in energy and nutrient supply can disrupt reproductive cycles and negatively impact maternal and offspring health.

During reproduction, the endocrine system is crucial in regulating changes in hormones and metabolism in response to environmental stimuli such as the availability of food (Erhuma et al., 2007). Some hormones that control metabolism and reproduction include GnRH Progesterone and other gonadal sex hormones, including testosterone, are essential for fertility. Maternal malnutrition can have an effect on fetal development and programming, which can influence the reproductive and productive consequences of the progeny. To control reproductive responses, the Kiss1-KP-GPR54 system combines internal and environmental inputs, including nutritional cues (Budak et al., 2006; Clarkson and Herbison, 2006). Gaining knowledge about the function of this system in metabolic regulation and seasonal reproduction can help manage diet and reproduction in a variety of animal species, increasing agricultural productivity.

1.7.1 Evidence that Feeding and Reproduction Are Connected in Fish

In fish, many appetite regulators control reproduction, e.g., NPY, orexin, MCH, and ghrelin (Blanco, 2020). For example, injections of orexin decrease spawning behaviour in goldfish (Hoskins et al., 2008), intracranial injections of NPY increase the number of GnRH fibers and LH cells in the pituitary in catfish, (Senthilkumaran and Kar, 2021), and brain or peripheral injections of ghrelin increase serum LH levels in goldfish (Unniappan and Peter, 2004). Appetite regulators in fish play an important role in the coordination between nutrition and reproduction through their effects on hormones and reproductive pathways.

Information on the effects of reproductive hormones on the regulation of appetite is scarce. In mammals several hormones have been shown to affect feeding, e.g., GnRH, kisspeptin, LH, FSH (Blanco, 2020). In fish, GnRH appears to regulate feeding. For example,

injections of GnRH2 decrease food intake in goldfish (Hoskins et al., 2008) and zebrafish (Nishiguchi et al., 2012) and increase hypothalamic orexin mRNA expression in goldfish (Hoskins et al., 2008) In addition, brain GnRH1 and GnRH3 expression levels decrease following fasting in the brain of mouthbrooding Nile tilapia (Das et al., 2019) and GnRH2 expression increases following overfeeding in zebrafish (Nishiguchi et al., 2012). Fasting increases the expression of brain KISS2 in zebrafish (London and Volkoff, 2019) and of hypothalamic kissr2 in pejerrey (Mechaly et al., 2018), suggesting a potential role in the regulation of food intake. The role of GnIH is still uncertain (Blanco, 2020; Muñoz-Cueto et al., 2017). Reproductive hormones may play an important role in regulating appetite in fish, but the exact mechanisms and extent of their influence still require further investigation.

1.8 Epigenetics

The word "epigenetics" refers to processes that result in modifications in gene expression without affecting the genome's DNA sequence (Herráez et al., 2022a). Epigenetic modifications are governed by three main mechanisms: (i) changes in chromatin compaction caused by DNA methylation, which involves DNA methyltransferases (DNMTs) and the Ten-Eleven-Translocation (TET) family of proteins (TET1, -2, -3); (ii) modifications in chromatin caused by histone post-translational modifications (PTM) and/or incorporation of variants; and (iii) regulatory actions by non-coding RNAs (ncRNAs), such as microRNAs (miRs) or long non-coding RNAs (lncRNAs) (Herráez et al., 2022b; Solís et al., 2022). These mechanisms may operate both within an animal's lifetime (intragenerational epigenetics) and across generations (transgenerational epigenetics) (Best et al., 2018). Epigenetics alterations are essential for controlling gene expression, which affects an organism's physiology, development, and susceptibility to disease.

During embryonic development, epigenetic modifications are essential for directing cell destiny, coordinating differentiation, and creating specialized cell types. During this developmental stage, deviations from the typical DNA methylation patterns might cause health problems in adulthood and poor health outcomes in the next generation. An epigenetic mechanism called genomic imprinting sex-dependently adds unique marks to DNA that cause differences in gene expression according to paternal ancestry (Skinner, 2011). Epigenetic

reprogramming is essential for basic developmental processes such as gametogenesis and embryogenesis.

In fish, relationships have been established among alterations in DNA methylation and life-history traits, such as early male maturation in salmonids (Morán and Pérez-Figueroa, 2011). Although these modifications frequently have long-term and intergenerational effects, they can have short-term effects in adults. For example, in adult guppies, exposure to an ectoparasite infection for 16 days causes epigenetic modifications, including methylation changes in the skin (Hu et al., 2018). Several mammalian studies highlight the function of epigenetic mechanisms in controlling the maturation and operation of reproductive processes during the course of an organism's life (Kim et al., 2008). Epigenetic control encompasses aspects including gonadogenesis, sexual determination, brain sexual differentiation, and the dynamic regulation of critical reproductive processes such as pubertal development and Gonadotropin-Releasing Hormone (GnRH) release (Rajendiran et al., 2021). Epigenetic mechanisms play a vital role in regulating reproductive processes throughout the life of organisms.

1.8.1 Proteins Involved in the Regulation of Methylation: DNMT and TET

Transcription factors bind to specific motifs in target gene promoters to control the expression of the target gene; CpG dinucleotides are frequently found in these binding motifs. Target gene repression is linked to promoter methylation, and it is essential to remove methyl groups from these promoters in order to facilitate transcription factor binding and subsequent gene activation (Joshi et al., 2022). This demonstrates the importance of methylation and demethylation dynamics in the fine regulation of gene expression through interactions with transcription factors.

Methylation is a regulatory process that affects chromatin shape, genomic imprinting, transcription cascades, and genome stability. One prominent example of an epigenetic regulatory feature that is frequently observed in CpG dinucleotides is the methylation of the fifth carbon of cytosine (5nC). Two major enzymes are involved in the dynamic adjustment of DNA methylation patterns: DNA methyltransferases (DNMTs), which add methylation, and the ten-eleven translocation (TET) family of dioxygenases, which aid in the removal of methylation from DNA (Gerecke et al., 2022; Zhang et al., 2023). DNMTs are essential to the process of epigenetic modification.

Histone methylation is directly influenced by Dnmt1, a maintenance methyltransferase that ensures proper inheritance of epigenetic marks during cell division (Campos et al., 2012; Firmino et al., 2017). Dnmt3, which includes Dnmt3a, dnmt3b, and Dnmt3L, regulates issue differentiation and epigenetic reprogramming throughout embryonic development which depends on de novo DNA methylation (Gerecke et al., 2022; Zhang et al., 2023). Mammalian DNMT1 is a maintenance methyltransferase that methylates newly produced DNA to maintain DNA methylation patterns throughout cell division. Conversely, de novo methylation is carried out by DNMT3A and dnmt3b, which creates new DNA methylation patterns in previously unmethylated DNA regions. Accurate tissue differentiation and the creation of novel cellular phenotypes by epigenetic reprogramming depend on this mechanism. Whole genome duplication events have resulted in several homologs of the DNMT family in fish. For example, eight DNMTs in zebrafish have been found, including one DNMT1, one DNMT2, and six DNMT3 (Campos et al., 2012; Del Castillo Falconi et al., 2022; Goll and Halpern, 2011). Similar to this, three variants of DNMT3 (dnmt3b.1, dnmt3aa, and dnmt3ab) and one DNMT1 have been identified in Senegalese sole (*Solea senegalensis*) (Firmino et al., 2017). DNMT enzymes are critical for the processes of tissue differentiation and epigenetic reprogramming, and this mechanism has become more diverse in fish.

TET proteins are essential for preserving a delicate balance among DNA hypermethylation and hypomethylation, which is required for a number of physiological functions in cells. Tet1, Tet2, and Tet3 are members of this family of enzymes that produce proteins with catalytic dioxygenase activity in their carboxy-terminal domain, indicating that they may play a role in the demethylation of DNA (Rasmussen and Helin, 2016; Zhang et al., 2023). Methylcytosine is specifically converted into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) by a methylcytosine dioxygenase that is produced by the TET2 gene. These derivatives of oxidized cytosine function as bridges in the demethylation pathway, which restores unmodified cytosine in the end (Albano et al., 2011; Rasmussen and Helin, 2016). TET2 is a methylcytosine dioxygenase that catalyzes the conversion of methylcytosine into 5-hydroxymethylcytosine (Albano et al., 2011; Morris-Blanco et al., 2019). TET3, which binds zinc ions and methyl-CpG, is an essential component of DNA demethylation, which impacts gene expression, developmental pathways, and a host of other biological processes (Beck et al., 2020; Yang et al., 2020; Yang et al., 2023). While TET3 is

highly regulated and responsive to developmental and environmental factors, suggesting its role in phenotypic diversification and adaptability, studies on zebrafish (Avagyan and Zon, 2016; Ortega-Recalde et al., 2019; Ross and Bogdanovic, 2021). TET proteins play an important role in regulating gene expression, evolutionary pathways, and phenotypic adaptations

1.8.2 Influence of Epigenetics on Reproduction in Fish

Epigenetic changes have a substantial effect on the regulation of gene expression in fish reproduction, as they affect critical processes such as gametogenesis, sex determination, and embryonic development. DNA methylation, histone alterations, and non-coding RNAs work together to control the patterns of gene expression that are essential for gametogenesis, sex choice, and embryonic development (Gavery and Roberts, 2017). These changes play a vital role in ensuring successful growth and development and are important tools in regulating the fundamental processes of fish reproduction.

Dynamic regulation of DNA methylation patterns takes place during fish gametogenesis. Variations in methylation within particular gene areas can either suppress or activate gene expression, which in turn affects the destiny of germ cells. Notably, the development of male and female gametes is significantly regulated by the methylation state of genes linked to sex determination in some fish species. For instance, sex determination in the Nile tilapia is linked to the methylation state of the anti-Müllerian hormone AMH) gene. The expression of the AMH promoter region and subsequent female development are caused by hypomethylation, whilst its silencing and subsequent male development are caused by hypermethylation (Baroiller and D'Cotta, 2018). Similarly, sex differentiation in the European sea bass (*Dicentrarchus labrax*) is influenced by the methylation state of the *aromatase gene (cyp19a1a)*[11]. Male development is linked to hypermethylation of the cyp19a1a promoter, while female development is linked to its hypomethylation (Navarro-Martín et al., 2011). In European sea bass , sex determination has been connected to the dynamics of DNA methylation, as distinct patterns of DNA methylation in genes involved in sex differentiation, like *sox9* and *dmrt1*, suggest a potential regulatory function of DNA methylation in governing the development of male and female sex differentiation (Ribas et al., 2013). Dynamic regulation of DNA methylation plays a fundamental role in the processes

[11] Aromatase, also known as estrogen synthase, is a crucial enzyme in the biosynthesis of estrogens, CYP19A1, a member of the cytochrome P450 superfamily.

of gametogenesis and sex determination in fish and acts as a key molecular mechanism in sexual differentiation.

Fish reproduction is impacted by histone changes. These changes tightly control genes related to fish reproductive functions, such as the manufacturing of hormones necessary for gonadal development (Yoshimoto et al., 2008). Non-coding RNAs, like long and microRNAs, are essential parts of the epigenetic machinery that affect fish reproduction at the molecular level. Through their binding to messenger RNAs, these short RNAs post-transcriptionally control the expression of genes by either encouraging or inhibiting their translation. Certain microRNAs have been found to be essential regulators governing the expression of genes involved in the development and differentiation of germ cells in fish gonads. For example, miR-202 plays a role in controlling the expression of genes linked to germ cell development in zebrafish. miR-202 affects gonadal development and fertility by targeting nanos1, a crucial gene for germ cell maintenance and differentiation (Fu et al., 2011). miR-202-3p affects sperm production by targeting the 3'-untranslated region (UTR) of the follicle-stimulating hormone receptor (fshr) gene, which modifies the *fshr* gene's expression (Wei et al., 2019). In conclusion, fish reproduction is impacted by epigenetics in a variety of ways at the molecular level.

1.8.3 Influence of Feeding on Epigenetics in Fish

Fish feeding patterns significantly alter the epigenetic landscape by influencing gene expression. There is a complex molecular relationship between feeding and nutrition, and epigenetics in fish. The regulation of gene expression patterns is influenced by alterations in DNA methylation, histone modification, non-coding RNA expression, transcription factor activity, and interactions with the gut microbiota. Foods can include methyl donors such as folate and choline, which supply methyl groups for DNA methylation thereby influencing both transient genomic responses and long-lasting epigenetic markers. In fish, these markings have a significant effect on the expression of genes linked to vital physiological processes such as growth, development, and metabolism (Choi and Friso, 2010). Another critical chemical pathway that is impacted by feeding is histone modification. The chromatin structure can be altered by changes in histone acetylation, methylation, or phosphorylation, which can either stimulate or inhibit gene transcription. Dietary variables are important (Landecker, 2011) because they affect the availability of substrates and cofactors in histone modification activities,

which in turn affects the patterns of gene expression associated with many physiological processes in fish.

Feeding schedules also affect non-coding RNA expression, particularly microRNAs (miRNAs). Distinct miRNAs can be modulated by specific dietary components, forming a regulatory network that precisely adjusts gene expression in response to nutritional cues (Farhud et al., 2010). The composition of the fish diet affects the gut microbiota, which in turn influences fish epigenetic regulation. Fish gut microbial populations generate compounds that act as signalling molecules, affecting the expression of host genes. Histone acetylation and DNA methylation can be changed by short-chain fatty acids, which are produced when gut bacteria ferment food fibers (Skjærven et al., 2022). Therefore, diet and gut microbiota play a key role in epigenetic regulation of fish and can influence gene expression through complex molecular pathways.

1.9 Effect of the Environment and Temperature Changes on Epigenetics in Fish

Epigenetics is greatly influenced by the environment, particularly in fish (Herráez et al., 2022a). Numerous factors, such as pH, water temperature, and rearing density, have been identified as potential contributors to epigenetic effects.

Temperature-dependent DNA methylation dynamics have been investigated in Senegalese sole (Campos et al., 2014), Atlantic salmon (Burgerhout et al., 2017), and zebrafish muscle tissue. Zebrafish seem to express more dnmt3a and less dnmt3b at higher temperatures (Campos et al., 2012; Han et al., 2016). When exposed to temperature fluctuations, adult zebrafish display modifications in their CH3 groups at 34 °C and disturbances in their Cys/Met metabolism at 18 °C. These modifications imply that heat stress may affect methylation processes (Nonnis et al., 2021), which may lead to modifications in chromatin organization, nucleosome assembly, and DNA methylation.

One well-known epigenetic marker that has been studied, particularly in fish studies, is DNA methylation, which is the addition of methyl groups to cytosines. Temperature variations during zebrafish embryonic development have been shown to impact dnmt3 expression (Campos et al., 2012; Dorts et al., 2016a). Rainbow trout's ontogenesis was recently used to assess the

expression patterns of genes linked to DNA methylation, such as *dnmt1* and *dnmt3* (Liu et al., 2020). Few studies, though, have looked at how fish, especially rainbow trout, respond to temperature changes in terms of the expression levels of *dnmt* genes. Understanding how different methylation profiles are established during early temperature stress may be possible by profiling the expression of *dnmt* genes. Numerous studies have shown that early exposure to temperature can have long-lasting effects on a variety of fish phenotypic traits, such as thermal acclimation capacity (Jonsson and Jonsson, 2019; Scott and Johnston, 2012), growth and muscle development (Albokhadaim et al., 2007; Steinbacher et al., 2011), sex differentiation (Valdivia et al., 2014) and intermediary metabolism (Seibert, 1985), as outlined in the review by Jonsson and Jonsson in 2019.

Moreover, fish undergo significant epigenetic modifications in response to temperature. For example, in sea bass (Navarro-Martín et al., 2011) Senegalese sole (Campos et al., 2013) and Atlantic salmon (Burgerhout et al., 2017). Sex determination is influenced by the differential methylation of certain promoters, such as aromatase and myogenin, in response to early temperature exposure. Furthermore, Metzger and Schulte (2017) demonstrated that exposure to developmental temperature has long-lasting effects on threespine stickleback genome-wide DNA methylation levels. However, during post-embryonic development in turbot, it was discovered that rearing temperature had no appreciable effect on genome-wide DNA methylation patterns (Metzger and Schulte, 2017; Suarez-Bregua et al., 2020). Therefore, temperature can have complex and species-specific effects on epigenetic regulation in fish and play an important role in biological processes.

