The Role of the Hypothalamus-Pituitary-Thyroid Axis in Appetite Regulation of Goldfish (*Carassius auratus*)

by

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Abstract

This thesis aimed to understand the role that the hypothalamus-pituitary-thyroid (HPT) axis plays in appetite regulation of goldfish (*Carassius auratus*). I altered nutritional and thyroid statuses to measure the response of thyroid axis components and appetite-regulating peptides. I predicted that fasting would downregulate the thyroid axis and trigger an orexigenic response, while overfeeding would upregulate the thyroid axis and trigger an anorexigenic response. Additionally, I predicted that hyperthyroid conditions would lead to negative feedback of the thyroid axis and an orexigenic response, whilst opposite under hypothyroid conditions. I uncovered for both experiments that the thyroid axis in goldish is most responsive to overfeeding and hyperthyroidism. Overfeeding led to a time-dependent increase in central thyroid transcripts while fasting decreased thyroid hormone degradation peripherally with no central response, no treatment altered levels of thyroid hormone in circulation. Hyperthyroidism resulted in negative feedback to the pituitary, but not hypothalamus, and did not lead to an increase in food intake despite an increase in the levels of thyroxine. The thyroid inhibitor, propylthiouracil, did not induce hypothyroidism or alter the expression of any thyroid axis transcript. Appetite-regulating peptides correlated weakly to changes in the thyroid, suggesting an overall poor association in goldfish between appetite regulation and thyroid status.

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List of Abbreviations

11-KT	11-Ketotestosterone
3β -HSD	3β-hydroxysteroid dehydrogenase
5'-MDA	5'-monodeiodination
ACTH	Adrenocorticotropic hormone
AgRP	Agouti-related peptide
AMPK	Adenosine-monophosphate activated protein kinase
ANOVA	Analysis of variance
BW	Body weight
Ca^{2+}-ATPase	Calcium ATPase
CART	Cocaine- and amphetamine-regulated transcript
CBP	CREB-binding protein
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CYP19	Aromatase
DA	Dopamine
DHP	17α-hydroxy-20β-dihydroprogesterone
DIO	Deiodinase
E_2	Estradiol
EF1α	Elongation factor 1α
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FPC	Fish protein concentrate
FSH	Follicle-stimulating hormone
FW	Freshwater
G6Pase	Glucose-6-phosphatase
GH	Growth hormone
GHRH	Growth-hormone releasing hormone
GIT	Gastrointestinal tract
GK	Glucokinase
GnRH	Gonadotropin-releasing hormone
GP	Glycogen phosphorylase
GS	Glycogen synthase
GTH	Gonadotropin
HPG	Hypothalamus-pituitary-gonad
HPS	Hypothalamus-pituitary-somatotropic
HPT	Hypothalamus-pituitary-thyroid
ICV	Intracerebroventricular
IGF	Insulin-like growth factor
IOP	Iopanoic acid
IP	Intraperitoneal
IRD	Inner ring-deiodination

LAT	Large neutral amino acid transporters
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MC4R	Melanocortin-4 receptor
MCH	Melanin-concentrating hormone
MCT	Monocarboxylate transporter
MT	17α-methyltestosterone
mTOR	Mammalian target of rapamycin
Na^+/K^+-ATPase/NKA	Sodium-potassium ATPase
NCoR	Nuclear-receptor co-repressor
NPY	Neuropeptide Y
OATP	Organic anion transporter polypeptides
OCF	Ovarian cavity fluid
OP	Organophosphorus pesticide
ORD	Outer ring-deiodination
P	Progesterone
PCB	Polychlorinated biphenyl
PI3K	Phosphatidylinositol 3-kinase
POMC	Proopiomelanocortin
PRL	Prolactin
PTU	Propylthiouracil
PYY	Peptide YY
qPCR	Quantitative polymerase chain reaction
RXR	Retinoid X receptor
SCN	Suprachiasmatic nucleus
SLC	Sodium/taurocholate co-transporting polypeptide
SMRT	Silencing-mediator for retinoid/thyroid hormone receptors
SPC	Soy protein concentrate
SRC	Steroid receptor coactivator
SS	Somatostatin
StAR	Steroidogenic acute regulatory protein
SV	Saccus vasculosus
SW	Seawater
T	Testosterone
T_3	Triiodothyronine
T_4	Thyroxine
TBG	Thyroxine-binding globulin
TG	Triglyceride
TH	Thyroid hormone
TMAO	Trimethylamine oxide
TPO	Thyroperoxidase
TR	Thyroid hormone receptor
TRE	Thyroid response element
TRH	Thyrotropin-releasing hormone

TRH-R	Thyrotropin-releasing hormone receptor
TSH	Thyroid-stimulating hormone
TSHR	Thyrotropin receptor
TTR	Transthyretin
UGT	UDP-glucuronosyltransferase
αMSH	α-Melanocyte stimulating hormone

Chapter 1. Introduction and Thesis Overview

1.1. Introduction

1.1.1. The control of appetite

Within vertebrates, a complex physiological system controls the ability/desire to consume food. Regulating appetite occurs by balancing energy – the maintenance of energy intake and expenditure (Hill, Wyatt, & Peters, 2012, 2013). When an organism is deprived of food – or in negative energy balance state – it will attempt to increase its food intake. Conversely, when an organism consumes food – and is in a positive energy state – signals derived from the meal nutrients or from the intestine inform the body that fullness has been reached and meal termination occurs (Druce & Bloom, 2006). This coordination happens via the central nervous system (CNS), which receives afferent signals of endocrine (hormones secreted into the circulatory system) or metabolic [carbohydrates (e.g., glucose) or fats (e.g., lipids)] nature (Chambers, Sandoval, & Seeley, 2013; Dubuc, Phinney, Stern, & Havel, 1998), released from peripheral organs dependent on the energetic state [e.g., glycogen breakdown and glucose release from muscles during fasting (Chandramouli et al., 1997); leptin released from adipose tissue during feeding (Shiraishi, Oomura, Sasaki, & Wayner, 2000)], and responds by producing output signals that dictate behaviours associated with feeding (Blouet & Schwartz, 2010). Central efferent signals are produced by different regions (groups of nuclei) in the brain and can be appetite-stimulating (orexigenic) or appetite-inhibiting (anorexigenic) (Austin & Marks, 2009; Benite-Ribeiro, Putt, & Santos, 2016). For example, proopiomelanocortin (POMC) and cholecystokinin (CCK) are anorexigenic signals that decrease food intake

when energy/food intake is high, while neuropeptide Y (NPY) and agouti-related protein (AgRP) are orexigenic signals that increase food intake when food supply is limited (or during extended periods of fasting) (Ahima & Antwi, 2008; Helfer & Stevenson, 2020; Rønnestad et al., 2017).

These orexigenic and anorexigenic signals of the brain act in a complex interactive manner (Kalra et al., 1999; Ueno & Nakazato, 2016) which is dependent on energetic state, i.e., fasting or feeding, and can regulate each other's expression/activity. For example, NPY neurons are able to inhibit the expression/activity of POMC neurons under fasting conditions (Paeger et al., 2017; Swart, Jahng, Overton, & Houpt, 2002) and AgRP acts as an inverse agonist to α-melanocyte stimulating hormone (α-MSH, the bioactive peptide of cleaved POMC) on melanocortin-4 receptors (MC4R) to induce feeding (Lima, Pedroso, Metzger, Gautron, & Donato, 2019). Appetite-stimulating or -inhibiting pathways have the ability to interact with hypothalamic neurons to induce or suppress the expression of genes involved in the hypothalamus-pituitary (HP) gonad, adrenal and thyroid axes (Martin, Smith, Bloom, & Small, 2006; Roa & Herbison, 2012; Vella et al., 2011; Watts, 2005). Central pathways that are involved in the control of food intake lie in close proximity to hypothalamic regulators of endocrine axis feedback loops, providing control for energy balance dependent on nutritional state.

While many HP axes play a role in energy balance [e.g., the HP-adrenal axis (Nieuwenhuizen & Rutters, 2008)], the hypothalamus-pituitary-thyroid (HPT) axis (hereafter referred to as the thyroid axis) is a major catabolic regulator in the body of vertebrates (Hill, 2012; Mullur, Liu, & Brent, 2014) and has been shown to be regulated

in part by orexigenic and/or anorexigenic pathways to maintain proper performance (Lechan & Fekete, 2006a).

1.1.2. The hypothalamus-pituitary-thyroid axis

Maintenance of the thyroid axis occurs through coordinated release and feedback loops aimed at the hypothalamus and pituitary in order to produce adequate amounts of thyroid hormone (TH) from the thyroid gland/follicles. Thyrotropin-releasing hormone (TRH) from the hypothalamus provides regulatory control over thyroid-stimulating hormone (TSH, also referred to as thyrotropin) synthesis and release from the anterior pituitary. TSH then binds to its receptors on the thyroid to stimulate the production and release of 3,5',3'-triiodothyronine (T_4, thyroxine) and 3,3',5-triiodothyronine (T_3, triiodothyronine) into circulation (Ortiga-Carvalho, Chiamolera, Pazos-Moura, & Wondisford, 2016). To maintain a proper TH set-point, both T_4 and T_3 feedback to the hypothalamus and pituitary in a negative fashion to control the expressions of TRH and TSH, i.e., if THs are in circulation at a concentration above normal levels, they inhibit TRH and TSH production, whilst the opposite occurs if circulating levels of THs are low (Costa-e-Sousa & Hollenberg, 2012). The metabolic action of circulating THs occurs in target cells, where entrance is mediated by TH-specific membrane transporters [e.g., monocarboxylate transporter 8, MCT8 (Visser, Friesema, & Visser, 2011)]. In order to elicit cellular action, conversion of T_4 to T_3 is required. Conversion occurs through iodine metabolism by deiodinase enzymes (DIOs) in various tissues (e.g., kidney, liver, brain) (Bianco & Kim, 2006). Iodine removal from the outer ring of T_4 by DIO type 1 and 2

(DIO1, DIO2) allows production of the bioactive T_3, while inner ring iodine removal of T_4 and T_3 by DIO type 3 (DIO3) and DIO1 results in the formation of inactive TH metabolites (e.g., 3,3',5'-triiodothyronine, reverse T_3; 3,3'-diiodothyronine, T_2) (Bianco & Kim, 2006; Ortiga-Carvalho et al., 2016). Once converted, T_3 binds and activates TH receptors (TR) – which exist as both ligand receptors and nuclear transcription factors (Glass, Holloway, Devary, & Rosenfeld, 1988) – that interact with DNA recognition sequences [i.e., thyroid hormone response elements (TREs)], resulting in *in vivo* transcription (Umesono, Murakami, Thompson, & Evans, 1991).

Within cells, THs elicit metabolic changes related to growth and development, reproduction and nutrient breakdown (e.g., lipid breakdown, glucose formation) (Kim, 2008; Ortiga-Carvalho, Chiamolera, Pazos-Moura, & Wondisford, 2011; Shkil, Siomava, Voronezhskaya, & Diogo, 2019) through stimulation or suppression of genes at the transcriptional level. For example, T_3 stimulates the mRNA expression and production of growth hormone (GH) from cultured rat pituitary cells (Martial, Baxter, Goodman, & Seeburg, 1977) while mice intraperitoneally injected with T_3 show decreased mRNA production of both pituitary TSH subunits (Shupnik, Chin, Habener, & Ridgway, 1985). In mammals, an increase in cardiac output and basal metabolic rate occurs through a T_3-mediated increase in the expression and production of ion channels [e.g., sodium-potassium ATPase (Na^+/K^+-ATPase) and calcium ATPase (Ca^{2+}-ATPase)] in order to increase heart contraction and oxygen consumption (Kahaly & Dillmann, 2005; Watanabe et al., 2003). In addition, THs are required for proper development of the musculoskeletal and central nervous (CNS) systems. For example, if tadpoles (aquatic

amphibious larvae) are thyroidectomized, or are experimentally rendered hypothyroid, they do not metamorphosize into juvenile or adult forms (Allen, 1938; Tata, 1998) unless they are re-exposed to T_4 and T_3 (Gilbert, 1968); within the CNS, T_3 downregulates important neurogenic pathways (Ma et al., 2019) in mouse astrocyte cultures (Morte, Gil-Ibáñez, & Bernal, 2018). Consequently, there can be additive or degressive actions by these hormones, which can be paradoxical with regards to how they act within an organism (i.e., increased TH levels do not always mean stimulation of gene expression).

1.1.2.1 Implication of the thyroid axis in the control of appetite

Periods of contrasting nutritional status can lead to a thyroid axis response in order to conserve or expend energy, i.e., in a positive energy balance (overfeeding) scenario, there is an increase in energy expenditure from increased digestion and processing of macro/micronutrients, episodic locomotor activity and resting energy expenditure (Hall et al., 2012). The energy expenditure increase associated with food consumption is in part due to anorexigenic peptides signalling to TRH neurons, increasing its expression and downstream TH production, whilst the opposite occurs during periods of food scarcity (Lechan & Fekete, 2006b). For example, when food is abundant, the anorexigenic α-MSH binds to MC4R on TRH neurons to stimulate TRH production and limit food intake, and conversely, during a fasting state, AgRP acts through the MC4R receptor (opposing α-MSH action) to induce an increase in food intake by repressing TRH production (and energy expenditure) (Fekete & Lechan, 2014; Joseph-Bravo, Gutiérrez-Mariscal, Jaimes-Hoy, & Charli, 2017).

Circulating THs may influence these appetite-regulating pathways in the hypothalamus, but it is unclear whether this action is direct or indirect. In rats, it has been shown that T_3-treated animals have increased mRNA expression of NPY and decreased POMC (Ishii et al., 2003), while fasting induces increases in hypothalamic T_3 levels and inhibition of TRH expression possibly by altering the interaction of orexigenic/anorexigenic neuropeptides on TRH neurons (Coppola et al., 2005). It likely that THs regulate appetite-related circuits by other pathways, such as increasing mitochondrial uncoupling leading to AgRP/NPY excitability (Coppola et al., 2007), or T_3 regulating hypothalamic neural plasticity (Herwig, Ross, Nilaweera, Morgan, & Barrett, 2008). There is growing evidence of T_3 regulating the phosphorylation of adenosine-monophosphate activated protein kinase (AMPK), which has been shown to control the sympathetic output of AgRP and POMC neurons (Claret et al., 2007; Hardie, 2010; López et al., 2010).

Changes within these central pathways, i.e., TRH modulation by either THs or orexigenic/anorexigenic neuropeptides, may be time dependent. For instance, during a period of sudden energy expenditure, e.g., an ingestion of food or physical activity, TRH stimulates the release of TSH, followed by a rise in T_4 levels in circulation until energetic balance is achieved, time at which THs feedback to the hypothalamus/pituitary to reduce the transient synthesis and release of TRH/TSH (Boelen, Wiersinga, & Fliers, 2008; Uribe et al., 2014). Thus, short-term energetic changes may not elicit a response in appetite circuits. Conversely, when fasting occurs for a long time period, the decrease in THs levels can lead to an inability to inhibit hypothalamic TRH, which instead may be

modulated through other signalling pathways (e.g., leptin from white adipose tissue) that signal AgRP/NPY neurons to repress TRH expression (Baver et al., 2014).

These appetite and thyroid interactive pathways are well established in mammalian models (Lenard & Berthoud, 2008), however, non-mammalian models, e.g., fish and amphibians, show limited evidence for the role of the thyroid axis in appetite regulation. Since the structure and function of thyroid axis components and appetite-regulating peptides are relatively well conserved across taxa (Elphick, Mirabeau, & Larhammar, 2018; Sower, Freamat, & Kavanaugh, 2009), insights into the interrelationships of these systems may provide better knowledge of differences in feeding mechanisms/regulation between ectothermic and endothermic organisms.

1.1.2. Historical perspective of thyroid research in fish

To highlight the importance of thyroid endocrine research in fish, I must pay homage to the pioneering work in the 1950s-70s of Drs. Martin Sage, Aubrey Gorbman, William Hoar, Richard Peter, John Geoffrey Eales and many more who contributed to the basic understanding of thyroid function in fishes (Eales, 1961; Gorbman, 1959; Hoar, Keenleyside, & Goodall, 1955; R. E. Peter, 1970; Sage, 1973). Much excitement into this field was due to the fact that although the thyroid system of fish shared similarities with that of endothermic vertebrates, major differences could be seen not only between fish and mammals but within fish, in particular between the most primitive forms (e.g., agnathans) and more "evolved" teleosts (e.g., salmonids), with the potential of providing insight into the evolution of thyroid function (Gorbman, 1969). Based on a review by

Sage (1973), one can understand how well researched this topic was during this time period and the realization of the diverse physiological roles of the thyroid axis:

> "The thyroid has been implicated in almost every aspect of teleost physiology including growth, differentiation, metamorphosis, maturation, reproduction … and several others. The resulting confusion has probably discouraged people from working in this field with the result that there has been very little published since the subject was last reviewed and no recent work has led to any new insight into the role of thyroid hormones in teleost" (p. 899).

This quote gives a breadth of the research done from 1949 up to this date (1973), however, none of the work cited in this review pertained to feeding or appetite – only a role of T_4 on various aspects of metabolism [citing (Higgs & Eales, 1971)]. Prior to this review by Sage (1973), the only known association between thyroid state and feeding in fish had come from a study in green sunfish (*Lepomis cyanellus*) showing a positive correlation between a hyperthyroid state and food consumption (Gross, Fromm, & Roelofs, 1963). Later on, a review by Higgs, Fagerlund, Eales, and McBride (1982) discussed the potential for T_4 and T_3 as growth agents in aquaculture, reviewing studies analyzing the responsiveness of THs to different food ration levels (Eales, Hughes, & Uin, 1981). Likewise, the administration of THs to this point had not been shown to have an effect on food consumption, but instead to aid in internal utilization of nutrients (Higgs, Fagerlund, McBride, & Eales, 1979).

From the 1970s to the 1990s, the majority of thyroid work was conducted on salmonids (Higgs et al., 1982; McBride, Higgs, Fagerlund, & Buckley, 1982), owing to

the development of commercial finfish companies in Canada, Norway, the United States and South America in order to compensate for declining populations of commercially important wild fishes and produce food for local consumption (Beamish, 2017; Flaherty, Reid, Chopin, & Latham, 2019; Hernández-Rodríguez et al., 2001; Liu, Olaf Olaussen, & Skonhoft, 2011; Milewski & Smith, 2019). While salmonid thyroid research was necessary to further our understanding of physiological processes related to animal culture, they represent poor models for other non-salmonid fish due (1) the fourth round (salmonid-specific) whole-genome duplication event 80 million years ago (Danzmann et al., 2008; Lien et al., 2016) creating paralogous genes with possibly multiple specific functions – compared to other fish that only underwent either two- [jawed vertebrates (Holland & Ocampo Daza, 2018)] or three-rounds [teleost specific (Meyer & Van de Peer, 2005)] – and (2) the use of transgenic salmonids in physiological research (Hallerman, McLean, & Fleming, 2007), which provide compounding factors not always comparable to wildtype fish when studying thyroid function [for examples see (Eales et al., 2004; Kang & Devlin, 2003)]. Some of the first non-salmonid models used for thyroid research were channel catfish (*Ictalurus punctatus*) and red drum (*Sciaenops ocellatus*), with several studies by the MacKenzie group showing large fluctuations in circulating THs related to daily cycles and to a minor extent, food rations (Gaylord, MacKenzie, & Gatlin, 2001; Loter, MacKenzie, McLeese, & Eales, 2007; MacKenzie, Moon, Gatlin, & Perez, 1993; MacKenzie, Vanputte, & Leiner, 1998). To date, thyroid research is still advancing in fish, with aquaculture practices established to aid with species conservation, e.g., sturgeon (Acipenseridae *spp*.) (Hoseini, Mirghaed,

Mazandarani, & Zoheiri, 2016; Li, Liu, & Xie, 2012), and the use of fish as models in biomedical research related to the thyroid, e.g., zebrafish (*Danio rerio*) (Jin et al., 2021; Lee, Moon, & Ji, 2021). However, as of today, there is limited information on what role(s) THs or thyroid axis components play in regulating/modulating food consumption (Deal & Volkoff, 2020).