Since *dnmt1* and *dnm3b* (but not *dnmt3a*) genes are downregulated at 21°C compared to 15°C, rearing temperature has an impact on the expression of *dnmt* genes in skeletal muscle in Senegalese sole larvae undergoing metamorphosis (Campos et al., 2013). Following heat stress, zebrafish showed similar changes in the expression of the *dnmt3* gene (two *dnmt3a* and four *dnmt3b*) (Campos et al., 2012; Dorts et al., 2016a). The paralogues of *dnmt3a* and *dnmt3b* responded differently, with the former exhibiting more fluctuation in expression than the latter (Campos et al., 2013). These results indicate different patterns and dynamics of gene expression in response to temperature among the *dnmt3a* and *dnmt3b* paralogues, which is in line with observations in Senegalese sole.

1.10 Overview of the Use of Fish as a Laboratory Model

Model organisms are essential to scientific research because they help us better understand how various biological processes affect human health and disease. Lab model fishes are one of these model organisms that have become essential tools because of their unique characteristics and genetic similarity to humans. Notably, zebrafish and medaka (*Oryzias latipes*) almost show a high degree of genetic similarity to humans, with zebrafish having homologs for about 70% of human genes (Yunus et al., 2022). Because of this quality, lab model fish are the best animals to study human diseases and developmental processes.

The quick development of lab model fish from fertilization to adulthood is a fundamental advantage. Zebrafish, for instance, reach sexual maturity in about three months, making research on them more effective than on other model species. Additionally, the transparency of fish embryos used as a laboratory model during early development gives scientists real-time access to internal functions and organ development. Understanding organogenesis and embryonic development is made possible by this trait (Gerlai, 2003). The significance of lab model fish as research tools is further increased by their prolific reproduction. Scientists can conduct large-scale research thanks to the ability to produce numerous offspring in a single mating event, greatly enhancing the statistical robustness of their findings.

Lab model fish's high throughput capabilities offer a platform for evaluating a wide range of substances as possible therapeutic candidates. This more efficient method speeds up the process of finding new drugs and uses fewer animals for testing, especially larger mammals (Crim and Lawrence, 2021). The translucent embryos of laboratory model fish provide a window into in-depth studies of developmental processes, illuminating the molecular and genetic principles driving embryogenesis, tissue regeneration, and morphogenesis. Researchers may investigate complicated behaviours in a controlled environment, advancing our knowledge of brain networks, learning, memory, and social interactions (Ostrander, 2000). Additionally, lab model fish usually are more affordable than other model organisms, making them available to a wider spectrum of research labs. Progress in many areas of study is made possible by the broad adoption of lab model fish as research organisms.

Considering their genetic resemblance to humans, quick development, and distinctive traits, lab model fish are crucial instruments in scientific research. Our understanding of

biological processes has been greatly improved by their numerous applications in disease modeling, drug development, toxicological investigations, and behavioural study, which has opened the road for medical improvements (Casebolt et al., 1998; Gerhard, 2007; Ostrander, 2000). Lab model fish will continue to be crucial in determining the future of scientific discovery and enhancing human health as ethical, affordable, and scientifically realistic solutions.

Guppies have emerged as significant laboratory models in various fields of research, particularly in behavioral ecology, evolutionary biology, and aquaculture. Their adaptability and rapid reproduction rates make them ideal for studying ecological and evolutionary processes. Guppies exhibit remarkable behavioral plasticity, allowing researchers to explore how environmental factors influence behavior. Studies have shown that guppies adapt their behavior in response to predation, parasitism, and environmental conditions, providing insights into the physiological and genetic mechanisms underlying these adaptations (Fox et al., 2024; Mohammed et al., 2020).

Research comparing guppies with other model organisms, such as zebrafish, has revealed significant differences in organ histomorphometry, suggesting that guppies may be more suitable for specific aquaculture studies due to their unique physiological traits. Guppies' growth and behavior are also affected by environmental variables, such as light color, which can influence their development and phototactic responses. This adaptability underscores their utility in experimental settings (Almaas and Harlita, 2023; Heckley et al., 2022).

1.11 Guppy Fish

1.11.1 The Guppy (*Poecilia reticulata*)

The live-bearing fish *Poecilia reticulata*, sometimes known as guppy, is native to Trinidad and Tobago, Venezuela, and Guyana. It is a member of the Poeciliidae family. It has spread worldwide during the last 150 years, establishing imported populations in at least 70 nations on six continents (Bragança et al., 2020; Farr, 1975). Notable introduction pathways include deliberate releases for mosquito control into artificial and natural water bodies. This technique originates in the early 20th century, albeit there is not much evidence to support its effectiveness. The second common pathway is the discharge of individuals, frequently from the large-scale commercial aquarium trade, as escaped animals or undesired pets (Deacon et al., 2011; Magurran, 2005). Because of its extraordinary ability to reproduce, *P. reticulata* can

produce broods of up to 40 fry after 4–6 weeks of gestation. Populations can grow quickly, averaging three to four months for each generation. Beyond its prodigious breeding habits, guppy adaptability is demonstrated by its persistence in various aquatic settings and situations. It quickly adjusts to new food sources, predators, and social dynamics. Guppies are polygamous animals; a single female can start a new colony by storing sperm from several males for up to eight months (Dolfi et al., 2021; Magurran, 2005). These abilities have made this species one of the most successful viviparous fish in global expansion and establishment in new habitats.

1.11.2 Guppy Anatomy

Guppies exhibit significant sexual dimorphism. Females of the wild species usually have a gray body tone, while males have a variety of patterns on their bodies such as spots, stripes, and splashes that come in different colors. Thyroid hormones are the main hormones that control the expression and development of these color patterns in male guppies (Prazdnikov, 2021). These hormones respond to environmental influences by modulating endocrine activities and influencing pigmentation.

Male guppies are typically 1.5 and 3.5 cm (0.6 and 1.4 inches) long, while female guppies are larger, ranging from 3 to 6 cm (1.2 to 2.4 inches). Males can be recognized by their pronounced dorsal and caudal fins. Many beautiful strains of guppy, such as the snakeskin and grass species, were created through selective breeding and are characterized by a variety of colors, patterns, and fin morphologies. Both sexes of these domesticated tribes typically have larger bodies and more elaborate ornamentation than their wild-type counterparts and often differ morphologically from one another (Dussault and Kramer, 1981). These differences in appearance and breeding patterns are the effects of the processes of artificial selection and natural evolution on the physical characteristics of this species.

The physical features of guppy are typical of teleost fish, with 7-8 soft rays in the dorsal fin, 8–10 in the anal fin, and 13–14 in each pectoral fin (Froese and Pauly, 2022). Guppies are notable for having a unique reproductive structure. In males, the anal fin changes to become a gonopodium, a sexual organ that is intromittent. Sperm bundles pass through a canal in the gonopodium to help the female get inseminated. Sperm bundles enter the female via a gonoduct that is located in front of the anal fin (Greven, 2005). Female guppies have a single, big ovary with a folded surface that has a "micro pocket" for storing sperm (SSP) (Kobayashi and

Iwamatsu, 2002; Magurran, 2005). This unique structure allows sperm from one or more males to be stored for a long time, often months until they are needed to fertilize the eggs.

1.11.3 Habitat of Guppy

P. reticulata has a wide distribution in its native habitat, which includes a range of conditions from clear alpine streams to murky, slow-moving bodies of water at lower elevations that frequently lack significant aquatic vegetation (Juliano, 1989). Guppies are mainly found on the shallow edges of pools and streams; they are rare in the deeper portions of the streams. *P. reticulata* is an adaptable species that can withstand a broad range of salinities and temperatures (18–28°C); it can even withstand concentrations that are 150% of normal seawater (Chervinski, 1984), but their common occurrence is usually in freshwater streams close to the coast. In non-native areas, guppies typically appear as the lone species in highly contaminated waterways (Barua et al., 2001). This ability to adapt to diverse environmental conditions makes *P. reticulata* a resilient and adaptable species.

1.11.4 Guppy Mating

Poecilia reticulata are asexual species that reach sexual maturity in 10–20 weeks, with females producing two to three generations per year. Predation levels influence the size at maturity (Van Wijk, 2011): animals from high-predation areas mature at 11 mm Standard length (SL) for females and 9 mm SL for males, while animals from low-predation areas mature at 15 to 18 mm SL for females and 15 to 16 mm SL for males.

The gonopodium, a unique anal fin found behind the ventral fin, is unique to male guppies. Sperm bundles, or spermatozeugmata, are made more accessible to pass through the female vaginal orifice by this structure (de Lira et al., 2021). Female guppies can retain the potential to fertilize for up to eight months after insemination by storing sperm in the folds of their gonoducts and ovaries. However, it is interesting to note that sperm that has recently been inseminated has a greater chance of fertilizing eggs than sperm that has been kept (Müller et al., 2018). These unique features provide significant adaptations to improve reproductive success and survival of the offspring.

The two main methods of mating that male guppies use are sneaky attempts for forceful copulation and wooing displays for cooperative copulation. Extensive activities are part of

courtship displays, such as sigmoid displays, in which males flex their bodies into "S" or "C" shapes to highlight colors that are sexually appealing. Females are drawn to these visual cues, which start cooperative copulation. On the other hand, sneaking attempts involve males quickly approaching females from behind in an effort to engage in covert copulation. A population's operational sex ratio, body size, and male phenotype can all have an impact on the decision between these strategies (Zhang et al., 2019). Because these displays serve as markers of male fitness and a willingness to commit to reproductive activities, females are more likely to choose males who engage in frequent and elaborate courtship displays (Dosen and Montgomerie, 2004). These diverse reproductive behaviors indicate different evolutionary strategies in *P. reticulata* males that contribute to mating success and sexual selection in their populations.

Guppies have a polyandrous mating system in which females mate with several males multiple times. Males with vibrant colors tend to attract more female attention, especially those with orange markings on their flanks—the bright coloring results from eating the carotenoid-rich fruits of the cab rehash tree (*Sloanea laurifolia*). Orange patches are indicative of higher levels of physical fitness, better swimming in strong currents, and robust male health with increased foraging capacity (Godin and Dugatkin, 1996; Houde, 2019). The thyroid affects the patterns and hues of male guppies (Prazdnikov, 2021). The frequency and length of a male guppie's courtship display have a significant impact on the mating preferences of the females. Fitness is determined by the strenuous physical effort required to maintain the sigmoid display (Cole and Endler, 2016; Ohlyan et al., 2012). A female guppy will increase mating activity and postpone the formation of her brood if she finds a more attractive second mate than her first (Rodd et al., 2002). The first two days after giving birth or when a females is a virgin are when she is most receptive to male wooing.

Guppies reproduce lecithotrophically, meaning that the embryos in their eggs get their food from the yolk the female deposits in the egg prior to fertilization. Until they are fully developed and prepared for birth, embryos go through internal development (Liu and Lee, 2014). Guppies have very variable litter sizes, ranging from one to more than one hundred offspring, and the size of the female has a direct bearing on the size of the offspring. Predation levels also have an impact on fry populations; females in high-predation locations give birth to more, albeit smaller, offspring than those in low-predation areas (Arendt and Reznick, 2005).

Although there is no sex disparity at birth, the adult sex ratio may favor females, indicating a higher rate of male mortality.

1.11.5 Nutrition of Guppy

P. reticulata is an omnivorous feeder that eats benthic debris, invertebrate larvae, and algae, which make up around half of its diet in the wild (Dussault and Kramer, 1981). There is evidence that *P. reticulata* may prey on both *Rivulus hartii* and its own species' larvae within its natural range. In areas where *P. reticulata* has been introduced, experimental captive trials of the closely related species *Gambusia holbrooki* have shown predation on a wide variety of larvae from other fish species (Howe et al., 1997), suggesting that *P. reticulata* may prey on these introduced species.

1.11.6 Economic Value of Guppy

P. reticulata is widely grown in commercial fish hatcheries and has significant economic value as an attractive aquarium species. A number of highly ornamented aquarium strains have been carefully selected and have become very popular in the retail aquarium industry, reaching up to 95% of pet stores in one area of Canada. Major producers of these decorative varietals are Taiwan, Malaysia, and Singapore (Deacon, 2023). Furthermore, the species is widely used in a variety of biological investigations as a model organism.

1.11.7 Social Benefit of Guppy

P. reticulata is a popular aquarium fish that has been around since the early 1900s, making it one of the most popular species. Its great leisure and aesthetic value are what makes it appealing as an aquarium fish (Deacon, 2023). But in its wild state, it has little to no social value and is not considered appropriate for fishing for pleasure.

1.11.8 Genetics of Guppy

P. reticulata has an XY sex-determination system, where males make up the heterozygotic sex, and has a karyotype with 23 haploid (gametic) and 46 diploid (zygotic) chromosomes (n = 23, 2n = 46) (Meffe and Snelson, 1989). Male pattern-determining genes are found on both gonosomes and autosomes, and they express themselves only when androgenic hormones are present (Greven, 2005; Kwan et al., 2013). Seventeen pigmentation traits with holandric (Y-linked) inheritance patterns were identified by Kirpichnikov (1981). Of these, 16

traits may be found on the X or Y chromosome, and five traits are autosomal (one of which is sex-limited) (Dick et al., 2018; Haskins et al., 1970; Kirpichnikov, 1981; Winge, 1938). The sex determination system and complex inheritance patterns in *P. reticulata* play a key role in the diversity of appearance traits and reproductive processes.

1.11.9 Reproductive Biology of Guppy

Internal fertilization happens in all poeciliids when the gonopodium is used to insert the male's spermatozeugmata into the female's reproductive tract. The female *P. reticulata* may retain sperm from a single insemination for as long as eight months, which helps with ova fertilization. After fertilization, eggs are stored in the reproductive tract of the female, where yolk deposits formed before fertilization provide nourishment for the developing embryos. Fry shows signs of feeding immediately after birth, and no further care from parents is given. Notably, there have been reports of parent predation on offspring in aquarium environments (Whitern, 1980). *P. reticulata* litter sizes vary greatly, from one to over one hundred offspring, and are strongly correlated with the size of the female (Travis, 1989). Differences in fry numbers among predation regimes are visible in *P. reticulata's* native habitat; females from high-predation areas give birth to more, albeit smaller, offspring than those from low-predation areas (Alkins-Koo, 2000). When *P. reticulata* and the predatory cichlid *Crenicichla alta* cohabit, an average-sized female's predicted litter size is 6.4; in places where *C. alta* is absent, the expected litter size is 2.8. Trained strains of *P. reticulata* that have been deliberately developed to have larger bodies regularly produce brood counts that are higher than this, frequently reaching up to several dozen. Female *P. reticulata* give birth to 2-3 generations per year (Alkins-Koo, 2000). Predation risk affects fish size at maturity. Females do not demonstrate a significant period of reproductive senescence; instead, they continue to reproduce until they are 20–34 months old. When a female is a virgin or in the first three to four days after giving birth, she is sexually receptive, but she avoids mating attempts at other times. Males engage in intense courtship behaviour, with females preferring those who exhibit higher rates of courtship—despite the fact that these males may be more vulnerable to predators (Endler, 1987; Himick and Peter, 1994a). In cases of female unresponsiveness, males may resort to sneak matings or monopodial thrusting to forcefully inseminate females. However, these attempts are generally unsuccessful, and females may expel male sperm before fertilization. The sex ratio at birth is equal, but adult sex ratios may favor females, indicating higher male mortality.

1.11.10 Egg Development

Research into embryogenesis in guppies has been constrained by their viviparous nature and internal fertilization process. However, by dissecting gravid female guppies at various stages of pregnancy, researchers have managed to observe and document the sequential development of guppy embryos. Guppy embryos develop internally within the mother fish, benefiting from the capacity to store sperm from multiple mates, leading to asynchronous fertilization and the presence of embryos at various developmental stages within a single gravid female.

Observations revealed a slight asynchrony in the development of different batches of eggs, likely attributable to asynchronous fertilization. Throughout the study, researchers identified eleven distinct stages of embryonic development (Martyn et al., 2006; Shaddock, 2008):

1. Fertilized Eggs: These eggs were fully swollen, brownish-yellow, rounded, and translucent, with oil droplets evenly distributed on the yolk surface.
2. Blastodisc: After fertilization, the oil droplets merged beneath the embryo proper, forming a blastodisc.
3. Gastrula: This stage featured a visible archenteron, discernible under a microscope.
4. Optic Cup Stage: Eyes remained unpigmented, while blood vessels of the portal system were visible in the lower part of the yolk sac.
5. Early-Eyed Stage: Eye pigmentation increased gradually, and pectoral fin buds emerged, along with the differentiation of somitic and non-somitic muscles.
6. Middle-Eyed Stage: Melanophores first appeared above the midbrain and subsequently behind the midbrain-hindbrain boundary.
7. Late-Eyed Stage: At this stage, a line of dark pigment cells demarcated the horizontal midline, and melanophores increased in number and density on the head.
8. Very Late-Eyed Stage: Embryo segmentation became apparent, and the flexure between the head and trunk gradually straightened.
9. Straightened Embryo: Approximately 22 somites formed the myotome.

10. Mature Embryo: Embryos developed jaws and absorbed their yolk, with some exhibiting retracted yolk sacs.
11. Neonate Fry: After birth, fry displayed swimming behaviour and were immediately capable of swimming, eating, and avoiding danger.

These developmental stages align with previous reports by Goodrich et al. (1944) (Goodrich et al., 1944), Tavolga (1949) (Tavolga, 1949), and Martyn et al. (2006) (Martyn et al., 2006). Shikano and Fujio (Shikano and Fujio, 1997) were documented similar observations of neonate fry behaviour.

1.12 Objectives

Biological processes in fish, especially processes related to reproduction, nutrition, and epigenetic regulation, are constantly influenced by environmental conditions. Temperature changes, fluctuations in food resources, and other biological and ecological factors are among the most important stimuli that can change gene expression patterns in these organisms. The guppy (*Poecilia reticulata*) species, as a viviparous fish with a short reproductive cycle, high sensitivity to environmental changes, and its importance in evolutionary and ecological biology research, is a very suitable model for studying these phenomena.

In the present study, the main goal is to investigate the dynamics of gene expression related to reproduction, methylation, and appetite regulation in guppy fish; especially since this study was conducted at different stages of pregnancy in females, and under different nutritional patterns (full nutrition versus long-term starvation), and at different temperatures. Understanding these dynamics can provide an answer to the fundamental question of how fish, especially viviparous species, adapt to changing environmental conditions through the regulation of gene expression and epigenetic mechanisms.

In this direction, molecular methods including RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction (qPCR) have been used, and methylation changes have been considered as one of the most key indicators of epigenetic regulation.

The importance of this study is not limited to its fundamental aspects in the field of gene and epigenetic regulation, but also has practical dimensions. Climate change, especially

temperature fluctuations, directly affects aquatic species, especially in tropical and subtropical regions. A more precise understanding of the molecular mechanisms of adaptation can help to better predict the biological responses of species to environmental changes. On the other hand, guppy is considered an important species in aquatic resource management and ornamental fish farming due to its economic value in the ornamental fish market and its role in ecological and evolutionary research. The results of this study can be used to optimize nutritional programs and manage temperature conditions in breeding farms, as well as design protection programs against climate threats.

Overall, this study, with an interdisciplinary approach, examines the link between nutrition, reproduction, and epigenetics within the framework of adaptive biology and attempts to take a step towards developing fundamental and applied knowledge in biology, aquaculture, and biodiversity conservation by providing a comprehensive picture of guppy molecular responses to environmental stimuli.

2 Chapter 2

Material and Methods

2.1 Experimental Animals

I purchased both male and female guppies (*Poecilia reticulata*) from MSR Imporium Importations Canada, located in Lasalle, Quebec, Canada. The fish were housed in 20L aquariums with filtered water, constant aeration, and a 12-hour light-to-12-hour dark photoperiod at 25°C. Submersible water heaters (Hagen, Baie d'Urfé, QC, Canada) were used to regulate the temperatures. A digital thermometer (model PH838, Dr. meter, www. drmeter. Com) was used to measure pH. Every day, the temperature and pH were measured. Fish were fed Fancy Guppy meal (Hikari Sales USA, Inc.) once daily at 11 a.m. It contained 5 percent protein, 8 percent fat, 2 percent fiber, 10 percent moisture, 18 percent ash, and 1 percent phosphorus (Hayward, CA, USA). Prior to the experiments, the fish were acclimated to these conditions for a week.

The studies followed protocols approved by the Animal Care Committee at Memorial University of Newfoundland, which followed the suggestions made by the Canadian Council on Animal Care Guidelines for the Care and Use of Experimental Animals.

2.1.1 Summary of Experimental Design

In this study, the expression of various genes in guppy fish was investigated. Three categories of genes were selected, including genes influencing feeding and fasting, genes related to reproduction, and genes affecting epigenetics. Experiments were designed and conducted under three different conditions.

In the first experiment, the effect of different reproductive stages on gene expression was examined. Sampling was performed at various stages of the reproductive cycle. In the second experiment, the impact of food intake variations and fasting on gene expression was studied. In the third experiment, the effect of temperature changes on the expression of the target genes was investigated.

All samples were collected and subjected to RNA extraction. Gene expression was measured using RT-qPCR. The obtained data were analyzed, and the results were evaluated.

Table 1 provides a summary of the different studies conducted, experimental conditions, and the number of specimens analyzed for gene expression.

Table 1: Overview of Experimental Conditions and Sample Allocation

Experimental Condition	Groups Studied	Type of Analysis
Different Reproductive Stages	Males and Females	Gene expression at appetite, reproduction, Epigenetic in brain, Intestine and Liver
Fasting Study	Males and Females	Gene expression at appetite, reproduction, Epigenetic
Temperature Study	Males and Females	Gene expression at appetite, reproduction, Epigenetic

2.1.1 Expression of Specific Genes at Different Reproductive Stages

To investigate gene expression across different reproductive cycles, the following experimental procedures were conducted. The body weight and total length of the fish were measured once at the end of the experiment. The average length and weight were 3.4 ± 0.29 cm and 0.39 ± 0.11 g, respectively. Before sampling, fish were given an overdose of MS-222 (TMS, tricaine methanesulfonate, Syndel Laboratories, Vancouver, BC, Canada) before being killed by spinal section. Brain, liver, and intestine were then removed and kept separate for subsequent use in 500 µL of RNAlater (Qiagen, Mississauga, Ontario, Canada) and were used to examine the expression of the studied genes.

All fish were dissected for observation of the gonads with the naked eye and with a compound microscope (10X). For gravid females, various embryonic stages were determined based on previous publications (Martyn et al., 2006; Shahjahan et al., 2013b).

2.1.2 Effects of Fasting on Gene Expression

Twenty male and twenty female guppies were divided into eight 20 L aquariums and acclimated at 25°C for one week. Fish were fed daily (11 am) with amounts ranging from 0.04 g to 0.180 g until the fish showed signs of satiety. Four tanks contained males, and four tanks contained females (5 fish per tank) (duplicated).

After the acclimation period, two tanks with females and two tanks with males continued to be fed, while the other 4 tanks were fasted for 14 days.

During the experiment, the average temperature was 25.3 ± 0.47 °C, and the average was pH of 7.78 ± 0. 172. Some mortality occurred during the experiment so that the final number of fish per tank varied from 3 to 5 [see appendix 1].

At the end of the experiment all surviving fish were killed, measured and weighed, and their brain, liver, and intestine were sampled. The sex and reproductive stage of the fish were determined.

2.1.3 Effect of Temperature on Food Intake and Gene Expression

In this study, 3 groups were tested: 1. The control group, where the temperature of 25°C was maintained in the tank during the entire test period; 2. The group in which, after 1 week of adaptation to 25°C temperature, the temperature was progressively (over a week) increased to 32°C and then kept at 32°C for 2 weeks, 3. After 1 week of adaptation at 25°C, the temperature was progressively lowered to 18°C and kept at 18°C for 2 weeks. There were three tanks with 6 fish in each group (duplicated). This experiment was conducted for both male and female fish.

2.1.4 Food intake quantification

Food intake was quantified in fed fish in the fasting experiment (to compare male and female feeding) and in the temperature experiments. Fish were fed once a day at 10:00 until they appeared satiated. Fish were hand-fed several rounds of pellets slowly and cautiously. The point at which fish ceased seeking out and ingesting pellets was called saturation. The weight of the food given to each tank was recorded. The average amount of food consumed was divided by the average weight of fish and the total number of fish per tank to determine the average daily food intake (g food/g fish) per tank.

2.2 Total RNA Extraction

With the help of the GeneJET RNA Purification Kit (Fermentas, Burlington, ON, Canada), total RNA was extracted from fifty milligrams of tissue samples, including the gut, liver, and brain. A NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc.) with an absorbance of 260 nm was used to measure the concentration and purity of RNA. USA (Wilmington, DE). For additional analysis, only RNA samples that showed a ratio of absorbance at 260 nm to that at 280 nm falling in the range of 1.8 and 2.1 were deemed appropriate.

2.3 cDNA Synthesis

One µg of isolated RNA was reverse transcribed using the SensiFAST cDNA Synthesis Kit (Meridian Bioscience Inc., Cincinnati, OH, USA) following the manufacturer's protocol. Briefly, the reactions consisted of RNA, one µL of reverse transcriptase and four microliters of TransAmp Buffer 5X. Distilled water was used to bring the total amount down to 20 µL. The reactions were then incubated for five minutes at 25°C, twenty-five minutes at 42°C, and fifteen minutes at 85°C.

2.4 Primer Design

Initially, searches on *https://www.ncbi.nlm.nih.gov/* using the gene name and the species *Poecilia reticulata* were used to obtain the mRNA sequences matching each targeted gene. Using the primer design tool found at *https://www.ncbi.nlm.nih.gov/tools/primer-blast*, primers were then created in accordance with the precise parameters needed for the real-time PCR method. Primers were located on exon-exon junctions and had a length in the range of 100 and 200 nucleotides (Table 2-1).

Table 2-1: Table of primers of studied genes with Gene Bank ID

Target Gene	Sequence Name	Orientation	Primer Sequence (5' to 3')	GeneBank ID
Gapdh	Pr gapdh_F1	Forward	CATTGAGAAGGCCTCCGCTC	XM_008431448.1
	Pr gapdh_R1	Reverse	TACTTCTCGTGGTTGACG CC	
EF-1a	Pr EF-1a_F1	Forward	CTA CAA GTG TGG TGG CAT CG	XM_012693672.1
	Pr EF-1a_R1	Reverse	CCA GGC GTA CTT GAA TGA GC	
Cck a	Pr CCKa_F2	Forward	TGG CTT CCA GAT CAG ATC CTC	XM_008432066.1
	Pr CCKa_R2	Reverse	TCC ATC CAG CCG AGG TAA TC	
Cck b	Pr CCKb_F2	Forward	GTG CGT AGA AAC TCC ATG GC	XM_008421465.2
	Pr CCKb_R2	Reverse	CGG CCA AAA TCC ATC CAT CC	
PYY a	Pr PYYa_F2	Forward	GGC CAA GTA CTA TAC GGC CC	XM_008415058.2
	Pr PYYa_R2	Reverse	CGA GCG CTT CCC ATA TCT CTG	
Dnmt 3a	Pr DNMT 3A_F2	Forward	GGA CTT GGG AAA ACT GGG AGA G	XM_008399113.2

	Pr DNMT3A_R2	Reveres	CGG ATG AAT GGC GGT GAG AT	
Dnmt 3b	Pr DNMT 3B_F2	Forward	GGC GGA AGG ATA CAC GTT TTG	XM_017310123.1
	Pr DNMT 3B_R2	Reverse	GGA ACA GTG CCA CGC CG	
Tet 2	Pr tet2_F1	Forward	ATC GCA TCA TGC CAC TGT GT	XM_008412058.2
	Pr tet2_R1	Reverse	CCC AGT GAT GCC AGA CCT TT	
Tet 3	Pr tet3_F1	Forward	CCT CTT ATG GGG ATT CGG TCA G	XM_008417664.2
	Pr tet3_R1	Reverse	TCT TCA AAA TAT CCC ACC CTC CA	
Orexin	Pr Orexin_F1	Forward	AGG AAG ACT CTG GCG TTC AT	XM_008429799.
	Pr Orexin_R1	Reverse	TGT GCT CTT GTT GCC AGA AC	
NPY	Pr NPY_F1	Forward	AGA GAC AAC CCA GAC ACT GT	XM_008421697.2
	Pr NPY_R1	Reverse	CGG GAG CCC TTC GTA TCT TA	
Npvf	Pr npvf F5	Forward	GAG CAG CAT CGA CGA CAG G	XM_008421653.2
	Pr npvf R5	Reverse	ATG TTG GCG TGC ATG TGG AG	
Kiss 2	Pr kiss2 F2	Forward	CTT GTG GAA AGA TGC GCC TG	XM_008434533.2
	Pr kiss2 R2	Reverse	GTC TTC TCC TGA GGG CTG TT	
GnRH 2	Pr GnRH2_F2	Forward	CGT GAG AGA TAC AGG CGT C	XM_008409509.2
	Pr GnRH2_R2	Reverse	AGA GCT GAG CGC TAA CAC AC	
GnRH 3	Pr GnRH3_F1	Forward	CAG GTG GAA AGA GAA GCG TG	M_014980758.1
	Pr GnRH3_R1	Reverse	CTT CAG GAA GCG ACA CCA CT	
AVP	Pr AVP F1	Forward	GCT GTA ACT CTG AGG GCT GC	XM_008418307.2
	Pr AVP R1	Reverse	GAA GCA GCA GGT CTG TAG GG	
IT	Pr IT F1	Forward	GTT CGG CGT GTT ACA TCT CC	XM_008418298.2
	Pr IT R1	Reverse	GGT AGT TCT CCT CCA CGC AG	

2.5 Real-Time PCR

Five µl of SensiFAST SYBR® No-ROX mix (Bioline), zero milliliters of water, zero milliliters of each 10µM sense and antisense primer, and four microliters of cDNA (diluted 1:10 with water) were used to prepare duplicate reactions. Next, the total reaction volume (10 µl) that

was produced was transferred into a 96-well plate. A CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada) was then used to perform cDNA amplification. The thermal cycling conditions were as follows: two minutes of initial denaturation at 95˚C; forty cycles of denaturation at 95˚C for five seconds; annealing at 60˚C for ten seconds; and elongation at 72˚C for fifteen seconds. Prior to data analysis, a serial dilution curve (1:2) was used to determine the efficiencies, R2 values (≥ 0.98), and ideal annealing temperatures for each primer pair. After every qPCR run, a melting curve analysis was carried out to verify primer specificity. The range of efficiencies for all reactions was 90–95 percent.

The $2^{-\Delta\Delta Ct}$ method was widely used as a relative quantification strategy for qPCR data analysis. This method uses the threshold cycles (CTs) produced by the qPCR system to make it easier to calculate the relative gene expression levels among different samples. The difference in the threshold cycle between the reference and target genes is represented by the first ΔCT.

ΔCT = CT (target gene) − CT (reference gene)

The subsequent ΔΔCT is the difference in ΔCT between a target sample and a reference sample:

ΔΔCT = ΔCT (target sample) − ΔCT (reference sample) = (CTD − CTB) − (CTC − CTA)

By using this method, the fold changes in target gene expression in a target sample compared to a reference sample are calculated and normalized to a reference gene. For reference samples, the relative gene expression is usually set to 1 because ΔΔCT equals 0 and yields a fold change of 1. The NormFinder Software (*https://www.moma.dk/software/normfinder*) was used to evaluate the stability of three candidate reference genes—GAPDH, β-actin, and elongation factor 1α—in each experiment. The most stable genes for the reproductive stage experiment were found to be EF-1α in the gut, GAPDH in the liver, and EF-1α in the brain. EF-1α was the most stable gene in the brain, GAPDH in the liver, and GAPDH in the intestine during the fasting experiment. β-actin was found to be the most stable gene in the brain, liver, and intestine of both males and females in the temperature experiment.

2.6 Statistical Analysis

The data was analyzed using GraphPad Prism version 9 software. The data is displayed as Mean ±SD. Shapiro-Wilk and Kolmogorov-Smirnov tests were utilized to determine whether the data had a normal distribution. An ANOVA was performed to compare daily food intake, which had a normal distribution, and then Tukey's post-tests were employed. Kluskal-Wallis tests and Dunn's multiple comparison tests were used to assess statistical differences among treatment groups because some of the data did not fit into a normal distribution. A significance threshold of $P<0.05$ was chosen.

3 Chapter 3
Results

3.1 Expression of Specific Genes in Males and Females at Different Reproductive Stages

3.1.1 Sample data

In total, 45 fish were sampled, of which 33 (73.33%) were females and 12 (34.61%) were males (Table 3-1). The body weight and total length of the fish were measured once at the end of the experiment. The average length and weight were 3.23 ± 0.30 cm and 0.40 ± 0.12 g, respectively in female and 3.35 ± 0.29 cm and 0.38 ± 0.07 g, respectively in male. The average weight of the gonad in the population (females and males) was 0.032 ± 0.037 g. Female stages were defined based on a previous the study (Haynes, 1995) and divided into 3 groups. Females with non-developed immature eggs, females with early-stage embryos (defined by the presence of yolk but no formed embryo) and females with late-stage embryos (formed embryos with visible eyes and pigmentation). Males all had mature testes with sperm and were all put in one category (Figure 3-1).