1.2. Thesis overview

The interest of using fish as models to study the interactions between the thyroid axis and appetite lies in the number of specific adaptations related to feeding and metabolism. For example, many genera/species have the ability to withstand extended periods of food shortages [e.g., seasonal fasting in Arctic charr (*Salvelinus alpinus*) (Striberny, Ravuri, Jobling, & Jørgensen, 2015)], while intra-species differences provide means to study divergent metabolic functions – Mexican blind cavefish (*Astyanax mexicanus*) have a lower standard metabolic rate and higher glycogen levels than their eyed-surface counterpart (Volkoff, 2016). Furthermore, fish may aid in the understanding of feeding and body weight regulation in mammalian vertebrates (Volkoff, 2019). In this thesis, I aimed to understand interactions between the thyroid axis and food intake in goldfish (*Carassius auratus*) by altering (1) nutritional status and (2) thyroidal state. This was first done through using food abundance as a proxy for energy intake and manipulating energy levels to see how the thyroid system might be regulated spatially and temporally. Secondly, by disrupting the thyroid system and keeping food abundance

constant, I was able to manipulate energy expenditure and analyze differences in food intake and feeding behaviour.

Goldfish have long been used in endocrinological research to assess feeding, metabolism, reproduction and more, and are useful to study these areas as they can be maintained in high stocking densities, have the ability to be handled/manipulated without causing chronic stress, and they display relatively high degrees of conservation with regards to some hormones and receptors compared to mammals (Blanco, Sundarrajan, Bertucci, & Unniappan, 2018; Popesku et al., 2008; Volkoff, 2019)

This thesis is divided into 5 chapters (including this one), 3 of which are written for, and intended for publication. In Chapter 2, I provide an in-depth review into the role of the thyroid axis in fish. This is the first comprehensive review to cover multiple aspects of this system, as previous reviews are currently outdated and/or singularly focus on growth and reproduction (Blanton & Specker, 2007), stress (M. C. Peter, 2011) and metamorphosis (Campinho, 2019). I argue that there is a major gap of knowledge missing in our understanding of this axis in non-mammalian vertebrates, as research in this field has mainly focused on rodents. I draw contrasts and comparisons between mammals and fish as it pertains to thyroid regulation – synthesis, secretion, transport and action within cells. As well, I delve into the interactions between the thyroid axis, growth and development, reproduction, osmoregulation, feeding and nutrient homeostasis, and the relevance of the thyroid system in aquaculture. In Chapter 3, I build upon past thyroid research in fish by examining the effects of food ration of the thyroid axis but fill a gap by examining its regulation centrally and examine the relationship between

central/peripheral thyroid transcripts and appetite-regulating neuropeptides. In Chapter 4, as little is known on how an altered thyroid status affects feeding and appetite regulation in fish, I attempted to create chronic hyper- and hypothyroid conditions by intraperitoneal implantation of osmotic pumps containing thyroxine (T_4) or propylthiouracil (PTU, a thyroid inhibitor) for 12 days. By examining food intake and feeding behaviour, along with thyroid and appetite regulating transcripts, I provide new insights on the role thyroid hormones play in appetite regulation in goldfish. Chapter 5 provides a concluding prospective on this thesis research and possible future directions it can progress.

While I compiled and formatted this entire thesis, pronouns used in Chapters 2, 3 and 4 may be presented plurally as “we” or “our” to reflect the collaborative nature of the research. Moreover, due to the nature of this thesis written in manuscript format, there may be repetition in information throughout. To the reader, I apologize if this causes any inconvenience.

1.3. Co-authorship statement

While I am the sole author of this thesis, certain chapters are co-authored by my supervisor, Dr. Helene Volkoff, who provided support in experimental design and manuscript revisions.

A version of Chapter 2 has been published in Frontiers in Endocrinology: Experimental Endocrinology:

Deal, C. K., & Volkoff, H. (2020). The role of the thyroid axis in fish. *Frontiers in Endocrinology*, *11*, 861. doi.org/10.3389/fendo.2020.596585

A version of Chapter 3 has been published in Molecular and Cellular Endocrinology:

Deal, C.K., & Volkoff, H. (2021). Response of the thyroid axis and appetite regulating peptides to fasting and overfeeding in goldfish (*Carassisus auratus*). *Molecular and Cellular Endocrinology. 528.* doi.org/10.1016/j.mce.2021.111229

A version of Chapter 4 has been submitted to Peptides:

Deal, C.K., & Volkoff, H. (submitted). Effects of thyroxine and propylthiouracil on feeding behaviour and the expression of genes related to appetite and thyroid function in goldfish (*Carassius auratus*). *Peptides.*

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Chapter 2. The Role of the Thyroid Axis in Fish

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Abstract

In all vertebrates, the thyroid axis is an endocrine feedback system that affects growth, differentiation and reproduction, by sensing and translating central and peripheral signals to maintain homeostasis and a proper thyroidal set-point. Fish, the most diverse group of vertebrates, rely on this system for somatic growth, metamorphosis, reproductive events and the ability to tolerate changing environments. The vast majority of the research on the thyroid axis pertains to mammals, in particular rodents, and although some progress has been made to understand the role of this endocrine axis in non-mammalian vertebrates, including amphibians and teleost fish, major gaps in our knowledge remain regarding other groups, such as elasmobranchs and cyclostomes. In this review, we discuss the roles of the thyroid axis in fish and its contributions to growth and development, metamorphosis, reproduction, osmoregulation, as well as feeding and nutrient metabolism. We also discuss how thyroid hormones have been/can be used in aquaculture, and potential threats to the thyroid system in this regard.

2.1. Introduction

The thyroid gland is a key metabolic regulator in the body of animals. An intact axis between the brain, thyroid and peripheral tissues is essential to modulate energy expenditure and homeostasis (McAninch & Bianco, 2014). An imbalance in energy homeostasis results in the release of brain or peripheral signals, which communicate to the thyroid to increase or decrease energy expenditure, by modulating the release of thyroid hormones (THs). In mammals, there is clear evidence that increased TH production/release induces increases in metabolic rate (Kim, 2008), weight loss (Reinehr, 2010) and cardiac output (Klemperer et al., 1995), while decreased TH production/release leads to opposite effects. In all vertebrates, THs are key hormones that influence a number of physiological processes including growth, development/morphogenesis and metabolism (Rabah, Gowan, Pagnin, Osman, & Richardson, 2019). However, in fish, the role of the thyroid is incompletely understood. Although homology in genetic mechanisms exists between mammals and fish (van de Pol, Flik, & Gorissen, 2017) and THs are generally conserved in structure and function (Zoeller, Tan, & Tyl, 2007), the thyroid system is not always analogous between groups.

Fish [Chondrichthyes (i.e., cartilaginous fish: sharks, skates, rays), Osteichthyes (i.e., bony fish: ray-finned and lobe-finned fish) and Agnatha (i.e., jawless fish: hagfish and lamprey)] (Benton, 2009) make up approximately 48% of all vertebrates (Fricke, Eschmeyer, & van der Laan, 2020), contributing to the 73,327 of total vertebrate species described (IUCN, 2020). This diversity has led to wide variations within ecological niches, physiological mechanisms and local adaptations. In the context of the thyroid,

major differences in terms of morphology, physiology and regulation are seen within and between species.

The thyroid was first described in fish in the 19th century (Simon & Green, 1844). Later studies compared the structure/location of the gland in different fish species [e.g., gill tissue in rainbow trout (*Oncorhynchus mykiss*) (Gudernatsch, 1911)], and uncovered the role of the thyroid as a regulator of metabolic activity (Lynn & Wachowski, 1951), and the role of the pituitary [sailfin molly (*Poecilia latipinna*) (Ball, 1962)] and hypothalamus [African lungfish (*Protopterus annectens*) (Kreider, Winokur, Manaker, Pack, & Fishman, 1988)] in the regulation of thyroid function. Despite over a century of research, our knowledge of the physiology of the fish thyroid is still incomplete, and previously published reviews focus on teleosts and on specific functions of the thyroid [e.g., metamorphosis (Campinho, 2019); reproduction (Blanton & Specker, 2007)].

This review provides a general overview of our current knowledge on the actions of thyroid hormones in fish (not only teleosts but other groups), including those on growth and development, reproduction, osmoregulation and feeding/metabolism, how thyroid function may be affected by intrinsic and extrinsic factors, and how this knowledge could be used by the aquaculture industry.

2.2. Thyroid hormones and the thyroid axis

2.2.1 Regulation of secretion

THs consist of two forms, thyroxine (or tetraiodothyronine, T_4) and the biologically active triiodothyronine (T_3) (Gavrila & Hollenberg, 2019). Although T_4 is

the predominant circulating form, T_3 is more biologically active (Stathatos, 2012). Conversion of T_4 to T_3 occurs in central and peripheral tissues (e.g., brain, gut, liver) by enzymatic removal (5'-monodeiodination, 5'-MDA) of an iodide unit on the outer ring of T_4 (Eales, MacLatchy, & Sweeting, 1993).

In vertebrates, the secretion of THs is regulated by the hypothalamus-pituitary-thyroid (HPT) axis (hereafter referred to as the thyroid axis). The prime stimulatory hormone for the thyroid gland/follicle is thyrotropin (TSH), from thyrotropes of the anterior pituitary. In higher vertebrates, thyrotropin-releasing hormone (TRH) is the main stimulator of TSH release, whereas some neurotransmitters, dopamine (DA) and somatostatin (SS), act as inhibitors (Fliers, Kalsbeek, & Boelen, 2014; Roelfsema, Boelen, Kalsbeek, & Fliers, 2017). Serum TH levels have direct inhibitory effects on the synthesis and release of both hypothalamic TRH and pituitary TSH (Costa-e-Sousa & Hollenberg, 2012). While it is clear in mammals that TRH stimulates release of TSH from the anterior pituitary, the role of TRH in activating the fish thyroid axis is not clear (Blanton & Specker, 2007).

In teleosts, there seems to be species-specific differences in TRH action on thyrotropes. In bighead carp (*Aristichthys nobilis*), TRH treatment of pituitary cells increases TSHβ mRNA expression levels (Chatterjee, Hsieh, & Yu, 2001). However, in common carp (*Cyprinus carpio*) (Geven, Flik, & Klaren, 2009; Kagabu, Mishiba, Okino, & Yanagisawa, 1998) and coho salmon (*Oncorhynchus kisutch*) (Larsen, Swanson, Dickey, Rivier, & Dickhoff, 1998), TRH does not directly affect TSH expression or release from the pituitary. It has been suggested that, in some teleosts, corticotropin-

releasing hormone (CRH) may play a greater role as a TSH stimulator than TRH (De Groef, Van Der Geyten, Darras, & Kühn, 2006; Larsen et al., 1998).

There is evidence that TRH stimulates the secretion of growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH) and melanocyte stimulating hormone alpha (α-MSH) in fish (Galas et al., 2009). TRH evokes release of proopiomelanocortin (POMC)-derived peptides (α-MSH and ACTH) (Tran, Fryer, Bennett, Tonon, & Vaudry, 1989) and GH (Trudeau, Somoza, Nahorniak, & Peter, 1992) from goldfish (*Carassius auratus*) anterior pituitaries, and PRL synthesis and release in common carp (Kagabu et al., 1998). It is possible that TRH-induced increases in T_4 plasma levels, as seen in rainbow trout and Arctic charr (*Salvelinus alpinus*) (Eales & Himick, 1988), might occur through stimulation of TSH release or other pituitary hormones such as GH and PRL.

Similar to mammalian TSH, fish TSH is a glycoprotein that comprises a hormone-specific β subunit (TSHβ) coupled to a glycoprotein α subunit (GSUα) [e.g., teleosts (MacKenzie, Jones, & Miller, 2009), elasmobranchs (Maugars, Dufour, Cohen-Tannoudji, & Quérat, 2014)]. The α subunit is common to TSH and gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] whereas the β subunit confers hormonal specificity (Maugars et al., 2014). TSH mRNA is mainly expressed in teleost pituitary tissue, although ectopic expression occurs, particularly in gonads (MacKenzie et al., 2009).

TSH exerts its actions by binding to TSH receptors (G protein-coupled receptors) on the basal membrane of thyroid follicles (MacKenzie et al., 2009). Two TSH receptor

sequences have been identified in most teleost groups but only one receptor gene has been identified in the coelacanth and elephant shark genomes (Maugars et al., 2014). Evidence suggests that, in fish, TSH has a stimulatory effect on the synthesis/release of THs and iodide uptake. For example, incubating thyroid glands from the sea catfish (*Galeichthys felis*) *in vitro* for 3 days with mammalian TSH increases T_4 release and thyrocyte height (Jackson & Sage, 1973); *in vivo* injections with mammalian TSH increase thyrocyte height and follicle proliferation in coho salmon (Nishioka, Grau, Lai, & Bern, 1987), and circulating T_4 levels in mummichog (*Fundulus heteroclitus*) (Grau & Stetson, 1977) and brook trout (*Salvelinus fontinalis*) (Chan & Eales, 1976).

The release of pituitary TSH is inhibited by DA (Scanlon et al., 1979) and SS (Tanjasiri, Kozbur, & Florsheim, 1976), neuropeptides, and by negative feedback actions by T_4 and T_3. In goldfish, treatment with SS suppresses radioiodide uptake by thyroid follicles but does not lower plasma T_4 in TSH-injected goldfish, supporting the role of SS as a TSH inhibiting factor in this species (Peter & McKeown, 1975). Appetite regulating peptides also affect TSH expression/release at the pituitary, as leptin and β-endorphin stimulate, whereas galanin and neuropeptide Y (NPY) inhibit TSH pituitary mRNA expression in bighead carp (Chowdhury, Chien, Chatterjee, & Yu, 2004).

In mammals, THs exert an inhibitory feedback action on TRH and TSH expression by binding to TRβ located on the TRH promoter in the hypothalamus (Dupré et al., 2004; A. N. Hollenberg et al., 1995), and inhibiting the transcription of both TSHα and TSHβ in the pituitary (Shupnik, Chin, Habener, & Ridgway, 1985). In fish, there is no clear evidence of TH inhibition on TRH. Injections of T_4 in common carp have no

effect on hypothalamic TRH expression, but increase hypothalamic CRH binding protein expression (Geven, Verkaar, Flik, & Klaren, 2006), which might result in CRH inactivation and in the modulation of TSH synthesis in the pituitary, as seen in mammals (Potter et al., 1991).There is however evidence in fish for feedback control of THs at the pituitary level, as THs decrease pituitary TSHβ expression both *in vivo* [e.g., goldfish (Yoshiura, Sohn, Munakata, Kobayashi, & Aida, 1999); turbot (*Scophthalmus maximus*) (Pradet-Balade et al., 1999); European eel (*Anguilla Anguilla*) (Pradet-Balade, Schmitz, Salmon, Dufour, & Quérat, 1997)] and *in vitro* [goldfish (Allan & Habibi, 2012)].

2.2.2. THs synthesis sites and peripheral regulation

Synthesis of THs occur in thyroid follicles – a single layer of epithelial cells (thyrocytes) enclosing a colloid-filled space (Power et al., 2001). In mammals, and most vertebrates, the thyroid gland is an encapsulated gland in the neck region. In fish, the thyroid gland can be either compact/encapsulated [e.g., Chondrichthyes or cartilaginous fish, such as sharks and rays, and Chondrostei, such as sturgeons] or more commonly diffusely arranged in the pharyngeal, heart and kidney regions [e.g., most teleosts with a few exceptions such as Tetraodontiformes and Lophiiformes] (Chanet & Meunier, 2014; Geven et al., 2007; Gorbman, 1969). In larval lampreys, the site of TH synthesis is the subpharyngeal endostyle, a filter-feeding apparatus, which transforms into typical follicular thyroid tissue during metamorphosis (Manzon & Manzon, 2017).

Synthesis of THs requires iodine, that, in most fish, is assimilated by diet or from water via the gills (Eales, 2019), and thyroid uptake of iodine requires TSH binding to

follicles. Evidence on TSH stimulation of iodide uptake in teleost fish is scarce as the spatial distribution of thyroid follicles makes it difficult to measure radioiodide uptake (Chan & Eales, 1976), but it has been shown in elasmobranchs, who have an encapsulated thyroid [e.g., lesser spotted dogfish (*Scyliorhinus canicula*) (Dent & Dodd, 1961)].

Once secreted from follicles, THs require peripheral regulation to exert their effects. Iodothyronine deiodinases are selenoenzymes that regulate TH availability and disposal. Several isoforms of deiodinases (DIOs) with different catalytic properties (type 1, 2 and 3, or DIO1, DIO2, DIO3) and tissue- and developmental stage-specific expressions exist (St Germain, Galton, & Hernandez, 2009). In mammals, DIO2 is part of the activating pathway [or outer ring-deiodination (ORD)] as it converts T_4 to T_3, whereas DIO3 is part of inactivation [inner ring-deiodination (IRD)] as it converts T_4 and T_3 to inactive metabolites [reverse triiodothyronine (rT_3) and 3,3′-diiodothyronine (T_2)] (Luongo, Dentice, & Salvatore, 2019; St Germain et al., 2009). DIO1 is capable of both activation (ORD) and inactivation (IRD), processing T_4 to T_3 and rT_3 to T_2, respectively (Gereben, Anikó, Dentice, Salvatore, & Bianco, 2008; Kelly, 2000). Similar DIOs have been shown in fish (Bianco, Salvatore, Gereben, Berry, & Larsen, 2002; Eales, 2019; Eales & Brown, 1993; García-G, Jeziorski, Valverde-R, & Orozco, 2004; Jarque & Piña, 2014). However, fish DIOs differ in some respects from their mammalian counterparts (Eales et al., 1993). For example, teleostean DIO1 is resistant to propylthiouracil (PTU, inhibitor of thyroperoxidase, TPO – responsible for iodide to iodine oxidation in thyroid

follicles) inhibition, and teleosts have relatively higher levels of hepatic DIO2 activity and expression compared to other vertebrates (Orozco & Valverde-R, 2005).

2.2.3. Regulation by circadian and seasonal rhythms

Several studies have shown circadian and seasonal cycles of THs and thyroid axis components. In mammals, circadian cycles of TRH and TSH are controlled by "pacemakers" within the suprachiasmatic nucleus (SCN) of the hypothalamus. These in turn regulate circulating TH levels (Philippe & Dibner, 2014). The pineal gland – which produces melatonin, and controls sleep patterns in a circadian and seasonal manner – has an inhibitory influence on circulating THs (Vriend, 1984). Studies in hamsters show that melatonin inhibits the release of TSH and increases DIO3 expression during winter months (short photoperiod), and stimulates TSH release in summer (long photoperiods), increases DIO2 expression and decreases DIO3 expression, thus controlling the availability and metabolism of THs (Milesi, Simonneaux, & Klosen, 2017; Sáenz de Miera, Sage-Ciocca, Simonneaux, Pévet, & Monecke, 2018).

Several studies in fish have shown that thyroid axis components respond to environmental cues (Grau, 1988) and undergo circadian and seasonal cycles (Cowan, Azpeleta, & López-Olmeda, 2017). Pituitary transcript expression levels of TSH and DIO exhibit distinct rhythms. In red drum (*Sciaenops ocellatus*), seasonal rhythms of T_4 correlate with pituitary TSH subunits (TSHα, TSHβ) and DIO3 gene expression cycles (Jones, Cohn, Miller, Jaques, & MacKenzie, 2013), and in Arctic charr, hypothalamic DIO2 expression is decreased during late summer (Striberny, Jørgensen, Klopp, &

Magnanou, 2019). In fish, there is evidence that the saccus vasculosus (SV, an organ only observed in fish, situated on the ventral side of the diencephalon, posterior to the pituitary gland) is the seasonal sensor in the brain. The SV expresses TSH and DIO2, suggesting that this organ might play a central role in seasonal changes in THs, albeit probably linked to reproduction (Ikegami & Yoshimura, 2016). In precocious male masu salmon (*Oncorhynchus masou*), the SV responds to changes in light, with salmon kept under long periods of light displaying high TSHβ and DIO2 protein levels, the opposite occurring with exposure to short periods of light (Nakane et al., 2013).

TH circadian cycles have been shown in several fish species [see (Cowan et al., 2017)], including Atlantic salmon (*Salmo salar*) (Ebbesson, Björnsson, Ekström, & Stefansson, 2008), winter flounder (*Pseudopleuronectes americanus*) (Eales & Fletcher, 1982), goldfish (Spieler & Noeske, 1979) and red drum (Leiner, Han, & MacKenzie, 2000), although the time of the peak of TH appears to be species-specific. There also appears to be sex-specific TH rhythms, as in rainbow trout, TH levels increase during the day and decrease at night in males, and increase at night and decrease in the morning in females (Ganzha & Pavlov, 2019). Seasonal variations in THs also exist, often related to migration and reproduction [e.g., channel catfish (*Ictalurus punctatus*) (Loter, MacKenzie, McLeese, & Eales, 2007); Atlantic cod (*Gadus morhua*) (Comeau, Campana, Hanson, & Chouinard, 2000).; rainbow trout (Cyr & Eales, 1988b)].

2.3. Mechanism of action and general actions of THs

The ability of THs to exert their many pleiotropic effects relies on efficient transport, bioactivation, and genomic/nongenomic actions at target tissues.

2.3.1. TH Transport

In higher vertebrates, THs are transported by plasma TH-binding proteins: thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin. The primary plasma TH-binding molecules in fish consist of albumin and prealbumin, the latter now identified as TTR (Power et al., 2000). A TBG-like protein has not yet been identified in fish. In contrast to mammals, fish TTR binds T_3 more avidly than T_4 (Eales, 2019), possibly making albumin the main T_4 binding protein (Power et al., 2000).

Due to the lipophilic nature of THs, it was previously assumed that passive diffusion across lipid bilayers of plasma membranes occurred. It is now believed that THs enter target cells via facilitated transport by several ATP-dependent transporters including the monocarboxylate transporters (MCTs) such as MCT8, organic anion transporter polypeptides (OATPs, predominately present in brain capillaries), large neutral amino acid transporters (LATs) and the sodium/taurocholate co-transporting polypeptide (SLC10A1, known as NTCP) (Bernal, Guadaño-Ferraz, & Morte, 2015; Heijlen, Houbrechts, & Darras, 2013).

With the exception of some studies on the role of MCT8 in zebrafish (*Danio rerio*) development, little is known about TH transporters in fish. The tissue distribution of TH transporters appears to vary between fish models. MCT8 mRNA is expressed in

brain, spinal cord and vascular system in zebrafish (Groeneweg, van Geest, Peeters, Heuer, & Visser, 2019) and mostly in the liver of fathead minnow (*Pimephales promelas*) (Muzzio, Noyes, Stapleton, & Lema, 2014). OATP1C1 is expressed primarily in the liver and brain in zebrafish (Admati et al., 2019; Zada, Blitz, & Appelbaum, 2017), and in the gonad, liver and brain in fathead minnow (Muzzio et al., 2014).

The expression of TH transporter transcripts shows an inverse relationship to circulating TH levels. In fathead minnow, exogenous T_3 administration leads to a reduction in liver OATP1C1 transcript abundance (Muzzio et al., 2014), while treatment with oral PTU increases brain MCT8 expression (Noyes, Lema, Macaulay, Douglas, & Stapleton, 2013). In zebrafish, MCT8 seems to mediate T_3 transport across the blood brain barrier (BBB) (Groeneweg et al., 2019) and MCT8-deficient zebrafish have altered nervous system development (Vatine et al., 2013). The role of OATPs in fish remains unclear but in zebrafish, OATP1C1 deficiency leads to hyperactivity of the thyroid and the development of goiter (thyroid follicle enlargement), possibly as a consequence of low TH levels as a result of reduced transport into target cells (Admati et al., 2019).

2.3.2. TH Nuclear Receptors

THs affect physiological processes by regulating expression of genes in target tissues (genomic actions) (S.-Y. Cheng, Leonard, & Davis, 2010). Within target cells, T_3 binds to thyroid hormone receptors (TRs). TRs are located on thyroid response elements (TRE) of the DNA, located at T_3 target gene promoter sites (Chiamolera et al., 2012). Nuclear TRs act as ligand-modulated transcription factors, In the absence of T_3, TR

represses transcription by recruiting corepressors [e.g., nuclear-receptor co-repressor (NCoR)/silencing-mediator for retinoid/thyroid hormone receptors (SMRT)], whereas in the presence of T3, TRs recruit coactivators [e.g. steroid receptor coactivator (SRC), p300/CREB-binding protein (CBP)] to facilitate transcription (Chiamolera et al., 2012). Therefore, the transcription rate of target genes depends on the binding of T_3 to TRs.

TRs are products of two different genes, c-erbAα and c-erbAβ (or TRα and TRβ) (Forrest & Vennström, 2000; Ortiga-Carvalho, Sidhaye, & Wondisford, 2014). The TR binds to a TRE as a monomer, a homodimer *(*α/α, α/*β*, *β*/*β*) or a heterodimer, in which a TR isoform dimerizes with the retinoid X receptor (RXR) (Bhagavan, 2002). TRα and TRβ each have different isoforms that have different tissue distributions (e.g., in mice, TRα1 and TRβ1 are expressed in all tissues, but TRα1 is predominantly expressed in the heart and brain, whereas TRβ1 is predominant in skeletal muscle, kidney and liver) and binding capacities (TRα2 and TRα3 isoforms are truncated and are unable to bind T_3) (Ortiga-Carvalho et al., 2014).

In fish, several species-dependent TR isoforms have been identified. For example, Japanese flounder (*Paralichthys olivaceus*), Atlantic salmon and Atlantic halibut (*Hippoglossus hippoglossus*) have two distinct TRα genes, while conger eels (*Conger myriaster*) have two subtypes of each TRα and TRβ genes (Kawakami, Tanda, Adachi, & Yamauchi, 2003; Marchand et al., 2001; Yu, Fu, & Shi, 2017). Goldfish have three unique TRα isoforms (TRα-1, TRα-2 and TRα-truncated) all similarly expressed in pituitary, brain, liver, gonads and gut (Nelson & Habibi, 2006). The goldfish truncated form may inhibit transcription of functional TRs by competition for TREs (Nelson &

Habibi, 2006; Nelson & Habibi, 2009). In tilapia, two isoforms of TRβ exist – a short (S-TRβ1) and long (L-TRβ1) isoform – differing by 9 amino acids. T_3 and T_2 bind to activate L-TRβ1, but not S-TRβ1, and regulate TRβ expression *in vivo* (Mendoza et al., 2013).

Differences in the number/type/specificity of isoforms, and tissues distributions might indicate species-specific differential splicing, target cells, and functions, although it must be noted that transcript expression levels might not reflect protein levels, for which information is lacking (S.-Y. Cheng et al., 2010).

2.3.3. Non-nuclear TH receptors

THs have the ability to act both non-genomically and extracellularly – within the cytoplasm or plasma membrane – in a very rapid manner. THs activate intracellular pathways and other transcription factors such as the mitogen-activated protein kinase (MAPK) (Cayrol, Sterle, Díaz Flaqué, Barreiro Arcos, & Cremaschi, 2019; Davis, Goglia, & Leonard, 2016) or phosphatidylinositol 3-kinase (PI3K) pathways (Hiroi et al., 2006; Moeller, Cao, Dumitrescu, Seo, & Refetoff, 2006) by binding to the integrin $\alpha v\beta_3$ TH specific plasma membrane receptor (Bergh et al., 2005). Non-genomic actions may have downstream long-term specific nuclear effects (cell proliferation, gene transcription) leading to cross-talk between non-genomic and genomic action of THs (De Vito et al., 2012).

There is very limited evidence showing direct non-genomic actions of THs in fish, as non-genomic and genomic effects can overlap in the nucleus. In embryonic

zebrafish, T_4, but not T_3, regulates sodium currents through the MAPK pathway requiring the integrin $\alpha_V\beta_3$ receptor (Yonkers & Ribera, 2009). It has been suggested that, in fish, THs regulate mitochondrial respiration (Oommen et al., 2006), similar to what is seen in rodents, for which TH binding sites have been shown in mitochondrial membranes (Hashizume & Ichikawa, 1982).

2.3.4. Actions of T2

Although most studies focus on the actions of T_4 and T_3, recent evidence shows that T_2, a product of T_3 ORD, is also biologically active and binds to TRβ in teleosts (Mendoza et al., 2013). In rodents, administration of T_2 increases metabolic rate and has hypolipidemic effects (Senese et al., 2018). In fish, T_2 regulates the transcription of genes associated with cell signalling and transcriptional pathways in the liver of Nile tilapia (*Oreochromis niloticus*) (Olvera et al., 2017) and stimulates mitochondrial respiration of liver and muscle in goldfish (Leary, Barton, & Ballantyne, 1996). T_2 (like T_4 and T_3) also decreases DIO1 and DIO2 activities in the liver of killifish (*Fundulus heteroclitus*) (García-G et al., 2004), and regulates thermal acclimation in zebrafish (Little, Kunisue, Kannan, & Seebacher, 2013) and growth in tilapia (Pamela, Maricela, Carlos Valverde, & Aurea, 2014). Therefore, while previously viewed as an inactive TH, T_2 may have a larger role than originally thought.

2.4. Role of the thyroid axis on somatic development and growth

In fish, as in all vertebrates, THs are crucial for the proper development of both embryos and adults, and are involved in major life transitions and metamorphosis in some species (Forhead & Fowden, 2014; Power et al., 2001; Stepien & Huttner, 2019).

2.4.1. Maternal origin of THs and importance in egg and larval development

In early mammalian development, an embryo relies solely on maternal THs as its thyroid gland is not yet fully functional (Stepien & Huttner, 2019). THs are actively transported from the mother to the embryo across tissue barriers – including the placenta and BBB – and act on embryonic target cells (Stepien & Huttner, 2019).

The diverse modes of reproduction in fish (Godwin & Phillips, 2018) result in species-specific thyroid-mediated development, due to the variety of mechanisms by which maternal transfer of THs into the egg/embryo occurs (Vergauwen et al., 2018).

Most fish have external fertilization and are oviparous [i.e., produce eggs that develop and hatch in the external environment (Sloman, 2011)]. Others have internal fertilization and the egg/embryo develops within the mother. In viviparity, eggs develop and hatch within the mother before being released as live young to the external environment (Sloman, 2011). In yolksac, or lecithotrophic viviparity, eggs are retained inside the female until fully developed, with no maternal chemical contribution beyond yolk. In matrotrophic viviparity, the embryos receive additional nutrition from the mother (e.g., maternal proteins and lipid-rich histotroph secreted from the uterus in histotrophy;

unfertilized eggs/other embryos in oophagy/adelphophagy; or through placenta-like structures) (Hamlett, 1993; Wourms & Demski, 1993).

In oviparous fish, there is evidence that THs are transferred from female fish to eggs (Lam, 1994). Fathead minnow and zebrafish eggs display high TH levels and high transcript levels of thyroid-related transcripts (TRα, TRβ, DIO1, DIO2, DIO3, TPO, sodium-iodide symporter, TRH-receptor, TSH-receptor, TG and TTR) before 2-3 days post-fertilization (dpf) – time at which endogenous TH production begins – suggesting a maternal transfer of THs (Vergauwen et al., 2018). In alligator gar (*Atractosteus spatula*) and spotted gar (*Lepisosteus oculatus*), injecting females with THs or TSH results in increases in the concentrations of T_4 and T_3 in early embryos (Castillo et al., 2015). As well, maternal injections and egg immersion have been shown to increase pigment concentrations in larval tissues, hatching and larval growth rate, swim bladder inflation, muscle development, larval metabolic capacity and metamorphosis [e.g., Sterlet sturgeon (*Acipenser ruthenus*) (Abdollahpour, Falahatkar, Efatpanah, Meknatkhah, & Van Der Kraak, 2018; Alinezhad, Abdollahpour, Jafari, & Falahatkar, 2020); piracanjuba (*Brycon orbignyanus*) (Landines, Sanabria, Senhorini, & Urbinati, 2010); matrinxã (*Brycon amazonicus*) (Urbinati, Vasques, Senhorini, Souza, & Gonçalves, 2008); zebrafish (D. D. Brown, 1997); goldfish (Reddy & Lam, 1992)]. Interestingly, it appears that T_4 concentrations are greater than T_3 concentrations in eggs of most freshwater (FW) fish, whereas T_3 concentrations are greater in seawater (SW) fish (Tagawa, Tanaka, Matsumoto, & Hirano, 1990), suggesting differential TH utilization during egg development.

Less is known about maternal transfer of THs in viviparous species. In the lecithotrophic viviparous dogfish (*Squalus acanthias*), 5′-MDA activity (an indicator of the production rate of the active thyroid hormone T_3) is present in yolksac embryos and may be of maternal origin (Leary, Ballantyne, & Leatherland, 1999), and in Korean rockfish (*Sebastes schlegelii*), maternal T_3 injections improve growth and survival of young *in utero* (D.-Y. Kang & Chang, 2004). In matrotrophic viviparity, there is an association between embryos and maternal structures, suggesting that maternal THs could be exchanged (Wourms & Demski, 1993). In surfperch (*Neoditrema ransonnetii*) – a matrotrophic teleost in which embryos are sustained by ovarian cavity fluid (OCF) ingestion and by nutrient absorption via enlarged hindgut – OCF and fetal plasma contain high TTR levels. TTR plasma levels are higher in pregnant fish than in non-pregnant fish, and large amounts of maternal TTR are taken up by fetal intestinal epithelial cells (enterocytes), indicating that maternal TTR is secreted into OCF and taken up by fetal enterocytes, presumably to deliver THs to developing embryos (Nakamura et al., 2020). In the viviparous bonnethead shark (*Sphyrna tiburo)*, yolk-dependent embryos undergo yolk-sac modification in which the fetal portion of a placenta attaches to the maternal uterine wall near mid-gestation, which facilitates direct exchanges of blood and nutrients between the mother and embryo (Wourms, 2015). In this species, T_3 in yolk increases from pre- to post-ovulation and peaks during the pregnancy stage, and maternal serum T_3 concentrations increase as development progresses, suggesting that maternal THs are needed for development of the egg/embryo (McComb, Gelsleichter, Manire, Brinn, & Brown, 2005).

2.4.2. The thyroid and growth axes

In fish, as in mammals, somatic growth is regulated by hormones of the growth (or hypothalamic–pituitary–somatotropic, HPS) axis, i.e., growth-hormone releasing hormone (GHRH) from the hypothalamus, and growth hormone (GH) produced by somatotrophs in the anterior pituitary. GH release is stimulated by GHRH and other secretagogues (e.g., ghrelin) and inhibited by SS (Rodriguez-Arnao, Miell, & Ross, 1993). GH has direct and indirect actions on tissues via the stimulation and release of insulin-like growth factors I and II (IGF-I, IGF-II) by the liver. These act on tissues to promote cellular proliferation and differentiation (Blanco, 2020; Triantaphyllopoulos, Cartas, & Miliou, 2019).

Embryonic differentiation/organogenesis and growth in teleosts is regulated by THs, likely by triggering both GH [e.g., THs increase GH mRNA transcription in rainbow trout (Moav & McKeown, 1992) and carp (Farchi-Pisanty, Hackett Jr, & Moav, 1995), and increase synthesis and release in hybrid tilapia (Melamed et al., 1995)] and IGF-I [e.g., THs induce *in vivo* and *in vitro* synthesis/release in Mozambique tilapia (*Oreochromis mossambicus*) (Schmid, Lutz, Kloas, & Reinecke, 2003)]. Since THs are crucial regulators of growth (Bolotovskiy & Levin, 2018; Keer et al., 2019), inhibition of thyroid function results in impairment in the development of brain, skeleton and other organs, as well as in pigmentation. For example, in zebrafish, treatment with T_3 increases IGF-1 expression and enhances swim bladder and eye development but IGF-1 receptor blockade supresses these effects of T_3 on swim bladder and eye (Molla et al., 2019).

2.4.2.1. Interactions between thyroid and growth axes

Components of the thyroid axis have been shown to affect the GH/IGF-I axis in vertebrates. TRH stimulates the secretion of GH by acting directly upon GH cells in amphibians (Gracia-Navarro, Castaño, Malagón, & Torronteras, 1991; Hall & Chadwick, 1984) and reptiles (Denver & Licht, 1988; Hall & Chadwick, 1984). In rodents, THs have been shown to stimulate GH synthesis and secretion (Dobner, Kawasaki, Yu, & Bancroft, 1981; Hervas, de Escobar, & del Rey, 1975), upregulate SS receptors (James et al., 1997) and increase SS immunoreactivity and release (Berelowitz, Maeda, Harris, & Frohman, 1980).

In fish, the effects of the thyroid axis on growth are not clear, as components have been shown to have both inhibitory and stimulatory effects. TRH increases GH secretion *in vivo* in goldfish (Cook & Peter, 1984) and tilapia hybrid (*Oreochromis niloticus x Oreochromis aureus*) (Melamed et al., 1995), and *in vitro* in common carp pituitary fragments (X. W. Lin, Lin, & Peter, 1993), but not in tilapia hybrid (Melamed et al., 1995) or sailfin molly (Batten & Wigham, 1984). TSH injections increase GH plasma levels in several species including Nile tilapia (Melamed et al., 1995), killifish (Grau & Stetson, 1979; Pickford, 1954), coho salmon (Higgs, Donaldson, Dye, & McBride, 1976), rainbow trout (Leatherland & Farbridge, 1992) and Indian carp (*Cirrhinus mrigala*) (Bandyopadhyay & Bhattacharya, 1993).

THs affect the growth axis in fish, although results are inconsistent. *In vivo* treatment with T_4 or T_3 decreases both pituitary and serum GH levels in female European eel (Rousseau et al., 2002) but has no effect on GH levels in goldfish (Allan & Habibi,

2012). T_4 administration to aquarium water increases somatotroph activity in red belly tilapia (*Coptodon zillii*) (Leatherland & Hyder, 1975), and *in vivo* T_3 injections increase pituitary GH mRNA expression in rainbow trout (Moav & McKeown, 1992) and GH plasma levels in hybrid tilapia (Melamed et al., 1995). THs also act on liver to stimulate IGF-I synthesis/secretion: T_3 increases hepatic IGF-I mRNA levels both *in vitro* and *in vivo* in Mozambique tilapia (Schmid et al., 2003) and zebrafish (Wang & Zhang, 2011), but not in coho salmon (Pierce, Fukada, & Dickhoff, 2005) or silver sea bream (*Sparus sarba*) (Leung, Kwong, Man, & Woo, 2008). T_3 may regulate IGF-I expression by binding to liver GH receptors [e.g., coho salmon (Pierce et al., 2005)] or TRs [e.g., rainbow trout (MacLatchy & Eales, 1992)], although this action seems species-specific.