Table 3-1: The number of male and female fish at different reproductive stages

Stage	Number
Immature	14
Early stage	9
Late stage	10
Male	12

Figure 3-1：Gonads of males and females: 1) ovary of a female at an immature stage; 2) female with early-stage eggs; 3) female with late-stage eggs; 4) testes of a mature male.

3.1.2 Brain Expression

There were no differences ($P<0.05$) in orexin and NPY brain gene expressions either among males and females or females at different stages of development (Fig 3.2)

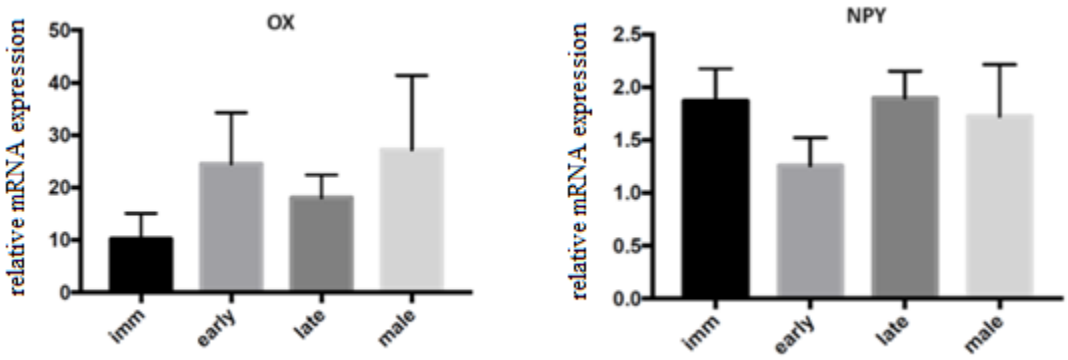

Figure 3-2: Relative changes in Orexin (OX) and NPY gene expression in brain in different groups are reported as mean ± standard deviation. Immature (n=8), Early stage (n=7), Late stage (n=7) and male (n=4).

Brain Kiss 2 expression was higher in late-stage eggs females compared to males, females with early-stage embryos and females with non-developed immature eggs, but there were no significant differences between the other groups (Fig. 3-3). Brain AVP expression was higher in males compared to both Females with non-developed immature eggs and females with late-stage eggs, and higher in females with early-stage eggs than in immature females (Fig. 3-3). There were no significant differences in GnRH2, GnRH3, IT or npvf across groups.

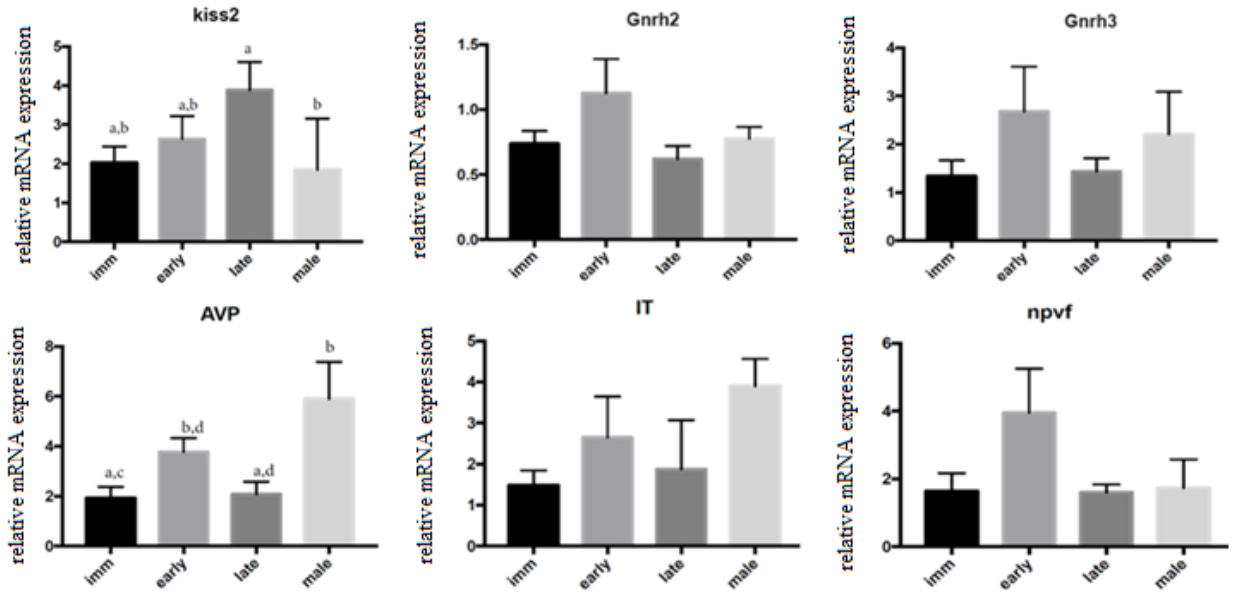

Figure 3-3: Brain relative changes of Kiss2, Gnrh2, Gnrh3, AVP, IT and npvf gene expression in different groups are reported as mean ± standard deviation. Dissimilar letters indicate a significant difference between two groups. Immature (n=8), Early stage (n=7), Late stage (n=7) and male (n=4).

3.1.3 Intestine Expression

In the intestine, there were no significant differences in CCKa, CCKb and pyy between groups ($p<0.05$).

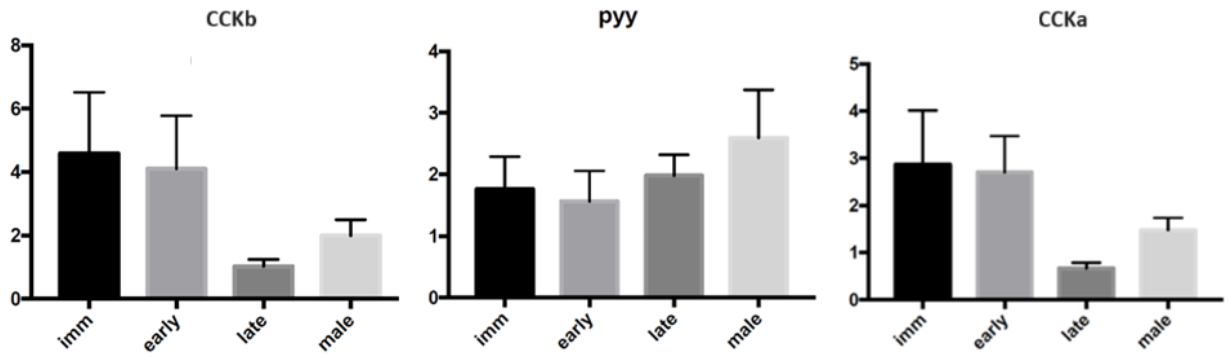

Figure 3-4: Relative changes of CCKb, pyy, and CCKa genes expression in intestine of different groups are reported as mean ± standard deviation. Immature (n=8), Early stage (n=7), Late stage (n=7) and male (n=4).

3.1.4 Liver Expression

In the liver, there were no significant differences in dnmt3b, dnmt3a, tet2 and tet3 across groups ($p<0.05$).

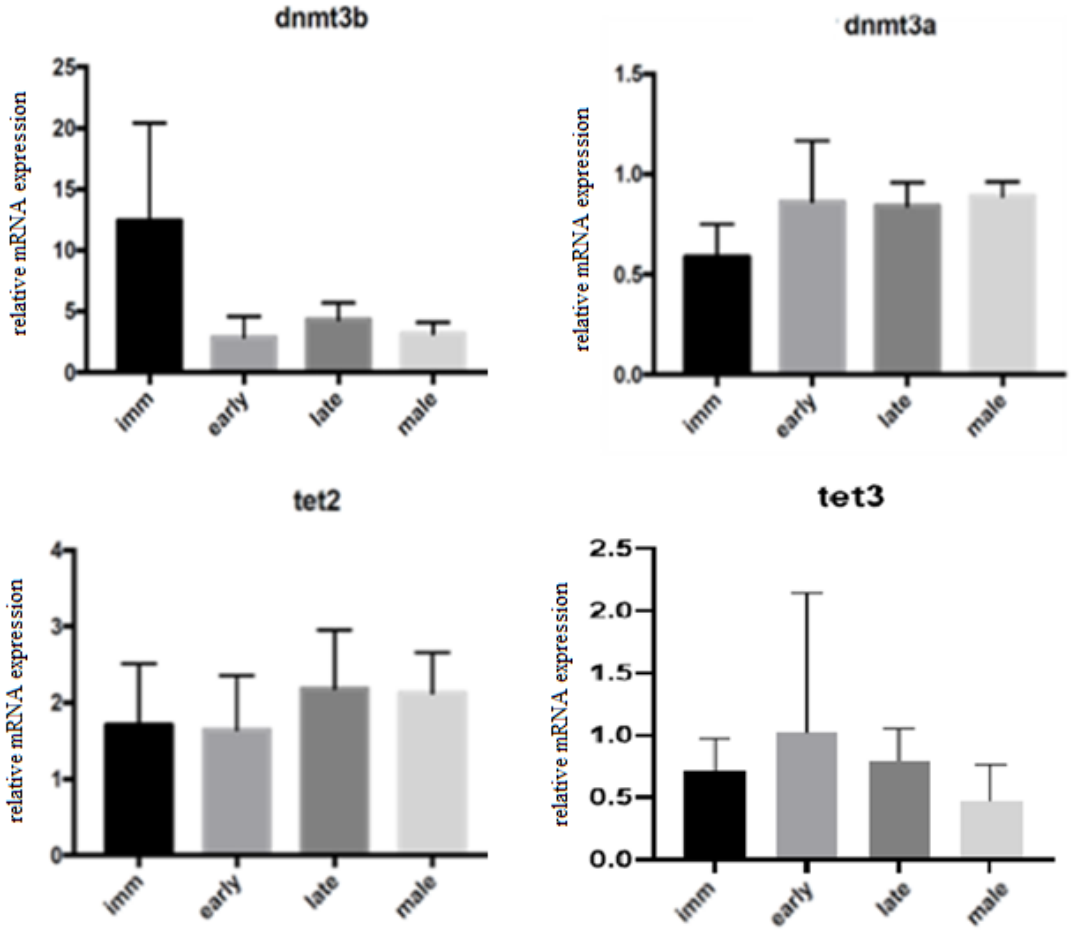

Figure 3-5: Relative changes of dnmt3b, dnmt3a, tet2 and tet3 genes expression in brain of different groups are reported as mean ± standard deviation. Immature (n=8), Early stage (n=7), Late stage (n=7) and male (n=4).

3.2 Effects of Fasting

3.2.1 Animals Used

A total of 14 female fish and 16 male fish were analyzed. The average weight was 1.14 ± 0.33 g and the average length of 4.5 ± 0.21 cm in females and 0.41 ± 0.09 g and 3.31 ± 0.16 cm in males. Female fish were in three developmental stages, namely immature, early stage, and late stage, while male fish were all mature, with sperm present in their testes (Table 3-2)

Table 3-2: Number and characteristics of sampled guppies

		Number	Weight (g)	Length (cm)
Female	Immature eggs	2	1.14 ± 0.33	4.5 ± 0.21
	Early-stage eggs	5		
	Middle stage eggs	2		
	Late-stage eggs	3		
Male	Mature with sperm	8	0.41±0.09	3.31±0.16

3.2.2 Food Intake

Figure 3-6 shows the average daily food intake (14 days) in female and male fish were 30.771 ±1.048 mg/g/fish and 18.92 ± 1.24 mg/g/fish, respectively. The food intake of females was significantly higher compared to that of males (Student's t-test).

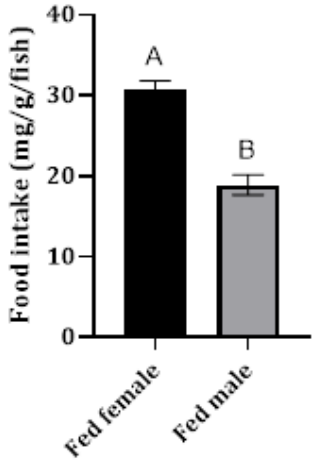

Figure 3-6: Average daily food intake in fed females (n=26 fish total) and fed male (n=29 total fish). Data are presented as mean ± standard deviation.

3.2.3 Expression of Appetite-Related Genes

Changes in the expression of transcripts involved in appetite are shown in Figure 3-7. Brain orexin expression was higher in fasted males compared to fed males, fed females and fasted females. Intestine pyya transcript expression was lower in fasted females compared to both fed females and fed males and lower in fasted males compared to fed males. There were no significant differences in intestine CCK or brain NPY expressions across groups.

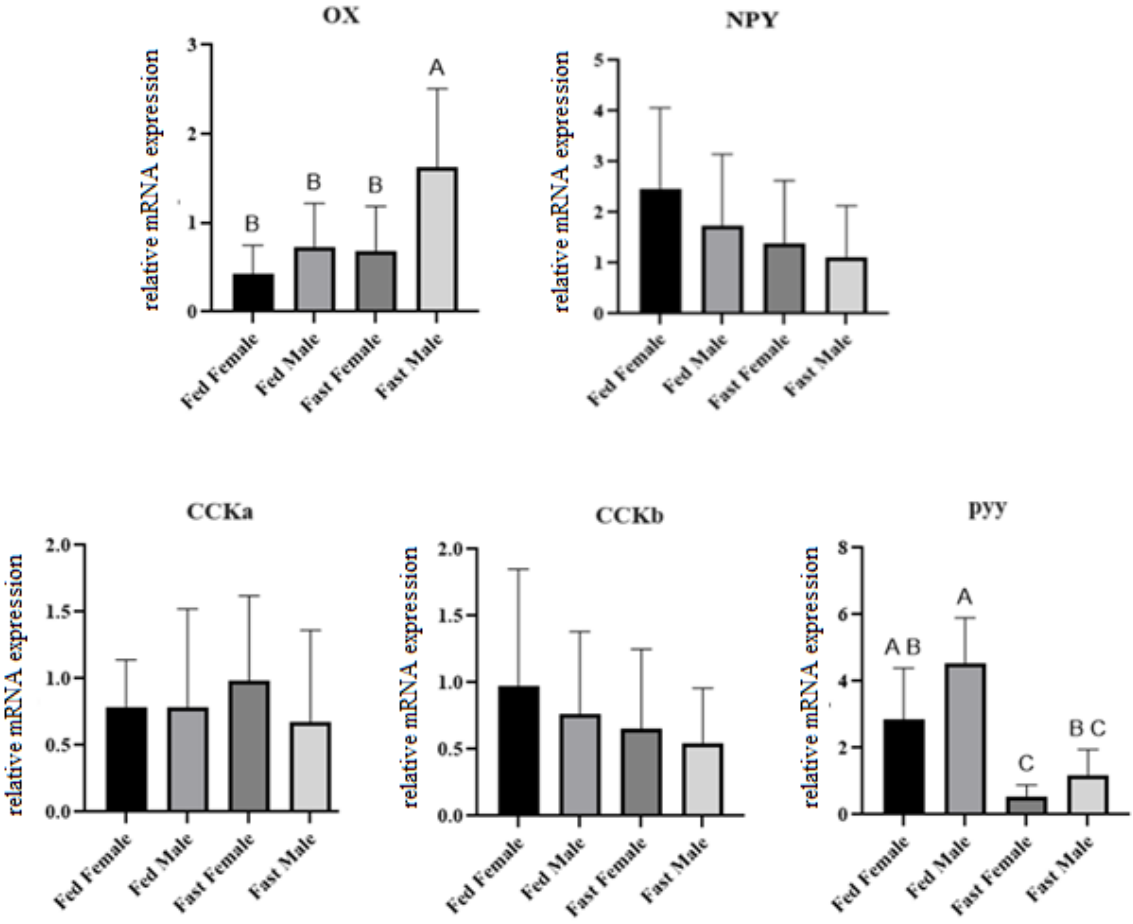

Figure 3-7: The relative changes of brain OX, NPY, and intestine pyya, CCKa and CCKb genes expression in different groups are reported as mean ± standard deviation. Dissimilar letters indicate a significant difference between two groups (Fed female (n=6), Fed male (n=9), Fast female (n=8), Fast male (n=7))

3.2.4 Expression of Reproduction-Related Genes

Changes in the expression of genes involved in reproduction are shown in Figure 3-8. Gnrh3 expression was higher in fasted male compared to fed males, fed females and fasted males. IT expression in fed female was higher compared to fed males, fasted females and fasted males. npvf expression in fasted males was higher compared to fed females and fed males. There were no significant differences in Kiss2, Gnrh2 or AVP between groups.

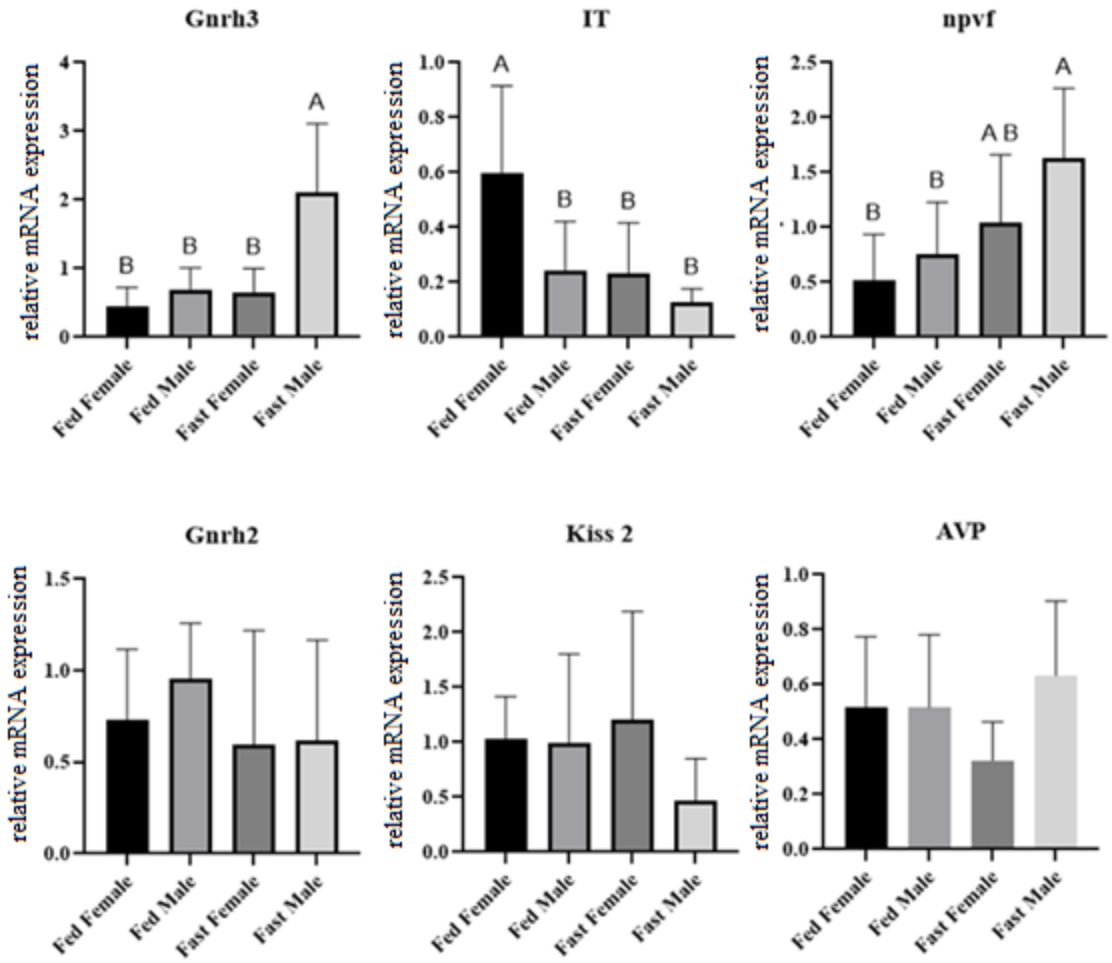

Figure 3-8 : Relative changes in brain Gnrh3, IT, npvf, Gnrh2, Kiss2, IT and AVP genes expression in different groups are reported as mean ± standard deviation. Dissimilar letters indicate a significant difference between two groups (Fed female (n=6), Fed male (n=9), Fast female (n=8), Fast male (n=7))

3.2.5 Expression of Epigenetic Related Genes

Changes in the expression of genes involved in epigenetics are shown in Figure 3-9. Hepatic dnmt3b expression levels were higher in fasted males compared to fasted females and fed females. There were no significance differences in dnmt3a, tet 2 or tet 3 between groups.

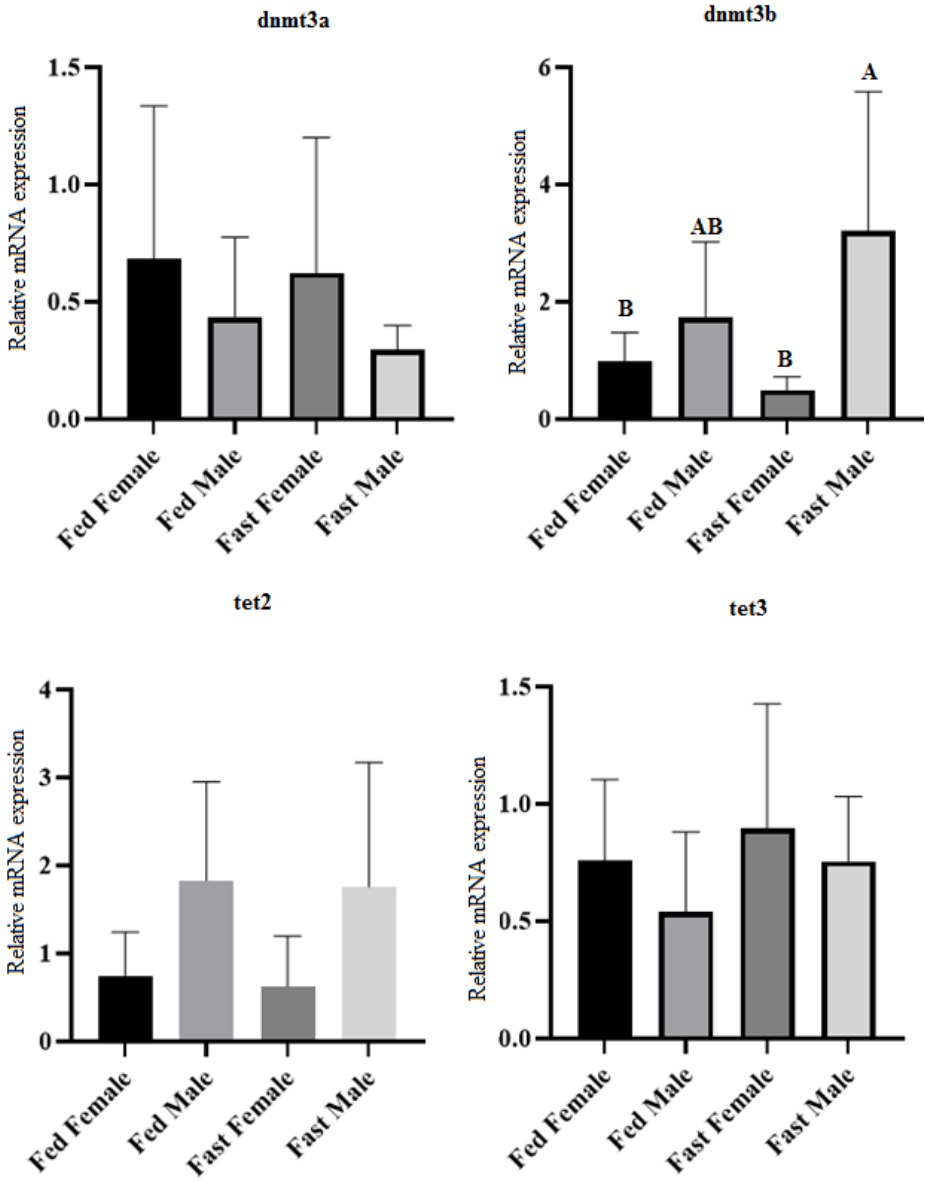

Figure 3-9: Relative changes in hepatic dnmt3a, dnmt3b, tet 2 and tet 3 genes expression in different groups are reported as mean ± standard deviation. Dissimilar letters indicate a significant difference between two groups. Fed female (n=6), Fed male (n=9), Fast female (n=8), Fast male (n=7).

3.3 Effects of Temperature

3.3.1 Effects on food intake

Figure 3-10 shows the effects of temperature on the amount of food consumed in males and females. In both males and females, food consumption was higher at 32 °C compared to 25 °C, and lower at 18 °C compared to 25 and 32 °C.

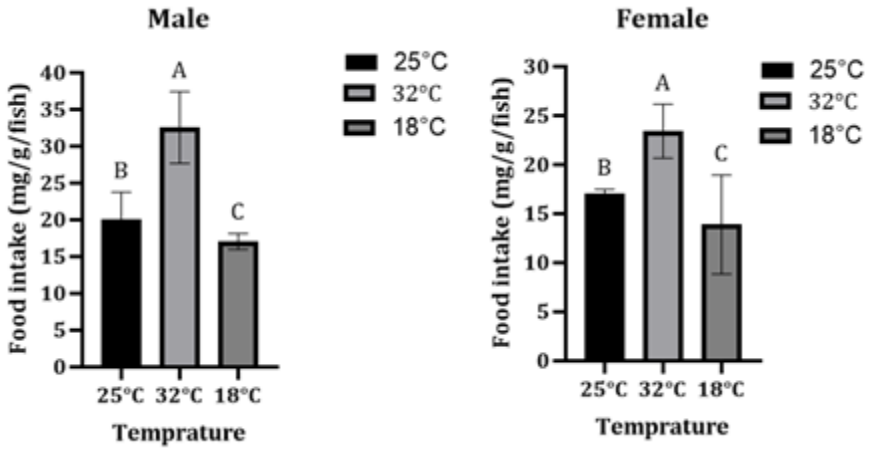

Figure 3-10: Effects of temperature on daily food intake in males and females. Data are represented as mean ± SD. Dissimilar letters indicate a significant difference between the two groups. n-= 6 fish per group.

3.3.2 Expression of Appetite-Related Genes

Changes in the expression of genes involved in appetite are shown in Figure 3-11. In males, OX (Orexin) expression levels were higher 32°C and 18°C than at 25°C. No other significant changes were observed in brain NPY or intestine pyy, CCKa and CCKb in either males or females.

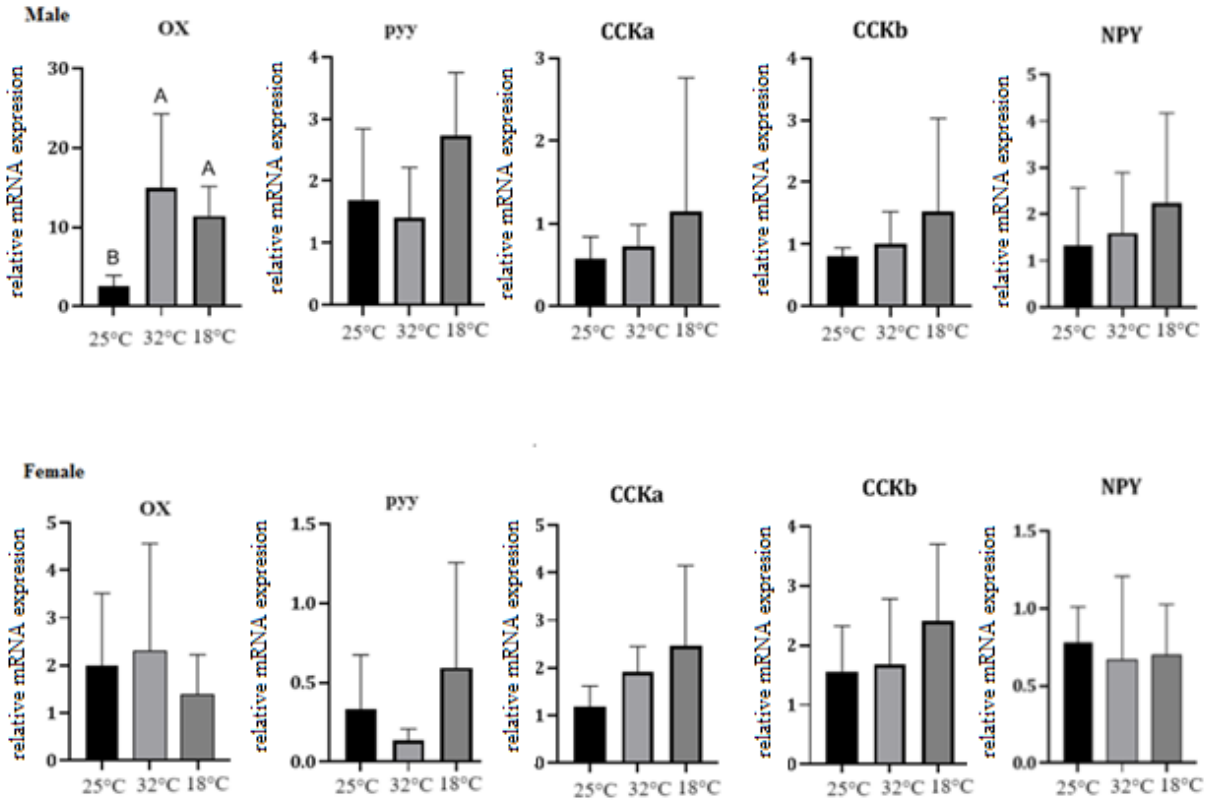

Figure 3-11: Relative changesin brain OX and NPY, and intestine pyy, CCKa and CCKb genes expressions in different groups in males and females are reported as mean ± standard deviation. Dissimilar letters indicate significant differences between groups. n-= 6 per group.

3.3.3 Expression of Reproduction-Related Genes

Changes in the brain expression of genes involved in reproduction are shown in Figure 3-12. In males, npvf levels were higher in fish at 32°C and 18°C compared to fish at 25°C. There were no other significant differences in expression across groups in either males or females.

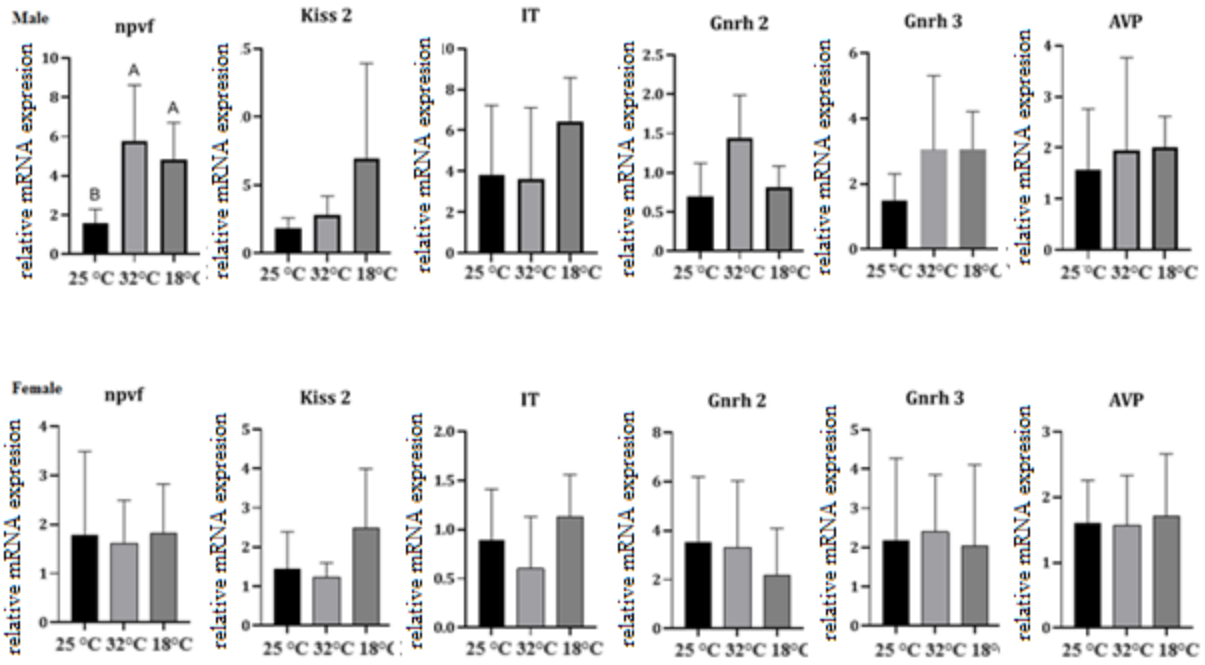

Figure 3-12: Relative changes of npvf, , Kiss2, IT, Gnrh2, Gnrh3 and AVP brain gene expressions in different groups in males and females. Data are reported as mean ± standard deviation. Dissimilar letters indicate significant differences between groups. n-= 6 fish per group.