Whereas the thyroid axis can affect growth, components of the growth axis affect the thyroid. In mammals, the thyroid axis is stimulated by GH, as seen by increases in TH levels following GH treatment (Yamauchi et al., 2018), and inhibited by SS (Lamberts, Reubi, & Krenning, 1997). In humans, ghrelin decreases TSH-induced production of thyroglobulin and mRNA expression of TPO in thyroid cells (Barington et al., 2017), while SS treatment decreases the volume of TSH-cells and serum concentrations of TSH in rats (Milosević, Sekulić, Brkić, Lovren, & Starcević, 2000) but has no effect on serum TSH and TH levels in humans (De Rosa, Corsello, Della Casa, De Rosa, & Raimondo, 1983).

In fish, there is evidence for a role of the GH axis in regulating thyroid function. TSH receptor expression is up-regulated in transgenic grass carp overexpressing GH (Chen et al., 2018), and in European eel, GH stimulates thyroid follicles to release T_4 and

enhances peripheral 5'-MDA activity (de Luze & Leloup, 1984). In mummichog, hypophysectomy prevents TSH-induced secretion of T_4 and treatment with ovine GH restores this response (Grau & Stetson, 1979). Information on the role of ghrelin and SS on the thyroid axis is scarce. Plasma TH levels are inversely correlated with SS plasma levels in rainbow trout (Holloway, Sheridan, Van Der Kraak, & Leatherland, 1999), and burbot (*Lota lota*) have decreased plasma ghrelin and TH levels pre-spawning (Nieminen, Mustonen, & Hyvärinen, 2003), suggesting an interaction between SS, ghrelin and THs.

2.4.3. Ecological importance of thyroid-mediated development

THs are particularly important for the development of the central nervous system (CNS) and for ecological/ecosystem shifts within fish. The plasticity of the fish nervous system allows it to regenerate after injury and be remodeled during life history shifts, processes in which THs are most likely implicated. This has been demonstrated in zebrafish submitted to optic nerve injury, in which the re-innervation of the optic tectum is accelerated when T_3 plasma levels are lowered with a TRβ antagonist and iopanoic acid (IOP, inhibits TH release and reduces peripheral T_4 to T_3 conversion) (Bhumika, Lemmens, Vancamp, Moons, & Darras, 2015).

In the case of migrating anadromous species, T_3 induces the proliferation of olfactory receptor neurons (which are crucial for natal stream imprinting) in olfactory epithelium (Lema & Nevitt, 2004) and T_4 induces a switch from UV to blue opsin photoreceptors in the retinas of young coho salmon and rainbow trout (C. L. Cheng, Gan, & Flamarique, 2009) – which allows better visual contrast for feeding before a SW

migration (Flamarique & Browman, 2001). In masu salmon, T_3 binding in the brain is tissue-specific during the parr-smolt transformation: At both life stages, T_3 binding is highest in the olfactory epithelium, and smolts show higher binding compared to parr in this region (Kudo et al., 1994). This suggests that THs play an important role in functional changes of the brain and olfactory epithelium, playing a preparatory role for shifting between aquatic habitats.

2.5. Metamorphosis

Fish metamorphosis refers to the dramatic changes seen in flatfish, lampreys and eels, but can be applied to any irreversible post-embryonic developmental event that affects multiple physiological or morphological traits (excluding those related to sexual maturation, reproduction or senescence) seen in several FW and marine species (Manzon & Manzon, 2017; McMenamin & Parichy, 2013). THs are key regulators of teleost metamorphosis, which involves cellular and molecular remodelling that lead to developmental changes (Campinho, 2019). Typically, thyroid activity is low during pre-metamorphosis (i.e., low TH levels, with reduced DIO and TR expression), increases during the metamorphic event, peaks during developmental changes (metamorphic climax), and decreases to pre-metamorphic levels (Campinho, 2019; McMenamin & Parichy, 2013).

In flatfish, pelagic larvae develop symmetrically with eyes on each side of the head, and morph into asymmetric benthic juveniles following the migration of one eye to the opposite side of the head to become right- or left-eyed, a species-specific distinction

[e.g, right-eyed Atlantic halibut (Alves et al., 2016), left-eyed Japanese flounder (Yu et al., 2017) and left- or right-eyed Starry flounder (Bergstrom, 2007)]. In Senegalese sole (*Solea senegalensis*), increases in TH circulating levels, pituitary TSHβ, and whole body thyroglobulin and TR transcript levels (Campinho et al., 2015) coincide with metamorphic climax and activity in thyroid follicles (Campinho et al., 2018). Similarly, during Atlantic halibut metamorphosis, the vast majority of transcripts expressed in the head transcriptome are related to the thyroid axis (Alves et al., 2016).

In sea lamprey (*Petromyzon marinus*), the blind, sedentary, filter-feeding larvae metamorphose into free-swimming juveniles. This involves major changes including the development/transformation of adult kidneys, GIT, gills, and the development of the eyes (Manzon & Manzon, 2017). Interestingly, as opposed to other fish, lamprey metamorphosis coincides with a drop in serum endostyle cells-derived TH levels, is blocked by TH treatment and is stimulated by goitrogens (which suppress TH levels), but the mechanisms by which this occurs are still unclear (Manzon & Manzon, 2017; Youson, 2015).

In diadromous species, which migrate between SW and FW, metamorphosis induces morphological and physiological changes (e.g., changes in body shape, pigmentation, kidneys, gut, eyes, osmoregulation, metabolism) that prepare the fish to survive in a new habitat (McMenamin & Parichy, 2013). In anadromous salmonids (e.g., *Oncorhynchus*, *Salmo* and *Salvelinus*), fish hatch and grow in FW before migrating to SW where most of the somatic growth takes place. Smoltification [or parr (FW fish)–smolt (SW fish) transformation] refers to the changes in physiology, behaviour and

morphology that occur in juvenile salmonids prior to this migration. These include pigmentation changes (i.e., body and darkening of fins) and changes in olfactory receptors and osmoregulatory adaptation (Björnsson, Stefansson, & McCormick, 2011; W. S. Hoar, 1988; Stephen D. McCormick, 2012; Stefansson, Björnsson, Ebbesson, & McCormick, 2008), all associated with a surge in TH levels. For example, TH treatment induces downstream migration in Atlantic (Godin, Dill, & Drury, 2011), coho, chum (*Oncorhynchus keta*) and sockeye (*Oncorhynchus nerka*) salmon (Iwata, 1995) and TSH injections or TH treatment increase purine synthesis, which is responsible for skin silvering in rainbow trout (Premdas & Eales, 1976) and brook trout (Chua & Eales, 1971).

In contrast to salmonids, eels hatch and develop as marine larvae [flat and transparent marine larvae (leptocephali)] and undergo a SW to FW (catadromous) migration. Larvae transform into transparent "glass eels", which move to FW and complete metamorphosis to become juvenile “elvers.” These then undergo a secondary metamorphic event (silvering) and return to the ocean for spawning. In Japanese eel, the change from leptocephalus larvae to glass eel is characterized by an increase in TH levels and TSHβ expression, with TSHβ levels peaking at the glass eel stage and THs increasing into the juvenile stages (Sudo, Okamura, Kuroki, & Tsukamoto, 2014).

Many teleosts undergo subtle irreversible post-embryonic morphological and physiological changes that have been defined as a metamorphosis and are regulated in part by THs (McMenamin & Parichy, 2013). These include the development of the fins and the appearance of adult stripes in zebrafish (D. D. Brown, 1997), and changes in

colouration and swimming behaviour marine fish such as red sea bream (*Pagrus major*) (Hirata, Kurokura, & Kasahara, 1989), grouper (*Epinephelus coioides*) (de Jesus, Toledo, & Simpas, 1998), surgeonfish (*Acanthurus triostegus*) and clown fish (*Amphiprion ocellaris*) (Roux, Salis, & Laudet, 2019).

2.6. Reproduction

THs regulate many aspects of the reproductive system, including formation of gametes and steroids, and sexual behaviour in both males and females. In vertebrates, the hypothalamus-pituitary-gonadal (HPG) axis regulates reproduction: Gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates the pituitary to release gonadotropins (GTH) [luteinizing hormone (LH) and follicle stimulating hormone (FSH)] which act on gonads to regulate gametogenesis and steroidogenesis [e.g., in mammals (Acevedo-Rodriguez et al., 2018) and fish (Biran & Levavi-Sivan, 2018)]. There is growing evidence of a crosstalk between the thyroid and HPG axes in several vertebrates (e.g., mammals, amphibians, fish) (Duarte-Guterman, Navarro-Martín, & Trudeau, 2014).

In mammals, the link between thyroid and reproductive function is well established. THs and TSH can affect gonadal development and sex steroid hormone synthesis and actions, and thyroid dysfunction is associated with decreased fertility, impaired gonadal function and disruption of seasonal cycles in both in males and females (Anderson & Barrell, 1998; De Vincentis, Monzani, & Brigante, 2018; Holsberger et al., 2005; Moenter, Woodfill, & Karsch, 1991). In fish, the link between THs and

reproduction is not clear, as inconsistent results have been reported, likely due to the diversity in reproductive strategies, and methods used to investigate TH actions (Raine, 2011).

2.6.1. TH and reproductive cycles

Several studies have shown correlations between circulating THs and reproductive cycles (e.g., gamete formation and maturation, and spawning/hatching events) in fish, but between species, the nature of these relationships vary. Among teleosts, some species display peaks in plasma THs during gametogenesis [e.g., rainbow trout (Osborn, Simpson, & Youngson, 1978); brook trout (White & Henderson, 1977) and/or during spawning [e.g., climbing perch (*Anabas testudineus*) (Chakraborti & Bhattacharya, 1984); sea lamprey (Sower, Plisetskaya, & Gorbman, 1985)], whereas others display decreases in TH levels during gonad maturation [e.g., Mozambique tilapia (Weber, Okimoto, Richman, & Grau, 1992)], before [e.g., sockeye salmon (Biddiscombe & Idler, 1983)] or during spawning [e.g., winter flounder (Eales & Fletcher, 1982)]. In the jawless Pacific sea lamprey, both males and females show peaks in plasma THs during gametogenesis and spawning (Mesa, Bayer, Bryan, & Sower, 2010; Sower et al., 1985).

In the Chondrostei stellate sturgeon (*Acipenser stellatus*) and lake sturgeon (*Acipenser fulvescens*), THs are correlated with increased gonad maturation during the spawning season (Dettlaff & Davydova, 1979; Plohman, Dick, & Eales, 2002), while in immature and previtellogenic individuals, changes in THs during the reproductive season

are more closely correlated with temperature, feeding and growth [e.g., great sturgeon (*Huso huso*) (Falahatkar, 2015) and lake sturgeon (Plohman et al., 2002)].

Very little is known about the role of THs in elasmobranch reproduction. In oviparous elasmobranchs, thyroid activity and TH levels are usually lowest in immature females in the non-breeding season, and greatest during egg development and vitellogenesis during the reproductive season [e.g., lesser spotted dogfish (Clements, 1957); brownbanded bamboo shark (*Chiloscyllium punctatum*) (Alimi, Savari, Movahedinia, Zakeri, & Salamat, 2015)]. Complete thyroid removal inhibits seasonal gonad development [e.g., spotted dogfish (Lewis & Dodd, 1974)]. A similar correlation between thyroidal function and female reproduction has been shown in viviparous elasmobranchs. In the Atlantic stingray (*Dasyatis sabina*), circulating T_3 levels and thyroid activity are low in immature individuals and high in females undergoing oogenesis, and, from ovulation throughout gestation (Sage, 1973; H. Volkoff, Wourms, Amesbury, & Snelson, 1999). Similarly, in the torpedo (*Torpedo ocellata*), thyroid activity is high in gestating females (Zezza, 1937). However, in female dogfish, thyroid activity does not seem to be associated with reproductive events, but rather with migration (Woodhead, 1966).

2.6.2. Evidence of expression of deiodinases, TH receptors and TSH receptors in gonads

2.6.2.1. Deiodinases

DIOs have been shown to be present in gonads [e.g., mammals (Wakim, Polizotto, Buffo, Marrero, & Burholt, 1993; Ślebodzińska, Ślebodziński, & Kowalska,

2000); amphibians (Duarte-Guterman & Trudeau, 2011); reptiles (H. Kang, Kenealy, & Cohen, 2020)] and to be involved in reproductive cyclicity. In mammals, 5'-MDA activity is elevated during gonad development and differentiation [e.g., horse ovary (Ślebodziński, 2005); pig testis (Ślebodzińska et al., 2000)]. In western clawed frog (*Silurana tropicalis*) gonads, DIO2 and DIO3 expressions increase and DIO1 expression decreases throughout the development into adult (Duarte-Guterman & Trudeau, 2011). Moreover, gender-specific roles of DOIs have been suggested in lower vertebrates. Adult western clawed frog testis show higher expression of DIO1, DIO2 and DIO3 than ovary (Duarte-Guterman & Trudeau, 2011), and in breeding green anole lizards (*Anolis carolinensis*), DIO2 and DIO3 expression levels are high in testes and ovaries, respectively (H. Kang et al., 2020).

Although DIO1, DIO2 and DIO3 activity/expression has been shown in the gonads of several fish [including striped parrotfish (*Scarus iseri*) (Johnson & Lema, 2011), European sea bass (*Dicentrarchus labrax*) (Isorna, Vallés, Servili, Falcón, & Muñoz-Cueto, 2008), goldfish (Marlatt et al., 2012), Nile tilapia (Coimbra, Reis-Henriques, & Darras, 2005), sapphire devil (*Chrysiptera cyanea*) (Hur et al., 2020) and rainbow trout (Sambroni et al., 2001)] their role in gonadal thyroid metabolism is not clear.

A gender-specific expression of DIO1 and DOI2 has been shown in parrotfish, with higher expression levels in ovaries than testes, suggesting that ovaries may require more bioactive THs than testes (Johnson & Lema, 2011). Whereas there is no evidence for a role of DIO1 in the gonads, DIO2 has been implicated in the regulation of gonad

maturation and gametogenesis. In zebrafish, DIO2 deficiency results in delayed sexual maturity and reduced gametogenesis and spawning in both males and females (Houbrechts, Van Houcke, & Darras, 2019). Conversely, high DIO2 activity/expression in gonads [e.g., female tilapia (Weber et al., 1992); male rainbow trout, (Sambroni et al., 2001)], may ensure appropriate levels of T_3 needed for gametogenesis. In the sapphire devil, transcript levels of ovary DIO3 increase as vitellogenesis progresses, suggesting that high DIO3 expression might prevent excess TH buildup (Hur et al., 2020).

2.6.2.2. TH Receptors

TRs are expressed in gonads of teleosts such as goldfish (Marlatt et al., 2012; Nelson & Habibi, 2006), striped parrotfish (Johnson & Lema, 2011), Korean rockfish (Muhammad, Wang, Wang, Jakhrani, & Qi, 2012), black porgy (*Acanthopagrus schlegelii*) (An, An, Nelson, Habibi, & Choi, 2010) and fathead minnow (Filby & Tyler, 2007), and their expressions appear to be gender-dependent and species-specific. The expressions of TRα and TRβ are higher in ovary than in testis in mature Korean rockfish (Muhammad et al., 2012), mature goldfish (Nelson & Habibi, 2006) and developing fathead minnow (Filby & Tyler, 2007), but higher in testis than the ovary in striped parrotfish (Johnson & Lema, 2011).

In fish that change sex as part of their life-history strategy, TR subtypes display expression changes in regard to gender. In protandrous (sex change from male to female) black porgy, TRα mRNA expression is low in immature testis and increases at maturation. During sex change, TRα expression decreases then subsequently increases

during ovary development and maturation and TRβ expression is highest in mature ovary after sex change than in any other gonadal or sex stage (An et al., 2010). These results suggest that TRα is critical for both testis and ovary development, and TRβ might only be required in the ovary of this species, similar to fathead minnow (Filby & Tyler, 2007). The significance of this differential expression is yet to be uncovered, but most likely important in cell-specific proliferation and differentiation in gonads, albeit, dependent on sex.

2.6.2.3. TSH Receptors

Thyrotropin receptor (TSHR) expression has been detected in gonads of several species, including European sea bass (Rocha et al., 2007), walking catfish (*Clarias batrachus*) (Bhat, Rather, Saha, Ganie, & Sharma, 2017), channel catfish (Goto-Kazeto, Kazeto, & Trant, 2009), striped bass (*Morone saxatillis*) (Kumar et al., 2000), biwa trout (*Oncorhynchus rhodurus*) (Hirai, Oba, & Nagahama, 2002) and sunrise sculpin (*Pseudobennius cottoides*) (Hirai et al., 2002).

TSHR expression levels increase during ovarian and testicular maturation in European sea bass (Rocha et al., 2007), channel catfish (Goto-Kazeto et al., 2009) and striped bass (Kumar et al., 2000), and peak during spermatogenesis in sunrise sculpin (Hirai et al., 2002), suggesting a direct role of TSH and TSHR in gametogenesis. In walking catfish, GnRH treatment increases TSHR mRNA expression in gonads, suggesting a positive correlation between TH levels and reproduction (Bhat et al., 2017).

2.6.3. Thyroid and HPG axes

In fish, as in mammals, the thyroid influences the HPG axis in a gender-, development- and species-specific manner. The effects of the thyroid axis on reproductive processes of fish occur via actions at all levels of the HPG axis, i.e., the hypothalamus, pituitary and gonads.

In the hypothalamus, the effects of THs on GnRH appear to depend on the species and the reproductive-stage considered, as well as the specific population of GnRH neurons. In male mature recrudescent (active gametogenesis) air-breathing catfish (*Clarias gariepinus*), thiourea-induced TH depletion reduces the number of hypothalamic GnRH immunoreactive neuronal cells and fibres (Swapna et al., 2006). In immature male Nile tilapia, T_3 treatment suppresses terminal nerve GnRH mRNA, but does not significantly affect preoptic or midbrain GnRH mRNA levels or the number of hypothalamic GnRH neurons (Parhar, Soga, & Sakuma, 2000), suggesting central-specific TH action dependent on reproductive stage.

Studies have shown that THs may act at the pituitary level to inhibit gonadotropin secretion. Hypothyroid conditions decrease pituitary LH immunoreactivity and LH circulating levels in male recrudescent air-breathing catfish (Swapna et al., 2006), and, in recrudescent goldfish, administration of T_3 decreases pituitary LH mRNA expression in males (Nelson, Allan, Pang, & Habibi, 2010) and attenuates GnRH-induced LH secretion in females (Ma, Ladisa, Chang, & Habibi, 2020).

Gonadal steroidogenesis occurs in Leydig cells of testes and thecal and granulosa cells of ovaries, and starts with the transport of cholesterol into the mitochondria

mediated by steroidogenic acute regulatory protein (StAR), where it is converted into pregnenolone, which is sequentially converted into active steroids such as progesterone (P), 17α-hydroxy-20β-dihydroprogesterone (DHP), the androgens testosterone (T) and 11-Ketotestosterone (11-KT, the predominant androgen in fish), and estradiol-17β (E_2) by several steroidogenic enzymes (Rajakumar & Senthilkumaran, 2020). In male vertebrates, Sertoli and Leydig cells are responsible for spermatogenesis and androgen biosynthesis, respectively, whereas oogenesis is stimulated by ovarian estrogen and progestins in females (Yaron & Levavi-Sivan, 2011).