3.3.4 Expression of Epigenetic-Related Genes

Changes in the hepatic expression of genes involved in epigenetic are shown in Figure 3-13. No significant changes were observed in any genes related to reproduction in either males or females.

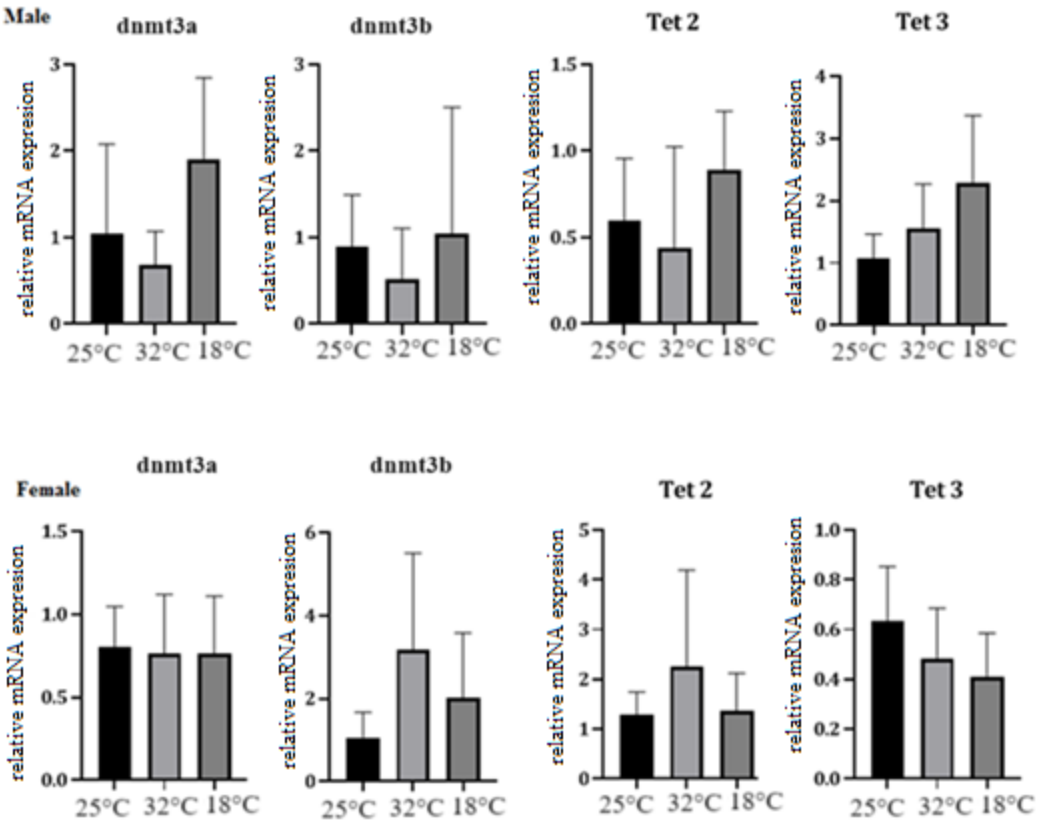

Figure 3-13: Relative changes of dnmt3a, dnmt3b, Tet2 and Tet3 genes hepatic expression in different groups in male and females. data are reported as mean ± standard deviation. n-= 6 fish per group

4 Chapter 4
Discussion and Conclusion

This study aims to elucidate the interactions among developmental stage, feeding, and temperature on gene expression dynamics in guppies (*Poecilia reticulata*), with a focus on genes involved in reproduction, appetite regulation, and epigenetic modifications. These findings provide a better understanding of the manner by which these factors influence gene expression to reveal insights into the molecular mechanisms that underly these physiological and behavioural adaptations in guppies. The differential expression of reproductive and appetite-related genes in response to these factors underscores the adaptive strategies employed by guppies to optimize reproductive success and energy balance in fluctuating environments.

4.1 Reproductive stages

This study sampled a total of 45 guppies, with a higher proportion of females (73.33%) compared to males (26.67%). Females were categorized into three reproductive stages based on the development of their eggs: 1) non-developed immature eggs; 2) early-stage embryos (yolk present but no formed embryo); 3. late-stage embryos (formed embryos with visible eyes and pigmentation). All sampled males had mature testes with sperm and were grouped into one category. This categorization facilitated the comparison of gene expression across different reproductive stages in females and across sexes.

4.1.1 Appetite Regulators

With regards to appetite regulators, no significant differences in brain gene expression of orexin and NPY were found males and females or among females at different developmental stages, to suggest that these genes may not play a significant role in sex-specific or stage-specific brain activity related to reproduction in guppies. In the intestine, no significant differences were observed in the expression of CCKa, CCKb, and pyy across groups. These similarities suggest that these genes, which are involved in appetite regulation and digestion, are not markedly influenced by sex or reproductive stage.

Orexin and NPY are well-known for their roles in appetite regulation across various vertebrate species, including fish. Generally, orexin increases food intake and wakefulness, while NPY is a potent appetite stimulator. For instance, studies (de Pedro et al., 2000; Matsuda et al., 2006) in goldfish (*Carassius auratus*) have shown that NPY and orexin expressions are upregulated by fasting and downregulated by feeding again.

Orexin and NPY have been shown to be related to reproductive processes in fish. In catfish, higher NPY expression is observed during the pre-spawning phase (Sudhakumari et al., 2017), to suggest a role for NPY in regulating reproductive timing. In addition, NPY is predominantly expressed in the brain and testis and silencing NPY leads to a decrease in GnRH and luteinizing hormone, which are crucial for gonadal function, gametogenesis and other reproductive processes. (Sudhakumari et al., 2017). In goldfish, NPY stimulates the release of GtH-II (Peng et al., 1993), to suggest it plays a role in coordinating growth and reproduction, potentially linking energy status with reproductive readiness. In the Siberian sturgeon (Amiya et

al., 2011), NPY-immunoreactive (NPY-ir) fibers are in close contact with GnRH cell bodies, suggesting that NPY might play a role in modulating GnRH activity.

In this study, no significant differences in NPY expression in the brain across different reproductive stages were observed nor across males and females, to suggest that in guppies, NPY's interaction with GnRH might not have the same prominent role in regulating reproductive processes, as seen in other fish. The absence of significant changes in NPY expression suggests that either NPY does not play a similarly critical role in guppies, or that reproductive hormones in the viviparous guppies may be regulated by other neuropeptides or pathways not directly influenced by NPY. Similarly, in platyfish, another viviparous species, there are differences in brain NPY expression between males and females (Pitts and Volkoff, 2017). It is conceivable that in viviparous fish, NPY expression might be less influenced by reproductive stages or other factors, such as environmental conditions or social interactions, could play a more substantial role. However, since we examined the whole brain, there may be differences in expression in specific brain regions such as the hypothalamus. It is possible that had we examined specific brain regions, we would have seen differences.

In goldfish, cGnRH-II administration decreased food intake and hypothalamic orexin mRNA expression and orexin A administration in females reduced spawning behaviour and decreased cGnRH-II expression (Hoskins et al., 2008), indicating a bidirectional interaction where orexin modulates feeding and reproduction through its influence on GnRH and vice versa. In Nile tilapia, orexin-B (but not orexin A) immunoreactive cells are present in the pituitary luteinizing hormone (LH) cells (Suzuki et al., 2009), to indicate that orexin-B may play a role in the regulation of pituitary functions related to reproduction.

In my study, I found no sex- or reproductive stage-related changes in whole brain orexin expression. Similarly, in platyfish, orexin brain expression is similar in males and females (Pitts and Volkoff, 2017). This might suggest that orexin's role in guppies could be less pronounced or different from that observed in other fish, or that the specific experimental conditions (whole brain vs. hypothalamus) or stages studied prevented us to see significant changes. This may reinforce the importance of species-specific studies to understand the mechanisms through which orexins function in different fish species. Guppies, with their different reproductive strategies

and life history traits, might utilize different neuropeptides or pathways to regulate feeding and reproduction compared to goldfish and tilapia.

In goldfish, CCK/gastrin-like immunoreactivity (IR) is present in the goldfish pituitary, and CCK8-s (a sulfated form of CCK) stimulates the release of GtH-II and GH in sexually regressed and recrudescing/sexually re-emerging goldfish (Himick et al., 1993). Notably, sexually regressed goldfish exhibited a greater GtH-II release response compared to sexually recrudescing fish (Himick et al., 1993). These results suggest that CCK may have a direct impact on reproductive processes and that CCK has a modulatory effect on reproductive hormones dependent on the reproductive stage.

In the guppy, no significant differences in the expression of CCKa or CCKb in the intestines at different reproductive stages or among males and females [similar to platyfish (Pitts and Volkoff, 2017)] were observed. The lack of significant differences in CCK expression may suggest that CCK's role in guppies might be less prominent compared to previously examined oviparous fish. This could be due to differences in experimental conditions or physiological variations the two species.

There is little evidence for a role of PYY in reproduction in either mammals or fish. PYY increases LH and FSH release in rats (Fernandez-Fernandez et al., 2005), but does not seem to affect LH, FSH or testosterone in human males (Izzi-Engbeaya et al., 2020). In the lamprey *P. marinus,* intestine PYY expression increases during the pre-spawning stage (Montpetit et al., 2005). Similarly, in common carp, adult fish have higher brain PYY expression compared to juvenile carp, males have higher brain PYY expression than females and highest PYY expression are seen in the spawning phase (Sudhakumari et al., 2024). This suggests that PYY might be involved in the regulation of gonadal cycles and the increase in expression might be associated with cessation of feeding during spawning. I did not find significant differences in PYY expression in the intestine of guppies at different reproductive stages or males and females. Similarly, in adult platyfish, CCK expression is similar between sexs in both brain and intestine. One possibility is that PYY produced by the intestine has only digestive functions and does not affect reproduction and differences might have been observed in brain CCK expression.

4.1.2 Reproductive Genes

There were no significant differences in GnRH2, GnRH3, or npvf expressions between groups, to indicate that they may not be significantly influenced by sex or reproductive stage.

The species-specific variations in the GnRH forms present and patterns in the expression of GnRHs in fish make it difficult to make comparisons. In seabream (*Pagrus major*), GnRH1 and GnRH2 mRNA levels increase during gonadal development and are highest during at spawning with no changes in GnRH3 (Okuzawa et al., 2003), in grass puffer (*Takifugu niphobles*), GnRH1 and GnRH3 mRNA levels increase in the spawning season with no change in GnRH2 (Shahjahan et al., 2010a) whereas in tiger puffer (*Takifugu rubripes*), GnRH1, 2 and 3 mRNAs are higher in mature fish compared to the immature fish (Zahangir et al., 2021). In the damselfish (*Chrysiptera cyanea*), high brain expression levels of GnRH1 and GnRH2, but not GnRH3, are seen during the late vitellogenic stage, suggesting their involvement in the physiological processes of vitellogenesis (Imamura et al., 2020). In Japanese flounder, increases in GnRH3 protein levels are seen in the telencephalon and hypothalamus in the pre-vitellogenic stage (Pham et al., 2006) . In my study, the lack of variations of GnRH might arise from the fact that expression was quantified in whole brain rather than specific brain regions, as different forms of GnRH have been shown to have distinct distributions and patterns in fish (Muñoz-Cueto et al., 2020).

Few studies have examined changes in GnIH with reproductive season. In sea bass, diencephalon GnIH expression is higher in the resting season compared to the reproductive season (Cowan et al., 2017). In grass puffer, GnIH brain and pituitary expression levels are elevated during the spawning and decrease post spawning in both sexes (Shahjahan et al., 2011). Studies (Moussavi et al., 2012, 2013; Wang et al., 2024) have shown that GnIH has season-dependent effects on the secretion of LH and FSH by gonadotropes. This indicates that GnIH can act as a negative regulator of reproduction in different seasons and affect the function of gonadotropes.

In guppy, brain Kiss2 expression was higher in females with late-stage embryos compared to males, females with early-stage embryos, and females with non-developed immature eggs. This indicates that Kiss2 might be involved in the regulation of advanced stages of embryo development in female guppies. Several studies in fish have shown sex and

reproductive stage-specific differences in kisspeptin expression. In chub mackerel, Kiss1 and Kiss2 are expressed in a wide range of tissues, with sexually dimorphic expression seen in adipose tissue (Selvaraj et al., 2010). In some fish species [e.g., chub mackerel (Ohga et al., 2014; Ohga et al., 2018), zebrafish (Kitahashi et al., 2009)], increases in Kiss gene expression are associated with pubertal onset. Kiss has been implicated in seasonal reproductive cycles, with increases in kiss expression during the spawning season [reviewed in (Ohga et al., 2018; Santhakumar, 2023; Sivalingam and Parhar, 2022; Wang et al., 2022a)]. Most studies seem to pertain to oviparous fish, and little is known about viviparous species. In the ovoviviparous male seahorse (*Hippocampus erectus*), brain *kiss2* mRNA increases at the early pubertal stage, and decrease during pregnancy (Zhang et al., 2018b). Consequently, kisspeptin expression may play a key role in regulating sex- and stage-dependent reproductive processes, although further research is needed to understand its mechanisms in viviparous species.

In my study, there were no differences in IT expression across males and females or among females at different stages. A previous study on guppies, shows that IT injections have similar effects on social behaviour in males and females, suggesting that isotocin may have a similar role in fostering social interactions in both sexes (Ataei Mehr et al., 2020). Studies in fish show that IT expression changes with the stages of reproduction, especially in females. For example, in both goldfish (Zhang et al., 2009) and catfish (Banerjee et al., 2018), peaks in brain IT expression are seen during the breeding season.

In my study, brain AVP expression was higher in males compared to females with non-developed immature eggs and females with late-stage embryos. Additionally, it was higher in females with early-stage embryos than in those with non-developed immature eggs. These findings suggest that AVP could be more active in males and early-stage pregnant females, potentially playing a role in the regulation of reproductive and social behaviours. In goby (*Neogobius melanostomus*) (Sokołowska et al., 2015) and catfish *Heteropneustes fossilis* (Singh and Joy, 2008), high brain protein AVT levels are seen in the pre-spawning period in both males and females. In catfish in the prespawing season, higher AVT levels are seen in females compared to males (Chaube et al., 2015). These results suggest that AVP expression in the brain may play an important role in regulating reproductive and social behaviors and is dependent on sex and reproductive stage.

4.1.3 Methylation Genes

No significant differences were found in the expression of dnmt3b, dnmt3a, tet2, and tet3 between groups. These genes are involved in DNA methylation and epigenetic regulation, indicating that such epigenetic mechanisms may not vary significantly with sex or reproductive stage in guppies. Although DNA methylation has been shown to play an important role during fish development [e.g., zebrafish (Jessop et al., 2018), little is known about its role in reproduction in adult individuals.

Male and female zebrafish do not differ in the liver expression of dnmt1 or dnmt3, which is consistent with my findings (Laing et al., 2018a). Dnmt1 is highly expressed in the gonad of the mandarin fish (Siniperca chuatsi), while Dnmt3a is highly expressed in the brain (Zhou et al., 2021). Dnmt3 is primarily expressed in the gonads of zebrafish (Smith et al., 2011). Research has indicated variations in the expression of Dnmts in the testes and ovaries of fish. Dnmt1 and Dnmt3 are both expressed more in the ovary of zebrafish than in the testis (Laing et al., 2018b). Nonetheless, dnmt3 expression is greater in the testes than in the ovaries in bluehead wrasse (*Thalassoma bifasciatum*), which experiences a female-to-male sex transition (Todd et al., 2019). It is feasible that guppy gonads would have displayed variations in dnmt expression.

Similar to dnmts, tet2 and tet3 have been shown to have a crucial role in fish development. For example, tet2−/− and tet3−/− double knockout zebrafish present abnormalities, including the number of promordial germ cells (embryonic precursors of sperm and egg) and usually die during the larval period (Li et al., 2015; Wang et al., 2021). There is however, to our knowledge, no evidence ot the role of tet in adult reproduction. My results seem to indicate that, at least in the liver of guppy, tet does not have a major role in either males or female in regulating adult reproductive processes. Similar to dnmt, tet expresison might be more variable in the gonads of adult fish.

In summary, among all genes studied, only kiss2 and AVP were influenced by sex and reproductive stage. However, this study has shortcomings. For example, only a few stages of female reproductive cycle were observed, and we did not assess changes in male at different reproductive stages. Additionally, the main focus of this study is gene expression analysis and protein levels might have revealed different changes.

4.2 Fasting

4.2.1 Food intake in males and females

In this study, food intake in females was higher compared to that of males. As most females were carrying eggs, the higher food intake in females was likely due to an energy investment in the production and maintenance of eggs. Similarly, female delta smelt *Hypomesus transpacificus* have higher stomach content than males when carrying developing eggs (Hung et al., 2014), with a decrease in feeding just before spawning.

Fish undergo alterations in their eating habits and body reserve dynamics during gonadal maturation and spawning. Captive females' ability to reproduce can be changed by dietary manipulations (Luquet and Watanabe, 1986). Fish physiological processes are governed by intricate regulatory systems that react to both internal and external cues (Douros et al., 2017; Reindl and Sheridan, 2012). Food is one of the most significant external cues that can influence a fish's feeding habits and growth (Conde-Sieira and Soengas, 2017). Food availability and composition both have a significant impact on these processes, mostly through altering the hormones in charge of their endocrine regulation.

4.2.2 Effects of fasting on appetite regulators

There were no significant differences in the expression of NPY in the brain observed among groups, suggesting NPY may not be major regulators of appetite in the context of this study.

Injection of NPY promotes food intake and supports an orexigenic role in a number of species, such as goldfish (Narnaware et al., 2000), grass carp (Zhou et al., 2013), zebrafish (Yokobori et al., 2012), and rainbow trout (Aldegunde and Mancebo, 2006). In the brains of goldfish (Narnaware and Peter, 2001), chinook and coho salmon (Silverstein et al., 1998), zebrafish (Yokobori et al., 2011), and winter skate (MacDonald and Volkoff, 2009b), food deprivation raises the expression of NPY mRNA. However, Atlantic cod's brain expression of NPY was unaffected by a 7-day fast (Kehoe and Volkoff, 2007). After six days of fasting, the brain NPY mRNA expression in Atlantic salmon remained unchanged (Murashita et al., 2009a), but it rose in the first nine hours following feeding (Valen et al., 2011). According to these studies, feeding and fasting in central NPY may have species-specific and time-sensitive effects. The effects of sex on fasting have been the subject of relatively few studies. Male zebrafish

(London and Volkoff, 2019) exhibit higher levels of NPY expression during fasting, whereas females do not. These findings suggest that the role of NPY in feeding regulation may be species, sex, and time-dependent, showing diverse responses to different nutritional conditions.

In the present study, brain orexin expression increased in the fasting group compared to the fed group. This increase was greater in females than in males. In addition, there was a significant difference in orexin expression among the fed female, fed male and fasted female groups compared to the fasted male group. This suggests that fasting has a more pronounced effect on orexin expression in males than females.

An increase in brain orexin expression has been shown in several fish species including pond loach (*Misgurnus anguillicaudatus*) (Kuhn et al., 2024), zebrafish (London and Volkoff, 2019), and cavefish (Wall and Volkoff, 2013). In contrast with my results, in zebrafish (London and Volkoff, 2019), fasting increases orexin expression in females but not in males.

In the intestine, pyya transcript expression was lower in fasted females compared to both fed females and fed males, and lower in fasted males compared to fed males. This indicates a downregulation of this pyya in response to fasting in both sexes, with a more pronounced effect in females. Similar fasting -induced decreases in intestine pyy expression have been shown in other fish including pond loach (Kuhn et al., 2024) and red-bellied piranha, *Pygocentrus nattereri* (Volkoff, 2014). Fasting decreases pyy expression in goldfish brain (Gonzalez and Unniappan, 2010) and Ya fish (*Schizothorax prenanti*) hypothalamus (Lin et al., 2014) However, in yellowtail, fasting increases intestinal pyy mRNA expression (Murashita et al., 2006) and has no effect on Atlantic salmon (*Salmo salar*) (Valen et al., 2011). These findings suggest that pyy's regulating mechanism and response to fasting may be species-specific, impacted by a range of extrinsic and intrinsic factors such as feeding patterns, physiological functions, and features of the digestive system. To my knowledge, there are no studies on sex-specific variations in pyy expression in fish.

There were no significant differences in the expression of CCK in the intestine between groups, suggesting CCK may not be a major regulator of appetite in the context of this study. Fasting-induced decreases in intestine CCK have been shown in several species, including pond loach (Kuhn et al., 2024), grass carp (Feng et al., 2012), winter flounder (MacDonald and Volkoff, 2009b; Volkoff et al., 2009), black tetra *Gymnocorymbus ternetzi* (Butt et al., 2019) and

platyfish (Pitts and Volkoff, 2017). However, fasting does not affect gut CCK expression levels in carnivorous fish such as Atlantic salmon (Murashita et al., 2009b), dourado (Volkoff et al., 2016) and piranha (Volkoff, 2014). Thes data suggests that, as pyy, intestine CCK's response to fasting is species- and time-specific.

4.2.3 Effects of fasting on reproductive genes

Fasting increased brain Gnrh3 expression levels in males but not in females, and no significant differences in Gnrh2 expression were found across groups, suggesting that in guppy, Gnrh3 but not Gnrh3 might be involved in appetite regulation, and that males might be more responsive than females.

These results suggest that the GnRH3 gene is more susceptible to metabolic changes than the GnRH2 gene in males under food restriction. Interestingly, in zebrafish, both GnRH2 and GnRH3 expression increases with fasting in females, but not in males (London and Volkoff, 2019). In mouthbrooding Nile tilapia, GnRH3 levels are lower in females brooding eggs (which do not eat) than in non-brooding feeding females, with no differences in GnRH2 across groups (Das et al., 2019). In winter flounder (*Pseudopleuronectes americanus*), fasting decreases GnRH2 expression in the hypothalamus and GnRH3 expression in the telencephalon (Tuziak and Volkoff, 2013a), but the study used a mix of males and females. In Atlantic cod (mixed sex juveniles), fasting does not affect either Gnrh2 or Gnrh3 hypothalamic expressions (Tuziak and Volkoff, 2013b). The discrepancies in the results are likely due to differences in protocols used (different sexes, reproductive stages, whole brain vs. different brain regions, different fasting times) as well as species-specific modes of reproduction (oviparity, viviparity, mouth brooding...).

No significant differences in the expression of Kiss2 were found between groups, suggesting it may not be significantly influenced by feeding status in guppy. Kisspeptin has been proposed as a molecular mediator that communicates metabolic status and influences reproduction, in addition to GnRH in mammals (Harter et al., 2018) and fish (Somoza et al., 2020; Wang et al., 2022b). Fasting increases the brain expressions of Kiss1 in females and Kiss2 in males in zebrafish (London and Volkoff, 2019), and hypothalamic Kiss1 and Kiss2 in both males and females in sea bass (Escobar et al., 2016) and Senegalese sole (Solea senegalensis) (Mechaly et al., 2011). However, in male tilapia fasting decreases Kiss2 brain expression (Park

et al., 2016) and no differences in kiss2 expression (Das et al., 2019) are observed when comparing non-feeding brooding females to non-brooding females.

In my study, fasting increased npvf expression in males but not in females. In contrast to my results, in zebrafish, fasting increases brain GnIH expression in females but not in males (London and Volkoff, 2019) and in female smooth tongue sole (*Cynoglossus semilaevis*) (LPXRFa) (Wang et al., 2023). Regulatory responses of the npvf or GnIH gene to starvation may be sex- and species-dependent and play different roles in physiological processes.

No significant differences in the expression of AVP were found across groups, suggesting AVP may not be significantly influenced by feeding status in the context of reproductive regulation. Little is known about the role of AVP in feeding in fish. In immature gilthead sea bream (*Sparus aurata*), a 14-day fasting decreases AVP hypothalamic expression (Skrzynska et al., 2017), suggesting AVP might act as an anorexigenic factor in this species. Injection of AVP induce decrease food intake in trout (icv), which is in part mediated by a stimulation of the hypothalamic-pituitary-internal axis and increases in cortisol levels (Gesto et al., 2014), in goldfish (icv) (Araishi et al., 2019) and in tiger pufferfish (ip) (Nagamine et al., 2024). Consequently, the role of AVP in regulating feeding and reproductive processes in fish may be complex and species-dependent, and still requires further investigation.

In guppies, the expression of IT was decreased by fasting in females but not males, suggesting a specific role for IT in fed female guppies, possibly related to reproductive processes. Similar to my results, in immature gilthead sea bream, fasting decreases brain IT expression (Skrzynska et al., 2017), although it does not affect IT plasma concentration (Mancera et al., 2008). These results suggest that IT expression may be more influenced by nutritional status in females and play a specific role in reproductive processes.

4.2.4 Effects of fasting on methylation genes

Liver expression levels of dnmt3b were not affected by fasting in either males or female guppy, although expression levels of dnmt3b were higher in fasted males compared to both fed and fasted females, possibly suggesting that male dnmt3b might be more sensitive to food restriction. There were no significant differences in the expression of dnmt3a, tet2, or tet3 across groups, indicating these genes may not be significantly influenced by feeding status in the context of epigenetic regulation. In mammals, caloric restriction has been shown to regulates (in

general inhibit) the expression of DNA methylation modulators. In female mice, 40% caloric restriction induces increased Tet3 and Dnmt3a and decreased Tet2, Dnmt1 and Dnmt3b expression in the liver (Hahn et al., 2017), suggesting that nutritional status can may modulate metabolic hepatic pathways via modulation of DNA methylation (Asif et al., 2020). To our knowledge, there are no published studies on the effects of fasting on methylation in adult fish.

In summary, the results provide insights into how gene expression in guppies is influenced by sex, developmental stage, and feeding status. Key findings include:

1. Females have significantly higher food intake compared to males, likely due to higher energy demands related to reproduction.

2. Brain orexin and intestine pyya show significant expression changes in response to fasting, with distinct patterns observed between sexes.

3. Reproduction-related genes such as Gnrh3, IT, and npvf are differentially expressed in response to feeding status, highlighting the complex interplay nutrition and reproductive physiology.

These findings contribute to the understanding of the molecular mechanisms underlying appetite regulation, reproduction, and epigenetic modifications in guppies, and underscore the importance of considering sex and developmental stage in such studies. Further research could expand on these results by exploring additional genes and environmental conditions to provide a more comprehensive understanding of gene expression dynamics in guppies.

4.3 Effects of temperature

4.3.1 Food intake

The study looked at how temperature affected the amount of food that both male and female guppies consumed. When food intake was compared 25°C and 32°C, it was higher in both sexes at 32°C and lower in both sexes at 18°C. This suggests that guppies eat less at lower temperatures—possibly because of decreased metabolic demands—and more at higher temperatures, perhaps because of increased metabolic rates.

Similar temperature-induced changes have been reported for other tropical fish including neon tetra (*Paracheirodon innesi*) (Kuhn et al., 2023), red-spotted grouper (*Epinephelus akaara*)

(Jeon et al., 2020) and anemone fish (*Amphiprion ocellaris*), as well as temperate fish [tench (*Tinca tinca*) (Guijarro et al., 1999); goldfish (Nadermann et al., 2019); pond loach (*Misgurnus anguillicaudatus*); catfish (*Ictalurus punctatus*) (Buentello et al., 2000)]. Likewise, the cold-water Atlantic cod *(Gadus morhua)* consumes less food at 2°C than it does at 11°C and 15°C (Kehoe and Volkoff, 2008). Fish models do, however, show species-specific differences in feeding responses. High temperatures, for instance, cause Atlantic salmon *(Salmo salar)* to voluntarily become anorexic (Hevrøy et al., 2012), indicating that the "temperature effect" on feeding can vary significantly across species found in warm and cold water.

4.3.2 Appetite regulators

In males, brain orexin expression levels were higher at both 32°C and 18°C compared to 25°C. This suggests that orexin may play a role in regulating appetite or energy balance in response to temperature changes, with heightened expression at extreme temperatures. In goldfish, higher temperatures (35 compared to 15°C) increase orexin mRNA levels (Nadermann et al., 2019). These results suggest that in these species, orexin might mediate the increase in feeding and locomotion at higher temperatures. In contrast, in black tetra and neon tetra brains, orexin mRNA levels are higher in fish at 20°C than in fish at 32°C (Kuhn et al., 2023) and in pond loach, brain orexin levels are higher at 20 than 30°C (Kuhn et al., 2024). These results indicate that orexin expression in the fish brain varies across species and may act as a regulatory mechanism for energy balance and feeding behavior in response to temperature changes.

No significant changes were observed in the expression of brain NPY. In grouper, brain NPY mRNA is higher at 25 °C compared to 15 and 20 °C (Jeon et al., 2020). However, consistent with my results, in many fish temperature seems not to affect NPY expression: NPY expression levels are similar in brains of Atlantic cod kept at 2, 4 or 11°C (Kehoe and Volkoff, 2008), in tambaqui mesencephalon/diencephalon at 25.6°C and 29.6°C (Lustosa do Carmo et al., 2023), in cobia brains at 30 and 34°C (Nguyen et al., 2023a; Nguyen et al., 2023b) and in goldfish brain, in the winter (15°C) and in the summer (28°C) (Chen et al., 2019). These results suggest that NPY gene expression in fish brains may be more dependent on other physiological or environmental factors and less affected by temperature changes.

No significant changes were observed in the expression of intestinal pyy, CCKa, CCKb in either males or females across different temperatures. This indicates that these genes may not

be significantly influenced by temperature changes within the studied range, or their regulation may not be temperature-sensitive in the context of appetite.

In contrast to my results, in lined seahorse, both CCKa and CCKb mRNA brain expression levels are higher at 30 °C compared to 22 and 26 °C (Zhang et al., 2018a), in both black and neon tetra (Kuhn et al., 2023), and pond loach (Kuhn et al., 2024), intestine CCKa expression increases at higher temperatures (32°C compared to 24°C in tetras, 30 °C compared to 20 °C in loaches). In goldfish, fish at 15°C have higher intestine CCKa levels than fish at 25 and 35°C (Nadermann et al., 2019) and fish in the winter (15°C) have higher brain CCKa expression than fish in the summer (28°C) (Chen et al., 2019). The results show that the expression of CCKa and CCKb genes changes differently at different temperatures in different fish species. Contrary to the results of this study, other species such as lined seahorse, black and neon tetra, and pond loach show increased CCKa and CCKb expression at higher temperatures, while in some species such as goldfish, lower temperatures lead to increased CCKa expression. These results indicate species differences in response to temperature.

Intestine PYY was not affected by temperature. Similarly, in black and neon tetra, there are no differences in intestine PYY mRNA expression at 20, 24°C, 28°C and 32°C (Kuhn et al., 2023). In contrast, in goldfish, fish at 15°C have higher intestine PYY levels than fish at 25 and 35°C (Nadermann et al., 2019). These differences indicate that the response to temperature in PYY expression depends on species differences.

4.3.3 Reproductive Genes

Brain npvf: In males, npvf levels were higher at both 32°C and 18°C compared to 25°C. This suggests that npvf may be involved in reproductive regulation in response to temperature changes, potentially influencing reproductive behaviour or physiology at extreme temperatures.

There are few published studies on the effects of temperature on NPVF expression in fish. However, for example, according to existing studies, NPVF plays a role in regulating behaviours such as feeding and sleeping, especially in different environmental conditions. For example, overexpression of NPVF leads to significant changes in activity and sleep patterns in zebrafish and mice, suggesting that NPVF may be critical in modulating physiological responses to environmental stimuli (Jaroslawska et al., 2015; Lee et al., 2017). While these studies focused

on zebrafish and mice, they provide insights into how temperature or environmental changes may indirectly affect NPVF-related pathways and potentially link npvf to temperature-regulated physiological processes such as feeding and reproduction. In mammals, temperature fluctuations modulate hypothalamic NPVF expression, affecting energy expenditure and behaviour, suggesting that similar temperature-dependent regulatory mechanisms may exist in fish and potentially influence reproductive or feeding behaviours under different thermal conditions (Boulant, 1981; Landsberg, 2012; Pankhurst, 1997a). More research is needed to explore these mechanisms in fish.

Other Reproduction-Related Genes: No other significant differences in gene expression were observed among groups in either males or females. This suggests that, apart from npvf, the studied reproduction-related genes may not be strongly influenced by temperature variations within the context of this study.