There is evidence in fish that THs increase spermatogenesis and androgen secretion in males and estrogen and progestin secretion in females. In zebrafish testis, T_3 stimulates spermatogenesis by increasing the division of spermatogonia and Sertoli cells (Morais et al., 2013; Safian, Morais, Bogerd, & Schulz, 2016), increasing the production of IGF-III (insulin-like growth factor-III, a stimulatory growth factor of spermatogenesis) by Sertoli cells, and enhancing the gonadotropin-induced synthesis and release of androgens by Leydig cells (Tovo-Neto, da Silva Rodrigues, Habibi, & Nóbrega, 2018). In male goldfish, treatment with T_3 decreases expression of CYP19 (aromatase, which converts androgens into estrogens) thus increasing the androgen to estrogen (A:E) ratio (Nelson et al., 2010), and inhibiting T_3 synthesis with monocrotophos (organophosphate pesticide) increases CYP19 expression and reduces the A:E ratio (Xiaona Zhang et al., 2018). In contrast, in cultured adult zebrafish testis, T_3 does not affect the release of 11-KT, or AR and CYP19 mRNA expressions (Morais et al., 2013), and in juvenile common carp, treatment with T_4 has no effect on testis diameter or number of spermatogonia

(Timmermans, Chmilevsky, Komen, & Schipper, 1997). In mid to late recrudescent male goldfish, T_3 decreases circulating E_2 levels and expression levels of testis estrogen receptor subtypes (ERα, ERβ1 and ERβ2) during mid-recrudescence (Nelson et al., 2010), but has no effect in late or regressed gonads (Allan & Habibi, 2012). This suggests that THs are essential for spermatogenesis in males but are reproductive stage-specific and seem to have the greatest effect in periods of active spermatogenesis.

In mid-recrudescent female goldfish, *in vivo* T_3 treatment decreases the expressions of estrogen receptors (ERα and ERβ1) and CYP19 in ovary (Nelson et al., 2010), and in recrudescent female air-breathing catfish, T_4 treatment decreases CYP19 immunoreactivity and E_2 levels in ovary (Supriya et al., 2005), while thiourea-induced TH depletion increase ovarian expression of CYP19 (Rasheeda et al., 2005). In oocytes of pre-spawning climbing perch, *in vitro* T_3 treatment increases progesterone release (Guin, Bandyopadhyay, Jana, & Bhattacharya, 1993) and 3β-hydroxysteroid dehydrogenase (3β -HSD, which converts pregnenolone to progesterone) activity (Datta, Nagendra Prasad, & Bhattacharya, 1999), and enhances gonadotropin-induced E_2 secretion in ovarian follicles from spawning rainbow trout (Cyr & Eales, 1988a).Therefore, similar to male testes, the actions of TH in ovaries appear more pronounced during active periods of gametogenesis. It has been suggested that in seasonal species such as goldfish, THs might inhibit oogenesis/vitellogenesis during non-spawning season, allowing fish to allocate their energy to somatic growth (Habibi, Nelson, & Allan, 2012; Nelson et al., 2010).

Very few studies have been performed in elasmobranchs. In the oviparous female dogfish, thyroidectomy impairs ovarian follicular development (Lewis & Dodd, 1974). Both male and female spiny dogfish show correlations between gonad follicle and thyroid growth, with female follicular cell height showing a positive relationship to thyroid weight (Woodhead, 1966).

While THs affect reproductive tissues, the thyroid axis is also regulated by reproductive hormones. In fish, treatment with E_2 appears to have inhibitory effects on TH levels, as seen by E_2 induced decrease in thyroid epithelial cell height and thyroid activity [e.g., European eel (Olivereau, Leloup, De Luze, & Olivereau, 1981) and rainbow trout (Leatherland, 1985)], decreases in plasma TH levels (usually T_3) [e.g., European eel (Olivereau et al., 1981), Atlantic salmon (Stephen D. McCormick, O'Dea, Moeckel, Lerner, & Björnsson, 2005) and southern hemisphere lamprey (*Geotria australis*) (Leatherland, Macey, Hilliard, Leatherland, & Potter, 1990)], decreases in hepatic T_3 production [e.g., trout (Cyr & Eales, 1988a; Flett & Leatherland, 1989) and masu salmon (Yamada, Horiuchi, Gen, & Yamauchi, 1993)], increases in TSH [e.g., rainbow trout (Flett & Leatherland, 1989) and masu salmon (Yamada et al., 1993)] and decrease in gonad TRα expression in male and female fathead minnow (Filby, Thorpe, Maack, & Tyler, 2007). Like estrogens, androgens might also affect the thyroid axis in fish (Leet, Gall, & Sepúlveda, 2011). Androgens have been shown to enhance thyroidal function in most teleosts examined [e.g., striped catfish (*Mystus vittatus*) (Singh, 1969); rainbow trout (Hunt & Eales, 1979); masu salmon (Ikuta, Aida, Okumoto, & Hanyu, 1985); coho salmon (Shelbourn, Clarke, McBride, Fagerlund, & Donaldson, 1992),

striped catfish (Singh, 1969)]. In Japanese medaka (*Oryzias latipes*) (León, Teh, Hall, & Teh, 2007) and coho salmon smolt (Shelbourn et al., 1992), 11-KT (medaka) and 17α-methyltestosterone (MT, coho) administration in larval males causes thyroid follicle hypertrophy and enhances 5'-MDA activity (Cyr & Eales, 1996). However, MT treatment induces a dose-dependent decrease in plasma T_4 and inhibits the smoltifying effects of T_4 in masu salmon (Ikuta et al., 1985).

2.7. Role of THs in osmoregulation

In mammals, the kidney is the major osmoregulatory organ, and THs influence renal development, kidney hemodynamics, glomerular filtration rate and ion and water homeostasis (Iglesias, Bajo, Selgas, & Díez, 2017) and thyroid dysfunction affects renal function (Iglesias et al., 2017).

In fish, osmoregulation is accomplished by the kidneys and GIT, but mainly by gills (via chloride cells) in teleosts and rectal gland in elasmobranchs (D. Evans, 2010). Compared to the outside water, the internal environment of marine fish is hypoosmotic, while that of a FW fish is hyperosmotic. Most species live in relatively constant habitats and can only survive within a narrow range of salinities (stenohaline). However, other species are able to adapt to a wide range of salinities (euryhaline) and some undergo drastic osmotic changes as they migrate [from SW to FW (anadromy) or from FW to SW (catadromy)] (D. H. Evans, 2011).

Several hormones control osmoregulation in fish. In euryhaline fish, cortisol (a glucocorticoid secreted by kidney) is considered the main SW adapting hormone whereas

prolactin (PRL, which promotes ion uptake and inhibits ion secretion) is viewed as a FW adapting hormone; GH and IGF-I have also been implicated in the control of SW adaptation (Stephen D. McCormick, 2001; S. D. McCormick, 2011). The thyroid axis has been shown to regulate osmoregulatory changes in fish, most likely through interactions with cortisol/GH and PRL (Stephen D. McCormick, 2001; S. D. McCormick, 2011).

2.7.1. Salinity tolerance in salmonids

Several studies have examined the role of the thyroid axis in determining tolerance to changing salinities in salmonids. Salinity tolerance (capacity to withstand SW) increases after TH treatment in FW coho salmon (Refstie, 1982; Young, Björnsson, Prunet, Lin, & Bern, 1989), Atlantic salmon (Stephen D. McCormick & Saunders, 1990; Saunders, McCormick, Henderson, Eales, & Johnston, 1985), pink (*Oncorhynchus gorbuscha*) and sockeye salmon (Baggerman, 1960), and sockeye salmon transferred from FW to SW have increased gill TRα, TRβ1 and TRβ2 mRNA and increased TH levels (Shin et al., 2014). In Atlantic salmon, T_3 increases the binding affinity of cortisol to gill cortisol receptors, an effect synergistic when co-injected with GH (J. Mark Shrimpton & McCormick, 1998) – indicative of increased SW tolerance. In amago salmon (*Oncorhynchus rhodurus*), T_4 treatment potentiates the action of GH on gill Na^+/K^+-ATPase (NKA, major ion pump) (Miwa & Inui, 1985), while there is a synergistic effect in gill NKA activity in Atlantic salmon (J. Mark Shrimpton & McCormick, 1998) and rainbow trout (J. M. Shrimpton & McCormick, 1999) when co-injected with T_3 and GH.

Atlantic salmon injected with PRL limits cortisol receptor binding affinity and decreases NKA activity, reducing SW tolerance. In coho salmon, PRL alone has no effect on plasma T_3 levels and decreases plasma T_4 levels, and when PRL is co-injected with TSH it prolongs the TSH-induced elevation of TH levels (Leatherland, 1982). In brook trout (*Salvelinus fontinalis*) co-injections of TSH and PRL increase plasma T_3 levels, hepatic T_3 content and 5'-MDA rates compared with TSH-treated animals (Leatherland & Flett, 1988), suggesting an interaction between TSH and PRL.

2.7.2. Evidence in other euryhaline fish

THs have also been shown to affect the osmoregulatory capabilities of other euryhaline species. In Mozambique tilapia, TH injections increase gill NKA activity (Subash Peter, Lock, & Wendelaar Bonga, 2000), potentiate the action of cortisol on gill NKA activity (Dangé, 1986) and increases chloride cell size (a function of ionoregulatory ability) (Subash Peter et al., 2000).

In summer flounder (*Paralichthys dentatus*), which move from high to low salinity ocean water during metamorphosis, SW tolerance increases after TH treatment in individuals undergoing metamorphosis, suggesting that, similar to anadromous salmon, THs regulate the development of osmoregulatory mechanisms necessary for the transition to FW to SW (Schreiber & Specker, 1999). In gilthead sea bream (*Sparus aurata*), exposure to low salinity increases T_4 levels and decreases gill DIO1 activity (Klaren, Guzmán, Reutelingsperger, Mancera, & Flik, 2007), while high salinity decreases T_4 levels and increases pituitary TSHβ and gill NKA activity (Ruiz-Jarabo et al., 2017).

However, in grass carp (*Ctenophayngodon idella*), an increased salinity decreases T_3 and TSH levels, and increases T_4 serum levels (Peyghan, Enayati, & Sabzevarizadeh, 2013).

Marine and euryhaline elasmobranchs in SW regulate urea and other body fluid solutes [trimethylamine oxide (TMAO), Na^+, Cl^-] such that they remain iso- or slightly hyperosmotic to their environment (Hammerschlag, 2006). While little information is available, it seems that the thyroid axis may contribute to elasmobranch osmoregulation. In Atlantic stingray, plasma urea levels and osmotic concentration increase following thyroidectomy and decrease after T_4 replacement therapy, possibly due to the regulation of urea efflux or metabolism (de Vlaming, Sage, & Beitz, 1975). In dogfish, 5'-MDA liver activity increases in the presence of TMAO (protein stabilizer that counteracts urea buildup) and TMAO + urea (Leary et al., 1999), suggesting a role of THs in urea metabolism, as seen in goldfish, for which T_4 increases ammonia production and excretion (William S. Hoar, 1958; Thornburm & Matty, 1963).

2.8. Feeding and nutrient homeostasis

The nutritional energy provided by food intake is essential for activity, growth and maintenance of bodily functions. In fish (Rønnestad et al., 2017) as in mammals (Klockars, Levine, & Olszewski, 2018), food intake is mainly regulated by brain feeding centres controlled by central and peripheral endocrine signals, which either stimulate [orexigenic peptides, such as orexin, agouti-related protein (AgRP) and neuropeptide Y (NPY)] or inhibit [anorexigenic signals, such as cocaine- and amphetamine-regulated transcript (CART) and α-melanocyte-stimulating hormone (α-MSH) derived from

POMC] feeding behaviour. Feeding centres receive information about nutritional status from the periphery [*e.g.,* gastrointestinal tract (GIT)] either via the general circulation or the brainstem/vagal complex. These peripheral signals include ghrelin, cholecystokinin (CCK), peptide YY (PYY) and leptin. Usually, when food intake is restricted, the expression of orexigenic hormones increases while that of anorexigenic hormones decreases (Rønnestad et al., 2017; Hélène Volkoff, 2016)

2.8.1. Role of the thyroid axis in feeding/food intake

In mammals, the thyroid axis regulates food intake, body weight (Amin, Dhillo, & Murphy, 2011) and metabolic/nutrient homeostasis (Kouidhi & Clerget-Froidevaux, 2018). The thyroid axis can influence feeding via the actions of TRH and THs in the brain, THs in the periphery, and also be influenced by endocrine appetite-regulating signals (e.g., NPY, leptin).

In rodents, central administration of TRH or TSH decreases food intake (M. T. Lin, Chu, & Leu, 1983; Vijayan & McCann, 1977) whereas TH injections increase feeding (Ishii et al., 2008; Kong et al., 2004). Conversely, food deprivation decreases hypothalamic TRH and pituitary TSHβ mRNA expression, and peripheral T_3 serum levels (Blake, Eckland, Foster, & Lightman, 1991), while refeeding increases hypothalamic TRH mRNA expression, increases plasma TSH and normalizes circulating T_3 levels (Rondeel et al., 1992).

Interactions between the thyroid axis and appetite-regulating signals have been shown in mammals. In rats, although TRH neurons contain NPY receptors (Toni,

Jackson, & Lechan, 1990), TRH does not stimulate NPY neurons (Xiaobing Zhang & van den Pol, 2012), but goats injected with NPY show a dose-dependent increase in TH levels (Moslemipour, Khazali, & Emami, 2006). TRH neurons excite orexin neurons (Xiaobing Zhang & van den Pol, 2012) and orexin has been reported to either increase (Cote-Vélez et al., 2017) or decrease (Mitsuma et al., 1999) hypothalamic TRH levels. Interestingly, some hypothalamic TRH neurons co-secrete CART but the nature of this interaction is unclear (Anthony N. Hollenberg, 2008). It has been suggested that the anorexigenic actions of TRH are mediated in part by the inhibition of melanin-concentrating hormone (MCH, an orexigenic neuropeptide) (Xiaobing Zhang & van den Pol, 2012), while the orexigenic effect of THs might occur via decreases in the expression of anorexigenic factors such as POMC, CART and MC4R (melanocortin 4 receptor, activated by α-MSH and AgRP to reduce food intake) (Decherf et al., 2010; Kouidhi & Clerget-Froidevaux, 2018; Sternson, Shepherd, & Friedman, 2005), and increases in the expression of appetite stimulators such as NPY (Ishii et al., 2003). Leptin (a adipose satiety signal) increases TRH expression directly by binding to its receptors at TRH neurons (Tartaglia et al., 1995), or indirectly via decreases in AgRP and NPY and increases in α-MSH (which innervate TRH neurons) (Amin et al., 2011; Fekete et al., 2000). There is no clear evidence of a correlation between THs and leptin expression and circulating levels (Kristensen, Pedersen, Langdahl, & Richelsen, 1999; Sreenan, Caro, & Refetoff, 1997).

In fish, interactions between feeding and thyroid status have been shown in several species. In green sunfish (*Lepomis cyanellu*s), high thyroid activity correlates with increased food intake (Gross, Fromm, & Roelofs, 1963), whereas in Amur sturgeon

(*Acipenser schrenckii*), low serum TH levels correlate to low feeding rates (Li, Liu, & Xie, 2012). In climbing perch, exposure to thiourea (TPO inhibitor) decreases food consumption (Pavlov, Zvezdin, & Pavlov, 2019). Reduced food ration in green sunfish (Gross et al., 1963) and long-term starvation in rainbow trout (Milne, Leatherland, & Holub, 1979) decreases the sensitivity of thyroid tissues to TSH, resulting in a decrease in TH levels. In winter flounder, hypothalamic TRH expression increases during fasting (Buckley, MacDonald, Tuziak, & Volkoff, 2010) but decreases in common carp (Huising et al., 2006), and in goldfish, TRH injections increase food intake (Abbott & Volkoff, 2011).

Little is known about interactions between the thyroid axis and appetite regulators in fish. In goldfish, TRH injections increase the brain expression of orexin, orexin receptor and CART (Abbott & Volkoff, 2011). In bighead carp pituitaries, leptin increases TSHα and TSHβ expression (Chowdhury et al., 2004), and in grass carp, leptin and ObRb expression levels increase in hepatocytes incubated with low doses of T_3 (although high doses inhibit expression) (Lu et al., 2015). In fasted burbot, plasma T_4 and TSH correlate with increased plasma leptin levels (Nieminen et al., 2003).

All together, these results suggest that in fish, the thyroid axis plays a role in regulating appetite, and responds to changes in feeding status.

2.8.2. Thyroid hormones, nutrient synthesis and metabolism

Nutrients and how efficiently they are metabolized have been shown to influence and be influenced by the thyroid axis. In mammals, hyperthyroidism is associated with

high metabolism – increased fat breakdown, weight loss, increased liver cholesterol synthesis and clearance and low serum cholesterol – while the opposite occurs in hypothyroidism (Liu & Brent, 2010). For example, in rats, T_3 increases caloric intake and leads to increased lipolysis (by fatty acid β-oxidation) (Oppenheimer, Schwartz, Lane, & Thompson, 1991), while hypothyroid female rats have reduced hepatic mRNA expressions associated with cholesterol uptake and lipid oxidation (Hapon, Varas, Jahn, & Giménez, 2005). Conversely, the quality of nutrients influences the thyroid axis and TH production. Rats fed fish oil diets have higher liver TR expression and increased thyroid signalling associated with lipid metabolism than rats fed soybean oil diets (Souza et al., 2010), and rats fed diets supplemented with *Yucca schidigera* (which contains saponins that decrease GIT nutrient absorption), have lower THs levels than control animals (Kucukkurt & Dundar, 2013).

In fish, THs influence nutrient metabolism of lipids, proteins and carbohydrates (Plisetskaya, Woo, & Murat, 1983) in a species-specific manner. T_4 treatment promotes lipolysis, stimulates lipid mobilization and decreases lipid stores (e.g., as seen by decreased total lipids and increased lipolytic enzyme activity) in coho salmon (Sheridan, 1986), and increases lipid efficiency, plasma cholesterol and triglyceride levels in Sterlet sturgeon (Abdollahpour, Falahatkar, Efatpanah, Meknatkhah, & Van Der Kraak, 2019). Body protein content decreases in European eel (glass stage) treated with THs (Degani & Dosoretz, 1986), and walking catfish exposed to thiourea (Tripathi & Verma, 2003). THs also affect glucose and related carbohydrate metabolism pathways. Following TH treatment, plasma glucose levels increase in red sea bream (Woo, Chung, & Ng,

1991), gilthead sea bream (Vargas-Chacoff et al., 2016), and European eel (Degani & Dosoretz, 1986), but decrease in rainbow trout (Matty & Lone, 1985). TH treatment increases liver gluconeogenic pathways in gilthead sea bream *in vivo* (Vargas-Chacoff et al., 2016), and expression of transcripts associated with glycolytic pathways [i.e., glucokinase (GK), glucose-6-phosphatase (G6Pase), glycogen synthase (GS), and glycogen phosphorylase (GP)] in silver sea bream hepatocytes *in vitro* (Leung & Woo, 2010). However, RNA-seq analysis conducted in liver of tilapia treated with T_3 shows a down-regulation of several pathways related to carbohydrate metabolism (i.e., amino sugars synthesis, galactose and mannose metabolism, tricarboxylic acid cycle) (Olvera et al., 2017).

The quality of the food (i.e., protein, carbohydrate or lipid content) also influences the thyroid axis in fish. For example, low protein diets reduce plasma T_4 levels and/or 5'-MDA activity in rainbow trout (Eales, MacLatchy, Higgs, & Dosanjh, 1992) and brook trout (Higgs, Fagerlund, McBride, & Eales, 1979). Similarly, in Japanese flounder, fish meal-fed fish have higher levels of T_3 than fish fed with fish protein concentrate (FPC) or soy protein concentrate (SPC) (Higgs et al., 1979). Rainbow trout fed a diet with low carbohydrates have low 5'-MDA activity compared to fish fed a carbohydrate-rich diet (Leatherland, Cho, & Hilton, 1984). Under a diet with low salmon oil content, rainbow trout have reduced plasma T_4 and increased plasma T_3 levels, while a high salmon oil diet leads to high plasma T_4 and low T_3 (Leatherland et al., 1984).

2.9. Relevance of the thyroid axis in aquaculture

The basic premise to aquaculture systems is to maximize growth at a minimum cost, producing an aesthetic product with high nutritional value (Higgs, Fagerlund, Eales, & McBride, 1982). The bottlenecks in aquaculture are often the survival of larval and juvenile stages, and successful spawning. Manipulations or disruptions of the thyroid axis could potentially have positive (e.g., increased developmental and reproductive success, hatching and growth rates) or negative (e.g., skeletal deformations, depressed food intake) effects in the aquaculture industry.

2.9.1. THs could be used to enhance early survival and development in fish

THs are important in the development and growth of fish, particularly during early life stages. In aquaculture settings, high mortality rates are seen in early life stages and several species develop skeletal deformities or abnormal pigmentations which might compromise the aspect of the fish and render it improper for sale [e.g., Atlantic salmon (Sadler, Pankhurst, & King, 2001); Atlantic cod (Opstad et al., 2013); flatfish (Yamano, 2005)].

Many studies have reported positive effects of TH treatment in newly fertilized eggs and larvae to enhance hatching, post-embryonic growth and larval survival. For example, immersion in T_4 reduces the hatching period, the number of physical deformities and mortality rate in Asian stinging catfish (*Heteropneustes fossilis*) eggs, (Nayak, Mishra, Mishra, & Pandey, 2004), and induces faster development (i.e., gut formation, swim bladder development, yolk absorption) in freshwater carp (*Catla catla*)

larvae (Nayak, Mahapatra, Mishra, & Mishra, 2000). Similar positive effects have been shown in Pacific threadfin (*Polydactylus sexfilis*) (C. L. Brown & Kim, 1995), spotted gar (Castillo et al., 2015), rainbow trout (Barrington, Barron, & Piggins, 1961), milkfish (*Chanos chanos*) (Lam, Juario, & Banno, 1985), grouper (de Jesus et al., 1998) and chum salmon (Dales & Hoar, 1954), as well as a number of South American fish [e.g., piracanjuba (Landines et al., 2010); matrinxã (Urbinati et al., 2008)*;* dourado (*Salminus maxillosus*) (Parra, 2003)].

However, negative effects of THs have also been reported. T_4 immersion results in reduced hatching, growth rate and yolk content in alligator gar (Castillo et al., 2015), decreased pigmentation in Atlantic salmon (Roche & Leblond, 1952), major abnormalities in Nile tilapia [i.e., abnormal shaped pectoral fins, lordosis and scoliosis (spinal curvature)] (Nacario, 1983) and albinism in Japanese flounder – possibly via inhibition of pigment production or impairment of melanophore development due to precocious metamorphosis (Yoo, Takeuchi, Tagawa, & Seikai, 2000).

Overall, these studies suggest that the effects of TH on eggs and larvae might be dose- and species-dependent.

2.9.2. THs can control and optimize the time of salmonid smoltification

As there are individual variations in growth rates in fish, THs (which are involved in stimulating both growth and smoltification) have been used to accelerate growth and promote the achievement of SW tolerance in several salmonids (Zohar, 1989). TH

treatments could also be useful in inducing promote out-of-season growth and smoltification.

Smoltification is controlled by environmental cues (mainly photoperiod and temperature), which induce changes in the thyroid axis (Prunet, Boeuf, Bolton, & Young, 1989; Wagner, 1974; Zydlewski, Haro, & McCormick, 2005) and only occurs when a threshold weight has been reached (Langdon, 1985). In aquaculture, the period following the transfer of fish from FW to SW is critical, as the performance (including optimal growth rates) of the fish after transfer depends upon a successful parr-smolt transformation (Jørgensen & Jobling, 1994).

A well-timed TH induction of smoltification may be advantageous in species which are released and recaptured [e.g., kokanee salmon (*Oncorhynchus nerka*) (Carr, Whoriskey, & Courtemanche, 2003)] to ensure the return of adult fish to release sites, as fish with the highest whole body T_4 content display increased odor attractions and more accurate homing behaviour compared to fish with low T_4 levels (Tilson, 1994). In Atlantic salmon smolts following transfer to SW, there is a transient suppression of appetite and growth (for up to 30 days) (Jørgensen & Jobling, 1994; Usher, Talbot, & Eddy, 1991), and THs treatment at the right time and the right dose during the parr phase might lessen this inhibition. However, T_4 administration in late Atlantic salmon parr depressed olfactory bulb response to L-alanine (nasal stimulant in salmon) and inhibited 5'-MDA, so timing of induction is critical (Morin, Hara, & Eales, 1995).

2.9.3. THs could enhance reproduction

THs may potentially be used to enhance reproduction in some aquaculture species by enhancing offspring survival and market value [e.g., increase quality of eggs for sturgeon caviar production (Y. Zhang, 2011)]. Higher embryonic/larval survival rates and hatching rates have been shown in fertilized eggs treated with THs [e.g., Pacific threadfin (C. L. Brown & Kim, 1995); Sterlet sturgeon (Alinezhad et al., 2020)] or following maternal TH injections [e.g., greater amberjack (*Seriola dumerili*), Japanese whitling (*Sillago japonica*), red spotted grouper (*Epinephelus akaara*), red sea bream and Japanese parrotfish (*Oplegnathus fasciatus*) (El-Zibdeh, Tachihara, Tsukashima, Tagawa, & Ishimatsu, 1996; Tachihara, El-Zibdeh, Ishimatsu, & Tagawa, 1997); striped bass (C. L. Brown, Doroshov, Cochran, & Bern, 1989)]. In medaka, administration of T_3 prior to spawning increases E2 production and oocyte growth, showing that T_3 administration can enhance final oocyte maturation (Soyano, Saito, Nagae, & Yamauchi, 1993).

The use of THs to enhance reproduction has been successfully used in large scale aquaculture production of some species [e.g., goldstriped amberjack (*Seriola lalandi*) (Tachihara et al., 1997); Korean rockfish (D.-Y. Kang & Chang, 2004)]. In goldstriped amberjack, maternal injections of T_3 reduce mortality during early development and growth, and larval survival increased from less than 1.0% when seed production began in 1985, to 7.3% by 1994 following implementation of T_3 injections (Tachihara et al., 1997).

2.9.4. Thyroid disruption by anthropogenic actions as a threat to aquaculture

2.9.4.1. Pollutants

Thyroid disruption by exposure to environmental toxicants such as metals [e.g., cadmium (Buha et al., 2018)], pesticides [e.g., organophosphorus pesticides (Leemans, Couderq, Demeneix, & Fini, 2019)] and pollutants [e.g., polychlorinated biphenyls, PCBs (Turyk, Anderson, & Persky, 2007)] could result in increased larval mortality and developmental deficiencies (Nugegoda & Kibria, 2017) depending on the aquaculture system and species.

With increasing anthropogenic and industrial activities, heavy metals can become soluble and accumulate to toxic levels, and potentially affect the thyroid axis (Cuesta, Meseguer, & Esteban, 2011). Cadmium decreases TH levels in rainbow trout (Ricard, Daniel, Anderson, & Hontela, 1998), while chromium exposure reduces TH levels in European eel (Teles, Pacheco, & Santos, 2005), and induces thyroid follicle hypertrophy and increases in serum TH levels in spotted snakehead (*Channa punctatus*) (Mishra & Mohanty, 2015). Exposure to mercury decreases circulating TH levels in spotted snakehead (Bhattacharya, Bhattacharya, Ray, & Dey, 1989) and increases the T_4:T_3 ratio – suggesting an inhibition of 5'-MDA activity – in yellowfin sea bream (*Acanthopagrus latus*) (Hedayati, Zare, & Abarghouei, 2012).

Organophosphorus pesticides (OPs) can inhibit growth and development of fish. Dimethoate decreases serum TH levels and increases TSH levels in roho labeo (*Labeo rohita*) (Dey & Saha, 2014), chlorpyrifos decrease serum TH and TSH levels in Asian stinging catfish (Khatun & Mahanta, 2014), and decreases in TH levels inhibits

development of sensory organs (eyes, olfactory organ and lateral line) and decreases survival rates in surgeonfish (Besson et al., 2020). In goldfish, monocrotophos decrease TH levels, and up-regulate pituitary TSHβ and hepatic DIO1 and DIO3 expressions (Xiaona Zhang, Tian, Wang, & Ru, 2013). In Senegalese sole, exposure to malathion affects growth patterns (eye migration, skeletal disorders), reduces thyroid follicle size and induces decreased thyroid signalling (as seen by low TRβ mRNA levels) (Ortiz-Delgado, Funes, & Sarasquete, 2019).

PCB exposure induces higher rates of thyroid metabolism (i.e., deiodination, glucuronidation and sulfation) and lower TH levels in European sea bass (Schnitzler, Klaren, Bouquegneau, & Das, 2012), coho salmon (Leatherland & Sonstegard, 1978) and rainbow trout (Leatherland & Sonstegard, 1980), but not in European flounder (*Platichthys flesus*) (Besselink et al., 1996).

Therefore, while some mechanisms of interaction between environmental toxicants and the thyroid axis are unknown, toxicants can have negative effects on thyroid economy of fish, and could potentially affect growth and production of aquaculture species.

2.9.4.2. Climate changes

Climate change brings about changes in the aquatic environment, such as increases in temperature and acidification, which deeply affect fish physiology (H Volkoff, 2020) and aquaculture practices (Barange et al., 2018), and might have potential effects on the thyroid axis.

Warmer temperatures have been shown to decrease the sensitivity of fish to THs in zebrafish (Little et al., 2013; Little, Loughland, & Seebacher, 2020) and mosquito fish (*Gambusia holbrooki*) (Le Roy & Seebacher, 2020), and in surgeonfish, a 3 °C increase in temperature induces lower TH levels and a disrupted development of sensory organ, an effect that can be reversed by treating the fish with THs (Besson et al., 2020). In addition, thermally challenged fish may produce less viable gametes, with fitness implications that could affect species at the population level (Fenkes, Shiels, Fitzpatrick, & Nudds, 2016). In Japanese medaka, high temperatures decrease the number of spawned eggs, an effect amplified by a reduction in TH levels (by sodium perchlorate exposure) (Lee, Ji, & Choi, 2014). Similarly, seasonal spawners such as goldfish exhibit high TH levels post-spawning in the summer (when water temperatures are the highest) as a way to inhibit pituitary LH and gonadal aromatase (Habibi et al., 2012). While these temperature-mediated effects have not held true for all fish species [e.g., Atlantic cod (Comeau et al., 2000; Cyr, Idler, Audet, McLeese, & Eales, 1998)], an earlier than normal increase in water temperatures as a result of climate change, might disrupt thyroid cycles and inhibit reproductive capabilities in some fish.

The thyroid axis is also sensitive to ambient acidity. For example, exposure to acid water increases T_4 plasma levels in the climbing perch (*Anabas testudineus*) (Subhash Peter & Rejitha, 2011) and brown trout (*Salmo trutta*) (J. A. Brown, Edwards, & Whitehead, 1989), and a decrease in T_3 levels in Atlantic Salmon (S. B. Brown, Evans, Majewski, Sangalang, & Klaverkamp, 1990).

Changes associated with climate may differentially affect specific life-history stages of fish (e.g., species that undergo substantial metamorphic events), which may result in plastic responses that lead to deficiencies later in life. These abiotic changes are poorly understood in the context of the thyroid axis and fish but require attention for future climate scenarios and aquaculture practices.

2.10. Summary and Conclusion

Thyroid hormones have diverse effects and play an important role in the maintenance of a normal physiological state in vertebrates. While similarities exist between fish and other vertebrates exist, fish thyroidal systems present unique features (see Table 1, Figure 1) and functions owing to the diversity in fish anatomies, habitats and life cycles.

The follicular structure of the thyroid is conserved in vertebrates, but most fish have diffuse glands making it more difficult to study. The mechanisms by which fish synthesize and metabolize THs is similar to those in mammals (i.e., THs requires thyroglobulin, iodine and TPO, and DIOs are needed to activate/inactivate THs), but fish might have different isoforms of enzymes which have different properties/actions/locations (e.g., DIO1 is insensitive to PTU and DIOs are located in various tissues), suggesting diverse TH metabolisms.

Evidence suggests that TRH may not be the major TSH-releasing factor at the pituitary in fish, but rather be responsible for the secretion of GH, PRL and ACTH, which in turn might affect TSH. THs appear to exert an inhibitory feedback action on TSH, but

there is no clear evidence for TRH. More advanced molecular techniques (e.g., RNA-sequencing) and *in vivo* studies may help to shed light on the true nature and interactions of TRH in fish.

Existing literature has highlighted the actions of TH in fish via genomic (binding to species specific isoforms of TRs) mechanisms. However, the non-genomic mechanisms by which THs act are poorly understood, as these processes can overlap with genomic actions. As in all vertebrates, T_3 is the main biologically active form of TH, but metabolized THs (e.g., T_2 and Tetrac) previously deemed inactive, are proving to have a role in regulating metabolism (Senese, Cioffi, de Lange, Goglia, & Lanni, 2014).

In fish, THs regulate many aspects of reproduction, including gonad maturation, steroidogenesis and sexual behaviour, and can affect the time of spawning, quality of eggs and fertilization rates and development of eggs/larvae. There are also deep complex interactions between the thyroid axis and growth (e.g., GH, IGF-1) and feeding/appetite (e.g., NPY, POMC) regulators, however, a good knowledge of these interactions is still lacking. A better understanding of the control of THs on reproduction, growth and development, and feeding might provide invaluable insights in aquaculture species/practices and may especially be important to maximize growth while reducing production costs in the ever-growing aquaculture industry.

Any alteration of the thyroid axis by environmental anthropogenic pollutants (effluents containing thyroid disrupting compounds) could have serious physiological and ecological consequences. Understanding specific mechanisms of action of these

pollutants might help to substantiate their potential long-term effects, and help fisheries managers regulate wild populations under threat from these compounds.

Finally, climate change is an additional stress to aquatic ecosystems, affecting both water temperature and shifting carbon dioxide concentrations through direct and indirect effects. Owing to the aquatic habitat of fish, the thyroid axis shows trends in seasonality (Holzer & Laudet, 2015), and is affected by external factors such as temperature, salinity and pH (Little et al., 2013), begging the question on how climate change might alter thyroid signalling.

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Figures

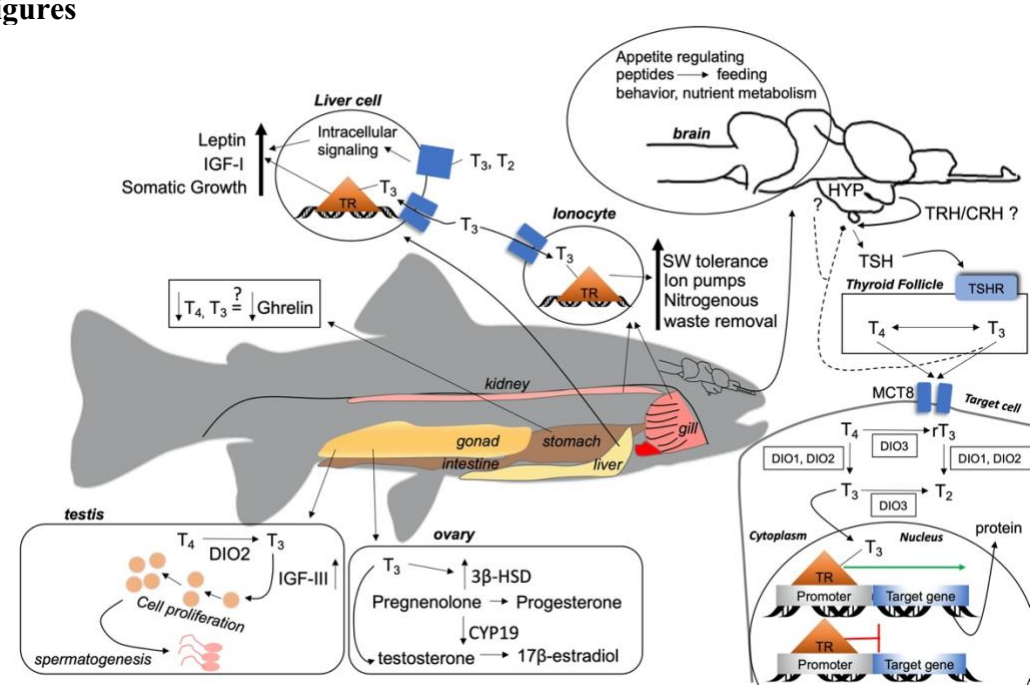

Figure 2.1. A summary of the general actions of THs in fish. A TSH-releasing factor (TRH/CRH) stimulates the anterior pituitary to release TSH, which binds to TSHR on the membrane of thyroid follicles. Intracellular processes produce T4 and T3 that enter the circulation to target cells (solid line) or feedback (dashed line) to the hypothalamus-pituitary axis. THs enter target cells through membrane transporters (e.g., MCT8), where bioactivation of T4 to T3 occurs through DIO1 and DIO2, or further metabolization to rT3 or T2 through DIO1, DIO2 or DIO3. THs enter the target cells nucleus from the cytoplasm and bind to TRs located on promoter regions of a thyroid hormone response element (TRE). When T3 is bound, gene transcription occurs (green arrow), otherwise transcription is repressed (red line). THs may act on various tissues in fish, as shown by general mechanisms in central and peripheral tissues. Question marks indicate evidence of effects of THs, but no known mechanism of action by THs in fish. Arrows that point up indicate that THs increase activity, production or synthesis. Down arrows indicate repression or reduction of synthesis/production. HYP hypothalamus, TRH thyrotropin-releasing hormone, CRH corticotropin-releasing hormone, TSH thyrotropin, TSHR thyrotropin receptor, MCT8 monocarboxylase transporter 8, T4 thyroxine, T3 triiodothyronine, rT3 reverse triiodothyronine, T2 diiodothyronine, DIO1 deiodinase I, DIO2 deiodinase II, DIO3 deiodinase III, TR thyroid receptor, IGF-I insulin-like growth factor I, IGF-III insulin-like growth factor III, 3β-HSD 3β-hydroxysteroid dehydrogenase, CYP19 aromatase

Tables

Table 2.1. Example effects of the thyroid axis on various physiological processes in fish. A (+) denotes the thyroid axis enhancing the physiological process while a (-) denotes a suppression or impairment.

Process		Effects
Egg/larval survival	+	Thyroxine increases egg viability, hatchability and survival [e.g., common carp (363)].
Egg/larval/juvenile development	+	TH immersion or injection increases pigmentation, hatching, growth rate, larval metabolic capacity [e.g., Sterlet sturgeon (125,130); zebrafish (133); goldfish (134)].
	-	Hyperthyroidism leads to arrested development of skeletal structures [e.g., zebrafish (149)].
Juvenile/Adult Development	+	T_4 induces opsin switch in juvenile coho salmon and rainbow trout (183). T_4 promotes intestinal and swim bladder development in freshwater carp larvae (360). T_3 and T_2 promote growth in tilapia (119).
Metamorphosis/Smoltification	+	THs increase olfactory bulb proliferation, body silvering and downstream migration in salmon (196, 197).
	-	Metamorphosis is blocked by THs in sea lamprey (191).
Reproduction	+	T_3 stimulates spermatogenesis in zebrafish by increasing IGF-III (255, 256). T_3 increases progesterone release in female climbing perch (262).
	-	T_3 treatment suppresses terminal nerve GnRH expression in Nile tilapia (Parhar et al., 2000) and administration of T_3 in male goldfish decreases pituitary LH mRNA expression (Nelson et al., 2010).
Osmoregulation	+	T_3 injections increase gill ion pump activity in Mozambique tilapia (296) and T_4 immersion increases salinity tolerance in summer flounder (Schreiber & Specker, 1999).
Feeding/food conversion	+	TRH injections increase food intake in goldfish (336). T_4 increases food, protein and lipid efficiency in Sterlet sturgeon (345).