In gourami, variations in the ideal temperature during non-reproductive periods were linked to a reduction in the number of eggs in the advanced vitellogenesis stage as well as a drop in the levels of GnRH3, GH, and βLH mRNA. These findings suggest that small variations in the surrounding temperature (±4°C) could have an impact on the promotion of vitellogenesis. βLH and GH mRNA levels are upregulated in blue gourami pituitary cells derived from high vitellogenin females, according to GnRH3. There were high levels of βLH mRNA in these females. This implies that the brain-pituitary axis, which controls oogenesis, is directly impacted by temperature (Levy et al., 2011). Consistent with the findings (Soria et al., 2008), silverside *(Odontesthes. bonariensis)* to higher temperatures has the potential to impact cytochrome P450 regulation, reduce pituitary hormone mRNA levels, and prevent the synthesis of E2 and vitellogenin and subsequent egg development.

The rate of development in *Mugil cephalus* is inversely proportional to temperature once the second stage of oocyte development has begun (Lee and Menu, 1981; Ojanguren et al., 1999). It is generally hypothesized that temperature variations in fish can suppress the GnRH3/GH LH axis at the level of gene transcription and play a role in controlling physiological processes during the last phases of vitellogenesis, which culminate in the oocyte's ultimate maturation. These may include increased LH responsiveness and stimulation of aromatase activity, E2 production, induction to promote oocyte maturation, and pituitary cell synthesis and

secretion of LH and GH, which stimulate IGF-1 synthesis in the ovary. Unlike females, non-reproductive males experience a decrease in brain and pituitary hormones linked to osmotic regulation (PRL), growth (PACAP, PRP, GH, and IGF-1), and reproduction (GnRH3, βLH, βFSH) when temperatures fluctuate. Impacts and demonstrates how these factors play a multifaceted role in both growth and reproduction (Pankhurst, 1997a; Soria et al., 2008; von Schalburg et al., 2005). Therefore, temperature, as a key factor, not only affects the growth and reproductive processes in fish, but also plays an important role in their hormonal regulation and physiological adaptation.

Research shows that high temperatures disrupt the hypothalamic-pituitary-gonadal axis, which leads to changes in hormone secretion, including IT (der Kraak, 1996). High temperature has been shown to decrease the expression of gonadotropin subunits, which are important for the regulation of reproductive hormones, including IT (Soria et al., 2008). Isotocin is produced in neurosecretory fibers in the pituitary gland and changes in temperature can affect the dynamics of secretion of these hormones and potentially alter reproductive behaviours (Batten, 1986; Urano et al., 1994). These findings indicate the direct and indirect effects of temperature changes on the expression of reproductive hormones and especially isotocin, which generally changes reproductive behaviours.

4.3.4 Methylation genes

Hepatic Epigenetic Genes: No significant changes were observed in the expression of genes involved in epigenetic regulation (dnmt3b, dnmt3a, tet2, and tet3) in either males or females across different temperatures. This suggests that these epigenetic regulators are not significantly affected by temperature changes, or that their expression is stable across the temperature range studied.

Rising temperatures have the potential to alter significant phenotypic characteristics in natural fish populations. One epigenetic mechanism that mediates phenotypic changes is DNA methylation. Research has demonstrated that European sea bass (Anastasiadi et al., 2017) exposed to elevated average temperatures at various stages of their larval development exhibit significant alterations in global DNA methylation and the expression of environmental genes linked to DNA methylation, stress response, and the formation of muscles and organs.

The temporal dynamics of dnmt3 gene expression patterns are observed in rainbow trout. Overall, temperature only moderately affected the expression of dnmt3 genes (Lallias et al., 2021). Campos and associates (Campos et al., 2013) demonstrated that the rearing temperature had an impact on the expression of the dnmt gene in metamorphosing Senegalese lone larvae. The expression of the dnmt1 and dnm3b genes was downregulated at 21°C as opposed to 15°C, while the expression of the dnmt3a gene was unaffected. Two additional zebrafish studies have demonstrated that exposure to heat stress changes the expression of the dnmt3 gene (Campos et al., 2012; Dorts et al., 2016b). Additionally, a study on zebrafish embryos revealed that the expression of dnmt3 was highly dynamic in the early stages of development. In particular, the two dnmt3a genes' mRNA levels significantly increased during development, and at the same developmental point, multiple significant differences in the levels of dnmt3a and dnmt3b transcripts were observed across temperature (Campos et al., 2012). Furthermore, Fellous's research (Fellous et al., 2022) on marine stickleback demonstrated that variations in temperature have an impact on TET3 expression. In order to identify potential "windows of opportunity" for adaptive epigenetic responses under future climate change, their study identified critical stages of gamete and embryo development with temperature-sensitive reprogramming and epigenetic gene regulation.

Collectively, these studies suggest that temperature changes can affect DNA methylation processes in fish, although the magnitude and direction of this modulation seem to vary depending on the species and developmental stage considered. While some species such as European sea bass and Senegalese sole show significant changes in DNA methylation or dnmt gene expression in response to temperature, others such as rainbow trout show only modest adjustments. This suggests that temperature may be a key environmental factor driving epigenetic regulation, but its effects can be species- and context-dependent, suggesting the need for more comprehensive studies to fully understand this phenomenon.

The results highlight the influence of temperature on food intake and specific gene expressions related to appetite and reproduction in guppies:

Food Intake: Both male and female guppies increase their food intake at higher temperatures (32°C) and decrease it at lower temperatures (18°C), indicating a temperature-dependent regulation of feeding behaviour.

Appetite-Related Genes: Brain orexin expression in males is higher at extreme temperatures (32°C and 18°C), suggesting a role in temperature-responsive appetite regulation. Other appetite-related genes (NPY, pyy, CCKa, CCKb) did not show significant temperature-dependent expression changes.

Reproduction-Related Genes: npvf expression in males is higher at both 32°C and 18°C, indicating a possible role in temperature-related reproductive adjustments. Other reproductive genes studied did not exhibit significant changes.

Epigenetic-Related Genes: The studied epigenetic genes (dnmt3b, dnmt3a, tet2, tet3) showed no significant temperature-dependent changes, suggesting stable expression across the temperatures studied.

These findings provide insights into how temperature influences physiological processes such as appetite and reproduction at the molecular level in guppies. Future research could explore additional genes and environmental factors to further elucidate the mechanisms underlying these temperature responses.

4.4 Limitations of Research

The present study, despite providing valuable data on the dynamics of gene expression in guppy fish and investigating its relationship with nutrition, reproduction, and epigenetic regulation, also had some limitations. All experiments were performed over a specific time period and under controlled laboratory conditions. Although this increases the reproducibility of the study, caution is required when generalizing the results to wild populations in natural habitats. The present study focused on reproductive stages, and other life stages such as infancy, early development, and full maturity were outside the scope of the study. As a result, gene expression dynamics throughout the entire life cycle were not fully reflected. Given the nature of the study and the available facilities, only a select set of genes related to nutrition, reproduction, and methylation were examined. It is possible that other key genes play a role in these processes

that were not evaluated in this study. Some environmental factors such as social interactions between fish, stress caused by maintenance in vitro, and intraspecific genetic differences can simultaneously affect gene expression. It is difficult to fully control these factors in practical conditions. In the present study, the focus of epigenetics was solely on methylation patterns and other epigenetic mechanisms such as histone modifications and non-coding RNA regulation were not evaluated. Although sexual differentiation was included in the analyses, individual variability and its interaction with sex and reproductive stage could affect the results, the full analysis of which requires larger sample sizes and longitudinal studies. Although guppy was chosen as a model species, the results of this study cannot necessarily be generalized to all fish due to the specific biological characteristics of this species (viviparity, high adaptability to environmental conditions, etc.).

4.5 Conclusion

The findings from my study underscore the critical roles of AVP and Kiss2 genes in the embryonic development of guppy, highlighting their significance in shaping early developmental processes. However, to fully elucidate the regulatory network governing embryonic development, further investigations involving additional genes are warranted. This study lays the groundwork for future research aimed at unraveling the intricate molecular mechanisms underlying embryogenesis in guppies and other related species.

My investigation reveals a nuanced relationship linking hunger, epigenetics, reproduction, and nutritional status in guppy, with notable differences in their responses compared to mammals. Fasting induces significant changes in gene expression related to hunger, epigenetic regulation, and reproduction, particularly in females, emphasizing the sex-specific nature of these responses. While my findings provide valuable insights, it is essential to acknowledge the study's limitations, including its reliance on singular cohorts and the use of shared gene expression data for comparisons between fed and fasting stages, as well as between male and female fish. Future studies should replicate these findings using diverse cohorts to validate my observations.

Despite elucidating the endocrine mechanisms governing feeding and reproduction in guppies, the broader understanding of these processes remains limited, especially in comparison to birds and mammals. This knowledge gap underscores the need for further research, particularly in non-mammalian vertebrates like fish. While my study contributes to clarifying the roles of hormones in hunger and reproduction, the diverse nature of fish species presents challenges in generalizing physiological concepts across taxa. Future investigations should continue to explore the intricacies of hormonal regulation in diverse vertebrate species to advance our understanding of evolutionary adaptations and ecological dynamics in response to changing environmental conditions.

The findings of this study, in addition to their scientific value in the field of molecular biology and evolutionary ecology, can also have practical applications. A more precise understanding of the molecular mechanisms of appetite and reproduction regulation in viviparous fish can help improve feeding programs, manage rearing conditions, and adjust the timing of feeding and reproduction in ornamental and commercial fish farming.

Also, this information can be used in conservation planning for species sensitive to climate and environmental changes. Understanding the relationship between nutrition, reproduction, and environmental changes, especially in ecologically important species such as guppy, can help develop knowledge-based conservation strategies and provide a scientific basis for assessing the vulnerability of natural populations to climate change.

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6 Appendix

Table 6-1 : The number of guppies in each tank, sex, pH and temperature, and feeding rate

Date	Tank	Number of Fish	Food intake (g)	Temperature (centigrade)	pH	Notes
June 21,2023	A	4 Females (Fed)		24.7	7.85	Experiment started
	B	5 Males (Fed)		26.2	7.71	
	C	5 Females (Fed)		25.4	7.73	
	D	4 Males (Fed)		25	7.8	
	E	4 Females (Fast)		25.3	7.8	
	F	5 Males (Fast)		25.3	7.68	
	G	5 Females (Fast)		25.2	7.79	
	H	4 Males (Fast)		25	7.78	
June 22,2023	A	3 Females (Fed)	0.045	24.8	7.87	1 Fish dead
	B	5 Males (Fed)	0.04	26.2	7.72	
	C	5 Females (Fed)	0.079	25.7	7.74	
	D	4 Males (Fed)	0.065	25.6	7.8	
	E	3 Females (Fast)		26.1	7.79	1 Fish dead
	F	5 Males (Fast)		26.2	7.7	
	G	5 Female (Fast)		25.7	7.75	
	H	4 Males (Fast)		25.3	7.8	
June 23,2023	A	3 Females (Fed)	0.089	24.1	7.81	
	B	5 Males (Fed)	0.082	25.7	7.67	
	C	5 Females (Fed)	0.15	25.6	7.66	
	D	4 Males (Fed)	0.091	25	7.78	
	E	3 Females (Fast)		24.61	7.92	
	F	5 Males (Fast)		25.9	7.81	
	G	5 Females (Fast)		25.4	7.85	
	H	3 Males (Fast)		24.9	7.86	1 Fish dead
June 24,2023	A	3 Females (Fed)	0.173	24.8	7.85	
	B	5 Males (Fed)	0.186	26.2	7.71	

	C	5 Females (Fed)	0.19	25.7	7.73	
	D	4 Males (Fed)	0.157	25.6	7.8	
	E	3 Females (Fast)		26.1	7.8	
	F	5 males (Fast)		26.2	7.68	
	G	5 Females (Fast)		25.7	7.79	
	H	3 Males (Fast)		25.3	7.78	
June 25,2023	A	3 Females (Fed)	0.067			
	B	5 Males (Fed)	0.069			
	C	5 Females (Fed)	0.051			
	D	4 Males (Fed)	0.041			
	E	3 Females (Fast)				
	F	5 Males (Fast)				
	G	5 Females (Fast)				
	H	3 Males (Fast)				
June 26,2023	A	2 Females (Fed)	0.074	24.8	7.84	1 Fish dead
	B	5 Males (Fed)	0.056	25	7.71	
	C	5 Females (Fed)	0.142	25.1	7.83	
	D	4 Males (Fed)	0.068	24.8	7.88	
	E	3 Females (Fast)		24.2	7.85	
	F	5 Males (Fast)		25.4	7.77	
	G	5 Females (Fast)		25.2	7.83	
	H	3 Males (Fast)		24.9	7.85	
June 27,2023	A	2 Females (Fed)	0.089	24.8	7.85	
	B	5 Males (Fed)	0.144	25.6	7.75	
	C	5 Females (Fed)	0.159	25	7.85	
	D	4 Males (Fed)	0.071	25.4	7.89	
	E	3 Females (Fast)		24.6	7.87	
	F	5 Males (Fast)		25.2	7.78	
	G	5 Females (Fast)		25.1	7.81	
	H	3 Males (Fast)		24.9	7.87	
June 28,2023	A	2 Females (Fed)	0.071	24.8	7.84	

	B	5 Males (Fed)	0.05	25.9	7.62	
	C	5 Females (Fed)	0.037	25.5	7.77	
	D	4 Males (Fed)	0.44	25.2	7.87	
	E	3 Females (Fast)		24.7	7.84	
	F	5 Males (Fast)		25.5	7.74	
	G	5 Females (Fast)		25.3	7.84	
	H	3 Males (Fast)		25.1	7.84	
June 29,2023	A	2 Females (Fed)	0.048	25.2	7.88	
	B	5 Males (Fed)	0.131	26.5	7.57	
	C	5 Females (Fed)	0.112	25	7.91	
	D	4 Males (Fed)	0.073	25.4	7.94	
	E	3 Females (Fast)		24.4	7.88	
	F	5 Males (Fast)		25.4	7.78	
	G	5 Females (Fast)		25.1	7.82	
	H	3 Males (Fast)		24.9	7.86	
June 30.2023	A	2 Females (Fed)	0.067	25	7.86	
	B	5 Males (Fed)	0.093	25.7	7.54	
	C	5 Females (Fed)	1.016	25.3	7.92	
	D	4 Males (Fed)	0.072	25.2	7.92	
	E	3 Females (Fast)		24.3	7.85	
	F	5 Males (Fast)		25.6	7.76	
	G	5 Females (Fast)		25.3	7.83	
	H	2 Males (Fast)		25	7.84	1 dead
July 1,2023	A	2 Females (Fed)	0.071	25.3	7.91	
	B	5 Males (Fed)	0.178	25.2	7.94	
	C	5 Females (Fed)	0.162	24.3	7.88	
	D	4 Males (Fed)	0.065	25.6	7.78	
	E	3 Females (Fast)		25	7.82	
	F	5 Males (Fast)		25.4	7.54	
	G	5 Females (Fast)		25.6	7.92	
	H	2 Males (Fast)		25.3	7.92	

July 2,2023	A	1 Females (Fed)	0.04	25.3	7.91	1 dead
	B	5 Males (Fed)	0.061	25.2	7.52	
	C	5 Females (Fed)	0.183	25.2	7.98	
	D	4 Males (Fed)	0.082	24.7	7.95	
	E	3 Females (Fast)		24.2	7.88	
	F	5 Males (Fast)		25.6	7.79	
	G	5 Females (Fast)		25.1	7.88	
	H	2 Males (Fast)		25	7.86	
July 3,2023	A	1 Female (Fed)	0.021	25.1	7.9	
	B	5 Males (Fed)	0.101	26.1	7.33	
	C	5 Females (Fed)	0.158	25.3	7.89	
	D	4 Males (Fed)	0.048	25.1	7.9	
	E	3 Females (Fast)		24.8	7.88	
	F	5 Males (Fast)		25.6	7.78	
	G	5 Females (Fast)		25.4	7.87	
	H	2 Males (Fast)		25.1	7.85	
July 4,2023	A	1 Female (Fed)	0.018	24.8	7.89	
	B	5 Males (Fed)	0.12	26.1	7.11	
	C	5 Females (Fed)	0.153	25.4	7.87	
	D	4 Males (Fed)	0.047	25.8	7.82	
	E	3 Females (Fast)		24.2	7.79	
	F	5 Males (Fast)		25.4	7.71	
	G	5 Females (Fast)		25.2	7.83	
	H	2 Males (Fast)		25	7.79	