- T_3 decreases body protein in European eel (346) and decreases plasma glucose in rainbow trout (350).

Chapter 3. Response of the Thyroid Axis and Appetite-Regulating Peptides to Fasting and Overfeeding in Goldfish (*Carassius auratus*)

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Abstract

The thyroid axis is a major regulator of metabolism and energy homeostasis in vertebrates. There is conclusive evidence in mammals for the involvement of the thyroid axis in the regulation of food intake, but in fish, this link is unclear. In order to assess the effects of nutritional status on the thyroid axis in goldfish, *Carassius auratus*, we examined brain and peripheral transcripts of genes associated with the thyroid axis [thyrotropin-releasing hormone (TRH), thyrotropin-releasing hormone receptors (TRH-R type 1 and 2), thyroid stimulating hormone beta (TSHβ), deiodinase enzymes (DIO2, DIO3) and UDP-glucuronosyltransferase (UGT)] and appetite regulators [neuropeptide Y (NPY), proopiomelanocortin (POMC), agouti-related peptide (AgRP) and cholecystokinin (CCK)] in fasted and overfed fish for 7 and 14 day periods. We show that the thyroid axis responds to overfeeding, with an increase of brain TRH and TSHβ mRNA expression after 14 days, suggesting that overfeeding might activate the thyroid axis. In fasted fish, hepatic DIO3 and UGT transcripts were downregulated from 7 to 14 days, suggesting a time-dependent inhibition of thyroid hormone degradation pathways. Nutritional status had no effect on circulating levels of thyroid hormone. Central appetite-regulating peptides exhibited temporal changes in mRNA expression, with decreased expression of the appetite-inhibiting peptide POMC from 7 to 14 days for both fasted and overfed fish, with no change in central NPY or AgRP, or intestinal CCK transcript expression. Compared to control fish, fasting increased AgRP mRNA expression at both 7 and 14 days, and POMC expression was higher than controls only at 7 days. Our results indicate that nutritional status time-dependently affects the thyroid axis and appetite

regulators, although no clear correlation between thyroid physiology and appetite regulators could be established. Our study helps to fill a knowledge gap in current fish endocrinological research on the effects of energy balance on thyroid metabolism and function.

3.1. Introduction

In mammals, nutrient excess or deficiency can lead to imbalances in metabolic status, internal energy reserves (e.g., adipose tissue and glycogen) as well as energy-controlling endocrine systems (Alberda, Graf, & McCargar, 2006; Muttarak, 2019). Signalling pathways in the central nervous system (CNS) respond to the presence or absence of food, and act to regulate energy intake and expenditure (Yousefvand & Hamidi, 2020). Peripheral endocrine factors [e.g., leptin, ghrelin, cholecystokinin (CCK)] communicate information related to nutritional status to the brain, in particular the hypothalamus [via receptors on the vagus nerve or by crossing the blood-brain barrier (BBB) and binding to central receptors] (López, Tovar, Vázquez, Williams, & Diéguez, 2007). The brain responds by producing central factors that are either orexigenic (appetite stimulating) [e.g., neuropeptide Y (NPY) and agouti-related peptide (AgRP)] or anorexigenic (appetite inhibiting) [e.g., proopiomelanocortin (POMC), the precursor of α-melanocyte stimulating hormone (α-MSH)] (Singhal & Ahima, 2008).

The hypothalamus-pituitary-thyroid (HPT) axis (hereafter referred to as the thyroid axis) has a key role in the regulation of energy homeostasis as it increases basal metabolic rate (B. Kim, 2008), weight loss (Reinehr, 2010) and cardiac output (Klemperer et al., 1995). In mammals, thyrotropin-releasing hormone (TRH) is synthesized in the hypothalamus and stimulates the anterior pituitary to release thyroid stimulating hormone (TSH). TSH binds to thyroid receptors and induces the release of thyroid hormones (THs), i.e., the prohormone thyroxine (T_4) and the biologically active triiodothyronine (T_3). Conversion of T_4 into the active T_3 occurs through two types of

deiodinase (DIO) enzymes (DIO1 and DIO2) in target tissues, selectively removing iodine from T_4 to produce T_3. T_3 can further be metabolized by deiodinase type three (DIO3) by iodine removal, as well as conjugation with glucuronic acid by UDP-glucuronosyltransferase (UGT) to become inactive and excreted by the body through the bile and intestine (Amin, Dhillo, & Murphy, 2011; Boelen, Wiersinga, & Fliers, 2008). The thyroid axis functions as a typical feedback loop. Both T_4 and T_3 provide negative feedback by decreasing synthesis of TRH and/or TSH from the hypothalamus and pituitary, and blocking the action of TRH on TSH release (Shupnik, Chin, Habener, & Ridgway, 1985; Sugrue, Vella, Morales, Lopez, & Hollenberg, 2010). These feedback loops regulate TH levels within a narrow physiological 'set range' (termed the homeostatic set point) and are affected by internal signals that provide information on energy status (Lechan & Fekete, 2006; López, Alvarez, Nogueiras, & Diéguez, 2013).

There is growing evidence in mammals that the thyroid axis regulates feeding and responds to changes in nutritional status (Amin et al., 2011; Boelen et al., 2008), as interactions between the thyroid axis and appetite-related factors have been shown to occur. In the hypothalamus, α-MSH and AgRP neurons project to TRH neurons which express MSH and AgRP (melanocortin) receptors to control activation or inhibition of the central thyroid axis (Kishi et al., 2003). In rats, administration of AgRP in *ad libitum* fed rats reduces TRH hypothalamic expression (Fekete et al., 2002), and α-MSH administration inhibits fasting-induced decreases in hypothalamic TRH transcript levels (Sarkar, Légrádi, & Lechan, 2002). In rats, NPY promotes positive energy balance in part by suppressing α-MSH and TRH (Cyr et al., 2013), and CCK stimulates thyroid follicular

cells and modulates TSH pituitary secretion (Ginda, 2001). It is shown in mammals that THs may regulate the expression of AgRP and NPY by activation/inactivation of AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) – common central nutrient sensors (López et al., 2010). In fish, AMPK and mTOR activation is also related to nutrient sensing (Comesaña et al., 2018), however, there are very few published studies examining the association between these appetite-regulating peptides and the thyroid axis (Abbott & Volkoff, 2011; Deal & Volkoff, 2020).

In fish, the mechanisms of action involved in the regulation of energy homeostasis in the presence or absence of food by the thyroid axis are unclear, largely because there are major differences in how mammals and fish regulate their thyroid axis (Deal & Volkoff, 2020). Whereas in mammals, TRH is the major TSH releasing factor, its actions on TSH release in fish are controversial (MacKenzie, Jones, & Miller, 2009). Moreover, in mammals, the thyroid gland is an encapsulated organ, whereas in fish, it is usually diffusely arranged throughout the body in the pharyngeal, kidney and heart regions (Gudernatsch, 1911). As well, DIOs in fish may catalyze THs differently than in mammals. For example, DIO1 is resistant to propylthiouracil [PTU, which inhibits TH synthesis in follicles of mammals (Nagasaka & Hidaka, 1976) and fish (Schmidt, & Braunbeck, 2011)], and, as opposed to mammals in which DIO2 is distributed in the central nervous system, heart, skeletal muscle, and appears absent from the liver (Salvatore, Bartha, Harney, & Larsen, 1996; St. Germain, Galton, & Hernandez, 2009), fish DIO2 is expressed largely in hepatic tissues (Orozco & Valverde-R, 2005). In addition, whereas in mammals, the thyroid axis adapts in response to increased energy

expenditure such as during pubertal growth spurts (Fleury, Van Melle, Woringer, Gaillard, & Portmann, 2001), increases in body weight (Reinehr, Isa, de Sousa, Dieffenbach, & Andler, 2008) and to maintain a constant body temperature (Danforth & Burger, 1984), fish are usually indeterminate growers (i.e., growth continues past maturation) and do not require metabolic energy to thermoregulate [but instead rely on behavioural thermoregulation (Schurmann, Steffebsen, & Lomholt, 1991)]. This suggests that fish may require a constant adjustment of their thyroidal set-point, which may be an evolutionarily advantage as a method to increase metabolic and locomotor activity (Little & Seebacher, 2014) and to reduce costs associated with bouts of feeding in unfavorable conditions.

In this study, we used goldfish (*Carassius auratus*) as a model to assess how varying food rations – fasting, satiation and overfeeding – influence the thyroid axis, and whether appetite-regulating peptides respond in concert to regulate energy balance. Goldfish (Cypriniformes, Cyprinidae) have long been used as models in neuroendocrinology, as they are tolerant to stress and allow for the accurate quantification of food intake (Blanco, Sundarrajan, Bertucci, & Unniappan, 2018; Volkoff, 2019). We analyzed transcript levels of thyroid axis components (brain: TRH; pituitary: TRH receptor (TRH-R) type 1 and 2, TSHβ; liver: DIO2, DIO3, UGT) and measured circulating serum total thyroid hormones (T_4 and T_3). In addition, we analyzed mRNA expressions of hypothalamic, telencephalic and intestinal appetite-regulating peptides (POMC, NPY, AgRP, CCK) that are known to regulate feeding and energy homeostasis in fish (Rønnestad et al., 2017), and might interact with the thyroid axis to

balance energy levels under different nutritional statuses (López et al., 2013). Furthermore, to understand temporal changes associated with the thyroid axis and appetite, we submitted goldfish to these different rations for 7- and 14-day periods to determine how energy homeostasis might change over time. We hypothesized that an increased food ration would lead to stimulation of the thyroid axis, indicated by upregulated transcript expression and hormone levels, while fish deprived of food (fasted) would have a suppressed thyroid axis (decreased transcript expression and hormone levels). We also hypothesized that orexigenic peptides would have increased transcript expression levels during fasting while the transcript expression levels of anorexigenic peptides would increase during overfeeding. Our study helps to fill a knowledge gap in current fish endocrine research on the effects of energy balance on thyroid metabolism and function.

3.2. Materials & Methods

3.2.1. Animals

Goldfish (n = 60, average body weight 15.64 ± 4.09 g and fork length 78.8 ± 7.28 mm) were acquired from Ozark Fisheries (Martinsville, IN, USA). Fish were acclimated for one week under a 16 h light: 8 h dark photoperiod at 20 ºC, being fed a 2 % wet body weight ration [number of fish x average weight (g) x 0.02] of 2 mm sinking pellets (35 % crude protein, 10 % crude fat, 3% crude fibre, 8.5% moisture, 8% ash; Omega Sea, Sitka, AK, USA) once a day (10:00). Both male and female fish were used. Fish were gonadal recrudescent, with gonadosomatic index (GSI) [GSI = weight of gonads (g) / total body

weight (g) x 100] of 1.93 ± 0.27 % for males and 2.77 ± 0.91 % for females (Peng, Trudeau, & Peter, 1993; Razani, Hanyu, & Aida, 1988)].

Goldfish were housed in 6, 65-liter stock tanks (10 fish per tank) and, upon the start of the experiment, each subjected to different food rations. Two tanks underwent fasting and received no daily food, two tanks were fed a normal ration to satiation (2 % wet body weight, control), while the last two tanks were overfed (4 % wet body weight). The satiation ration of 2 % (defined as the moment fish stop searching for and consuming pellets) has been determined by previous studies using goldfish (Feliciano et al., 2011; Mandic & Volkoff, 2018; Volkoff, 2013), and doubled to represent an overfed ration. Food intake per group was calculated as food consumed per average body weight per hour (mg food/g average BW/60 min), based on the weight of pellets consumed (average 3 mg each) at one hour after the feeding period, and converted to percentage relative to the control group (100 %). Nitrates and pH were measured daily to ensure overfeeding did not lead to water toxification (i.e., ammonia and nitrite production). After 7 and 14 days under these feeding conditions, fish were fed their rations at the standardized feeding time (10:00) and one hour later, 5 fish from each tank (2 tanks per treatment, 6 tanks total) were immersed in 0.3 mg/L tricaine methanesulfonate (MS222) (Syndel Laboratories, Vancouver, BC, Canada), killed by spinal section, weighed and measured, and sampled for serum and tissues.

All experiments followed animal care protocols approved by Memorial University of Newfoundland Animal Care Committee following the guidelines of the Canadian Council on Animal Care guide to the care and use of experimental animals.

3.2.2. Intraperitoneal injections of thyroxine and saline

Thyroxine (T_4) was purchased from Sigma Aldrich (St. Louis, MO, USA) and injected into fish for enzyme-linked immunosorbent assay (ELISA) physiological validation. Fish physiological saline (0.11 M NaCl, 2.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM $NaHCO_3$, 0.5 mM NaH_2PO_4) injections were also performed as a control to establish basal TH levels. A T_4 injection solution was prepared as a 5 μg/μL stock solution in 0.01 M NaOH and frozen at -20 °C. The 5 μg/μL T_4 stock was subsequently diluted in freshwater fish physiological to a dose of 10 ng of T_4 per g of fish [concentrations based on (Goodyear, 2012)]. Solutions were injected intraperitoneally (midline, slightly posterior to the pelvic fins) using a 250 μL Hamilton syringe (Hamilton Company, Reno, NV, USA) with a 27-gauge needle (Becton Dickinson and Company, Franklin Lakes, NJ, USA), as described in previous studies [e.g., (Goodyear, 2012; Volkoff, 2013)]. On the day of injections, fish were fed at the standard feeding time (10:00) to satiation. Eight fish were randomly netted from a stock tank, lightly anesthetized in MS222 and weighed to determine concentration of the injected dose. Fish were then injected with 100 μl of saline injections (n = 4) or 100 μl of T_4 (10 ng/g, n = 4). Following injection, fish were returned to observation tanks and sampled for blood one-hour post-injection as detailed below.

3.2.3. Serum and Tissue Collection

Blood was collected from anesthetized fish from the caudal peduncle with 27-gauge syringed needles, let clot for three hours at room temperature and centrifuged at

5000 rpm for 15 min. Serum was collected and stored at -80 °C until hormone analysis. Whole brain, pituitary, liver and proximal intestine tissues were collected and stored in RNAlater (Qiagen, Mississauga, ON, Canada) at -20 °C until RNA extractions were performed. Hypothalami and telencephalons were dissected from whole brains at time of RNA isolation. Given the small sample size of our experiment, we attempted to reduce our type 2 error rate by randomly allocating fish to duplicate tanks and randomly sampling tanks to minimize any bias.

3.2.4. RNA extraction and cDNA synthesis

RNA extractions were conducted using a GeneJET™ RNA Purification Kit (Fermentas, Burlington, ON, Canada) following the manufacturer's protocol. Final RNA concentrations were determined by optical density at 260 nm using a NanoDrop ND-2000 (NanoDrop Technologies Inc., Wilmington, DE, USA). Quality of RNA was assessed by measuring the ratio of the sample tissue at 260 and 280 nm, only samples with a ratio between 1.8 and 2.1 were used in subsequent quantification.

Total RNA was then reverse transcribed to cDNA using a SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK). 500 ng of total RNA was mixed on ice with 4 μL 5x TransAmp Buffer, 1 μL Reverse Transcriptase and RNase-free water for a reaction volume of 20 μL. In a Bio-Rad C-1000 Touch Thermal Cycler, the following program was set: 25 °C for 10 min (annealing), 42 °C for 30 min (reverse transcription) and 85 °C for 5 min (inactivation). The cDNA product was diluted 1:5 with RNase-free water and

frozen at -20 °C until quantitative polymerase chain reaction (qPCR) analyses were performed.

3.2.5. Quantitative polymerase chain reaction (qPCR)

To initially validate transcripts of interest, three sets of specific primer pairs (forward and reverse) for TRH, TRH-R type 1 and 2, TSHβ, DIO2, DIO3, UGT, NPY, AgRP, POMC and CCK were designed using Primer 3 software (https://primer3.org/), based on coding regions for the gene of interest (see Table 1 for sequences and accession numbers) and synthesized by Integrated DNA technologies (IDT, Coralville, IA, USA). Primer design ensured the spanning of an exon-exon junction and product size range of 150-250 base pairs (AgRP was designed with a range of 100-300 base pairs after initial optimization tests showed high cycle values, i.e., Ct > 32). To optimize primers, each primer pair was run with serial diluted cDNA samples (1:2, 1:4, 1:8, 1:16) and a no template control (water) in triplicate using a Bio-Rad CFX96 Real-Time System on a C1000 Touch Thermal Cycler (Bio-Rad, Mississauga, ON, Canada) to determine the primer pair with highest efficiency and correlation followed by melt-curve analysis. If primer pairs had efficiencies close to 100 % (0.97 for TRH, 0.98 for TRHR type 1 and 0.93 for TRHR type 2, 1.0 for TSHβ, 0.98 for DIO2, 0.91 for DIO3, 1.0 for UGT, 0.98 for NPY, 0.97 for POMC, 1.1 for CCK, 0.97 for AgRP, 0.98 for EF1α, 0.98 for β-Actin) and adequate correlations ($0.9 < R^2 < 1.0$), the primer sets were deemed adequate. Specificity of primer pairs was determined by a melt curve analysis, and all amplicons

gave way to a single peak. There was no amplification of the no template control (water instead of cDNA).

To determine the most stable reference gene for expression analysis, sampled tissues (brain, liver and intestine) from each group (fasted, satiated and overfed rations) were amplified with three housekeeping genes (elongation factor 1α, EF1α; β-actin; ribosomal 18S) and their inter-sample stability – characterized by the lowest cycle threshold variation – determined using the Normfinder software (Andersen, Jensen, & Ørntoft, 2004) in RStudio (V 1.2.5001, RStudio Team, Boston, MA). For central tissues (hypothalamus, telencephalon and pituitary) β-actin was the most stable gene and EF1α had the highest stability in peripheral tissues (intestine and liver).

Following primer optimization and selection of a reference gene, relative expression analyses were carried out for genes of interest. Relative mRNA expression quantification was carried out in 96-well plates, using a Bio-Rad CFX96 Real Time System on a C1000 Touch Thermal Cycler with the following program set: 95 ºC for 2 min followed by 40 cycles of 95 ºC for 5 s, 60 ºC for 10 s and 72 ºC for 20 s. All cDNA samples were run in duplicate, including a no template control (water instead of cDNA). Expression levels were compared using the relative Ct (ΔΔCT) method using the CFX Maestro Software (Bio-Rad). The average Ct of the reference gene (β-Actin or EF1-α, depending on the tissue) was subtracted from the average Ct of the transcript of interest to determine the ΔCT for each sample. The ΔCT of the calibrator (tissue of a satiation ration fish) was then subtracted from the ΔCT of each of the samples to determine the ΔΔCT. This number was then used to determine the amount of mRNA relative to the

calibrator and normalized by β-Actin or EF1-α. The relative mRNA expression of control groups (2 % ration) was set at 100 % and other ration levels were displayed relative to the control group by the formula: [(100 x mean Ct of each ration level) / average Ct of 2 % ration fish].

3.2.6. Total thyroxine and total triiodothyronine ELISA

Total T_4 and T_3 ELISA kits were purchased from Monobind Inc. (Lake Forest, CA, USA) and run following the manufacturer's protocol. Serum samples were validated with the ELISA kits by running serial dilutions of serum samples adjacent to standards – a parallel relationship between samples and standards ensured kit validation. Samples were run in duplicate and read at 450 nm using a Biotek Synergy Mx Fluroescence plate reader (BioTek Instruments Inc., Winooski, VT, USA). A positive ELISA control consisted of serum from thyroxine injected fish (low background), and ultrapure analytical grade distilled water (high background) was used as a negative ELISA control.

3.2.7. Statistics

All statistical tests and graphing were done in GraphPad Prism 8 (version 8.4.3), with significance set at $p < 0.05$. All data are expressed as mean ± standard error of the mean (SEM). All data were tested for normality using the Kolmogorov-Smirnov normality test. If mRNA expression or hormone concentration data failed to pass normality, it was logarithmically transformed prior to analysis. Parametric data was then analyzed by two-way analysis of variance (ANOVA) models comparing interactions between the factors “time” (7 and 14 days) and “food ration” (fasted, satiated, overfed)

on transcript expression data of thyroid axis components (TRH, TSHβ, DIO2, DIO3 and UGT) and appetite regulators (NPY, AgRP, POMC and CCK), followed by Tukey's multiple comparison test for pairwise comparison within and between groups. If a significant interaction was seen between factors, then only interactive effects were considered regardless of significant main effects. In some instances, for mRNA expression data, unpaired t-tests were carried out to analyze significance between two groups. As BW and food intake data failed to meet normality after being logarithmically transformed, multiple t-tests between time points and groups were done, correcting for multiple comparisons using the Mann-Whitney method. As the Mann-Whitney method only accounted for differences between groups, non-parametric Kruskal-Wallis tests were carried out to test differences within groups.

3.3. Results

3.3.1. Food Intake

There were no significant differences in food intake between 7 and 14 days for either satiated or overfed fish. Overfed fish had significantly higher food intake levels than controls at both time periods (Mann-Whitney t-test) (Suppl. Fig. A1).

3.3.2. Morphometrics

Within groups, the BW of fasted fish decreased significantly from T0 to 14 days. The BW of satiated fish significantly increased between T0 and 7 days, with overfed fish

increasing their weight from the start (T0) to the end (14 days) of the experiment (Kruskal-Wallis t-test) (Fig. 3.1).

There were no significant differences in BW between groups at T0. BW for fasted fish was lower than both control and overfed fish at 7 and 14 days. Overfed fish had similar BW's than control fish at both 7 and 14 days (Mann-Whitney t-test) (Fig. 3.1).

3.3.3. Effects of food ration on the expressions of TRH, TRH receptors and TSHβ

Overall variations in hypothalamic TRH transcripts were explained by the interactive effect of food ration and experimental time ($F_{2,40} = 9.284$, $p = 0.0005$) (Fig. 2A). At 14 days, overfed fish had significantly higher TRH levels than overfed fish at 7 days and control and fasted fish at both 7 and 14 days.

In the telencephalon, there were interactions between expression TRH and food ration and time ($F_{1,39} = 4.491$, $p = 0.0405$) (Fig. 3.2B). There were no significant differences in expression between groups at either 7 or 14 days. Within groups, overfed fish had higher TRH expression at 14 days compared to 7 days.

Pituitary TRH-R type 1 expression was influenced by differences in food ration ($F_{2,38} = 12.45$, $p < 0.0001$) (Fig. 3.2C). Fasted fish had significantly higher TRH-R type 1 mRNA expression compared to controls at 7 and 14 days and overfed fish at 14 days. TRH-R type 2 expression was influenced by neither food ration nor experimental period (Fig. 3.2D).

There was an interaction between food ration and time and pituitary TSHβ expression ($F_{2,42} = 5.741$, $p = 0.0063$) (Fig. 3.2E). At 7 days, there were no significant

differences in TSHβ expression between feeding groups. At 14 days, overfed fish had higher expression than controls at 14 days, and all feeding groups at day 7. Fasted fish at 14 days had higher TSHβ expression than fish overfed for 7 days.

3.3.4. Effects of food ration on circulating serum levels of thyroid hormones

Circulating total thyroid hormone levels (total triiodothyronine, tT_3; total thyroxine, tT_4) and tT_3/tT_4 ratios were not affected by either the amount of food consumed or time and showed no pairwise differences between or within groups (Fig. 3.3A-C).

T_4-injected fish had higher serum levels of tT_4 (42.20 ± 3.92 ng/mL) and tT_3 (6.25 ± 1.21 ng/mL), and a lower tT_3/tT_4 ratio (0.146 ± 0.03) compared to saline injected fish [tT_4 (0.93 ± 0.17 ng/mL); tT_3 (2.02 ± 0.34 ng/mL; tT_3/tT_4 ratio (2.40 ± 0.62) (unpaired t-test)]. Interassay coefficients of variation (CV) of the ELISA were 13.1 % and 7.30 %, while intraassay CVs were 1.99 % and 5.02 % for tT_4 and tT_3. There was no gender-specific difference in tT_4 or tT_3 levels (data not shown).

3.3.5. Effects of food ration on the brain and hepatic expression levels of deiodinases and UGT

In the liver, DIO2 transcript levels showed no significant interaction between food ration and time, and no significant pairwise differences (Fig. 3.4A).

Hepatic DIO3 transcript levels showed a significant interaction between food ration and time ($F_{2,35} = 3.772$, $p = 0.0318$) (Fig. 3.4B). DIO3 expression in fasted fish at

14 days was lower than that of both fasted and overfed fish at 7 days. There were no significant variations between experimental groups at either 7 or 14 days.

Expression of liver UGT showed a significant interaction between food ration and time ($F_{1,42} = 9.941$, $p = 0.0030$) (Fig. 3.4C). In pairwise comparisons, fasted fish at 7 days had significantly higher expression levels of UGT than both fasted and satiated fish at 14 days. There were no significant variations between experimental groups at either 7 or 14 days.

In the brain, hypothalamic DIO2 expression showed no interaction between food ration and time (two-way ANOVA) (Fig. 3.4D). Hypothalamic DIO2 expression decreased significantly at 14 days in overfed fish compared to controls (unpaired t-test).

3.3.6. Effects of food ration on the expression of appetite regulators

AgRP and POMC expression were assessed only in the hypothalamus, as their expression levels in the telencephalon were too low to confidently analyze mRNA expression changes (Ct > 32 cycles). NPY expression was assessed in both hypothalamus and telencephalon.

There was a strong interaction between food ration and time in POMC transcripts in the hypothalamus ($F_{2,37} = 7.203$, $p = 0.0023$) (Fig. 3.5A). Between groups at 7 days, fasted fish displayed higher POMC expression relative to controls, whereas there no significant differences between food ration groups at 14 days. Fasting or overfeeding for 14 days resulted in decreased expression of POMC compared to 7 days for both groups.

Differences in food ration resulted in significant variations in AgRP transcript expression in the hypothalamus ($F_{2,40} = 6.747$, $p = 0.0030$) (Fig. 3.5B). At both 7 and 14 days, AgRP expression was higher in fasted fish than in controls (unpaired t-test). Fish fasted for 14 days had increased AgRP expression relative to controls at 7 days.

Food ration and experimental time periods had no significant effect on either hypothalamic or telencephalic NPY expression levels (Fig. 3.5C-D).

Intestinal transcript expression of CCK showed a significant response to changing food ration ($F_{2,42} = 4.444$, $p = 0.0178$). CCK expression in overfed fish at 7 days was lower compared to both fasted and satiated fish at 14 days (Fig. 3.5E). There were no significant differences in CCK expressions between ration group at either 7 or 14 days.

3.4. Discussion

Our results show that both fasting and overfeeding leads to time-dependent differential thyroid axis regulation and metabolism. Fasted fish displayed a decrease in hepatic DIO3 and UGT, suggesting a decrease in peripheral degradation of THs, whereas overfeeding increased TRH and TSH expressions, suggesting an activation of the thyroid axis under abundant food conditions by possible reduced action of THs at the hypothalamus or inhibition of a satiation signal. The expression of appetite-regulating peptides was affected little by nutritional status, with the exception of an increase in the orexigenic AgRP in fasted fish, and time-dependent decreases of the anorexigenic POMC in both fasted and overfed fish (Table 2). We acknowledge that the lack of significant differences in some of our data might have been due in part to the relatively small sample

sizes, which might have resulted in low power to detect small, variations (Krzywinski & Altman, 2013). However, given the tightly controlled nature of our experiment, it is likely that large variations in our data can be attributed to inter-individual variations, and not purely associated with the low power of the study.

3.4.1. Effects of fasting and overfeeding on body weight

In our experiment, BW tended to decrease in fasted fish and to increase in satiated and overfed fish.

Similar changes/trends have previously been shown in other fish, including goldfish (Mennigen, Sassine, Trudeau, & Moon, 2010; Tinoco, Nisembaum, Isorna, Delgado, & de Pedro, 2012) and zebrafish (Ghaddar et al., 2020; Jia et al., 2019; Montalbano et al., 2019). The small changes seen in our study would likely have been more pronounced had the fasting period been longer [e.g., 28 days in (Mennigen et al., 2010)], or overfed fish fed a higher ration [e.g., 6 % (Tinoco et al., 2012)]. However, at 14 days, fasted fish had significantly lower BW than both satiated and overfed fish, which is not surprising, as fasting may have induced mobilization of energy reserves [in particular lipids, as seen in rainbow trout (*Oncorhynchus mykiss*), in which liver protein content is lower in fish after four weeks of food deprivation (Farbridge & Leatherland, 1992), zebrafish, for which hepatic triglyceride (TG) content decreases after 3 weeks of fasting (Jia et al., 2019) and in European sea bass (*Dicentrarchus labrax*) for which 15 days of fasting activates fat breakdown in liver, muscle and adipose tissue (Rimoldi, Benedito-Palos, Terova, & Pérez-Sánchez, 2016)]. Similarly, satiated (at 7 days) and

overfed (at 14 days) fish showed increases in BW compared to T0, possibly due to increased fat deposition, as seen in overfed zebrafish (*Danio rerio*) (Landgraf et al., 2017) and rainbow trout (Roh et al., 2020).

3.4.2. The thyroid axis responds to different food rations

3.4.2.1. Central thyroid axis transcripts

Our findings show a time- and ration-dependent regulation of the thyroid axis, as seen by changes in TRH, TRH receptors and TSH expressions at the central level.

In our study, TRH expression levels were not affected by fasting. Previous studies in goldfish have shown that fasting for 3, but not 10 days, increases hypothalamic TRH expression, and intracerebroventricular (ICV) injections of TRH increases food intake (Abbott & Volkoff, 2011), suggesting that TRH acts as an orexigenic factor in this species. In contrast, in common carp (*Cyprinus carpio*), fasting for 6 weeks decreases hypothalamic TRH expression, and re-feeding to satiation for 6 weeks restores these levels to basal values (Huising et al., 2006). Similar to carp, rats fasted for 3 days show suppressed hypothalamic TRH expression (Légrádi, Emerson, Ahima, Flier, & Lechan, 1997), and fasting results in decreased TRH release into circulation compared to control (fed) or refed rats (Rondeel et al., 1992). The lack of decrease in TRH expression in the hypothalamus or telencephalon of fasted fish compared to satiated fish in our experiment could be a long-term energy saving mechanism. It has been suggested that a short-term increase (after 3 days) in TRH expression might induce locomotor/searching behaviour (Abbott & Volkoff, 2011), followed by a return to basal levels (after 10 days) to promote

energy saving if fasting is prolonged, likely a consequence of a "shut-down" of the thyroid axis. Similar TRH expressions between fasted and control fish at 7 and 14 days in our study are consistent with this hypothesis.

Although it is not clear why a decrease in TRH expression was not observed in fasted fish, it is possible that the maintenance of basal TRH levels might be needed to maintain a minimal neural activity (i.e., central nervous system activity), and reducing TRH expression past a certain level would lead an unnecessary metabolic depression [as seen following a 3-month estivation period in African lungfish (*Protopterus annectens*), where TRH is depressed is the diencephalon region of the brain (Kreider, Winokur, Pack, & Fishman, 1990)].

The time-dependent increase in TRH expression in both the hypothalamus and telencephalon seen in overfed fish is consistent with previous studies in mammals. Early-life overfed rats have 30 % higher TRH mRNA expression levels than fasted individuals (de Gortari, Alcántara-Alonso, Matamoros-Trejo, Amaya, & Alvarez-Salas, 2020), and long-term overfeeding (100 days) in humans increases resting metabolic rate and the response of TSH to TRH (Oppert et al., 1994). To our knowledge, there is no published data on the effects of overfeeding on TRH levels in fish. In goldfish, TRH ICV injections increase locomotor behaviour (Abbott & Volkoff, 2011), so, the increased TRH expression seen in our study may occur to increase metabolism, locomotor activity and energy expenditure in times of high food abundance.

Very few studies have examined TRH receptors in fish. Two TRH receptor subtypes have been cloned in goldfish, and seen in other species such as white sucker

(*Catostomus commersoni*) (Harder et al., 2001), whereas four types have been identified in sockeye salmon (Saito et al., 2011) and Japanese medaka (*Oryzias latipes*) (Mekuchi et al., 2011), and all show species and form-specific binding affinity and tissue expression. In goldfish, (Burt & Ajah, 1984) and rainbow trout (Schwartzentruber & Omeljaniuk, 1995), high affinity TRH binding sites are present in the pituitary, while African lungfish show a weak concentration of pituitary TRH-Rs (Pack, Caine, Winokur, Manaker, & Fishman, 1989).

Our results show that fasting up-regulates pituitary TRH-R type 1 expression after 7 days and a similar but non-significant decrease was observed at 14 days for TRH-R type 2 expression. However, overfeeding did not affect TRH-R expression. It is unclear whether functional differences occur in fish between the two TRH receptor types. In mammals, both TRH-R1 and TRH-R2 exhibit similar affinities for TRH, but TRH-R2 may show more TRH-independent signalling activity compared to type 1 (Sun, Lu, & Gershengorn, 2003; Wang & Gershengorn, 1999). In our study, the reason for an increase in TRH-R type 1 during fasting is unclear, as the expression of these receptors have never been analyzed as a function of nutritional status. It may be that at the pituitary level, the increased expression of TRH-R type-1 may sensitize the pituitary to growth hormone (GH), as TRH is known to induce GH release [common carp (X. W. Lin, Lin, & Peter, 1993)] and fasting induces an increase in GH levels [Chinook salmon (*Oncorhynchus tshawytscha*) (Pierce, Shimizu, Beckman, Baker, & Dickhoff, 2005)]. Further work is required to determine differences between TRH-R type 1 and 2 in fish, and the extent (if any) of their thyrotropic role.

Pituitary TSHβ expression was not affected by fasting but was higher in overfed fish than controls after 14 days, mirrored by elevated TRH expression, suggesting a possible TRH-dependent TSH release at the pituitary.

Although in mammals (Chen & Meites, 1975; Escobar del Rey, Garcia, Bernal, & Morreale de Escobar, 1974), reptiles [e.g., green anole (*Anolis carolinensis*) (Licht & Denver, 1988)] and birds [e.g., domestic fowl (Scanes, 1974)], it is well established that TRH binds to pituitary TRH receptors to stimulate the release of TSH from the anterior pituitary, the exact role of TRH in activating the fish thyroid axis is not clear, and species-specific differences in TRH action on thyrotropes exist (Deal & Volkoff, 2020). For example, TRH injections increase TSHβ expression *in vitro* in bighead carp (*Aristichthys nobilis*) (Chatterjee, Hsieh, & Yu, 2001), but not in common carp (Geven, Flik, & Klaren, 2009), coho salmon (*Oncorhynchus kisutch*) (D. A. Larsen, Swanson, Dickey, Rivier, & Dickhoff, 1998) or Atlantic salmon (*Salmo salar*) (Fleming, Maugars, Martin, Dufour, & Rousseau, 2020). It is thought that a corticotropin-releasing factor (CRF) is responsible for inducing TSH release at the pituitary [e.g., Atlantic salmon (Fleming et al., 2020); common carp (Geven et al., 2009); coho salmon (D. A. Larsen et al., 1998)]. To our knowledge, there are no reports on the action of TRH on TSH release in goldfish. The concomitant increases in TRH and TSH expressions in overfed fish might suggest a direct role of TRH at the pituitary level to stimulate TSH secretion. However, it must be cautioned that mRNA expression levels do not always correlate directly with protein levels, i.e., an increased tissue expression does not indicate

increased protein production [owing to factors such as efficiency of translation, or post-transcription processing (Haider & Pal, 2013)].

In mammals, fasting decreases the response of TSH to TRH time dependently [e.g., human (Vinik, Kalk, McLaren, Hendricks, & Pimstone, 1975)] providing evidence that TSH levels are dependent on nutritional status. In fish, little is known on how TSH responds to changes in food availability. In salmonids, chronic fasting reduces levels of plasma THs compared to fed fish, suggesting less thyroid stimulation by TSH [e.g., rainbow trout (Leatherland & Farbridge, 1992); coho salmon (Leatherland, 1982)]. Whether this decreased TSH production is fish is mirrored by a decrease in TRH release is unknown.

3.4.2.2. Circulating thyroid hormones

We found no significant differences in TH levels between experimental groups. The range of circulating TH levels in our study are consistent with previously reported ranges in goldfish [T_3 levels: 0.5-9.2 ng/mL; T_4 levels: 0.9-12.4 ng/mL (de Pedro, Gancedo, Alonso-Gomez, Delgado, & Alonso-Bedate, 1995; MacKenzie, Sokolowska, Peter, & Breton, 1987; Sohn, Yoshiura, Kobayashi, & Aida, 1999)]. We found significantly higher TH levels in TH-injected fish, validating our ELISA assays.

In mammals, food deprivation is usually associated with reduced TH levels. For example, in humans and rodents, fasting decreases TSH levels (Azizi, 1978), TH receptor binding capacity (but not affinity) (Burman, Lukes, Wright, & Wartofsky, 1977; Schussler & Orlando, 1978) and DIO activity (Araujo et al., 2008; Diano, Naftolin,

Goglia, & Horvath, 1998; Kaplan & Yaskoski, 1982), resulting in reduced circulating levels of TH (Herlihy, Stacy, & Bertrand, 1990; Martinez & Ortiz, 2017).

Previous studies in fish point to decreases in TH levels during fasting and ration-dependent increases in TH levels. In goldfish, plasma free T_3 and T_4 levels are lower in fasted (after 8h) than fed fish (de Pedro et al., 1995), and in juvenile rainbow trout, 3 and 8 weeks of fasting result in lower plasma TH levels and T_3 liver content (Farbridge, Flett, & Leatherland, 1992; Raine, Cameron, Vijayan, MacKenzie, & Leatherland, 2005). In both Atlantic salmon (McCormick and Saunders 1990) and red drum (*Sciaenops ocellatus*) (MacKenzie, Moon, Gatlin, & Perez, 1993), plasma TH levels increase with ration levels (T_4 and T_3 levels for 0 % - 1.6 % BW rations after 6 weeks; T_3, but not T_4, for 0.5 % - 6.0 % BW rations, respectively). In Arctic charr, where T_3 levels increase up to a 2 % ration and reach a plateau, T_4 levels only increase after the ration exceed 2 % (Eales & Shostak, 1985a), suggesting that levels of T_3 and T_4 might differentially respond to rations.

In our study, the lack of change in TH levels with nutritional status may be due to the fact we measured bound versus free THs in serum. Total T_4 (tT_4) and T_3 (tT_3) concentrations constitute both THs in free dissociated forms, as well as THs bound to carrier proteins [e.g., transthyretin (Power et al., 2000)]. Therefore, total TH measurements may not be sensitive enough to see circulating changes as a function of food ration. In goldfish, total T_4 and T_3 significantly increase after TSH injection (Miller, Jaques, Szkudlinski, & MacKenzie, 2012), but central CRF administration decreases thyroid T_3 free fraction in lower jaws containing thyroid tissue without affecting either

TH bound fractions (T_3 and T_4) (de Pedro et al., 1995). Interestingly, in Arctic charr (*Salvelinus alpinus*), the proportion of bound T_3 to plasma proteins correlates directly to total T_3, suggesting that total THs are also a physiologically relevant measure in fish (Eales & Shostak, 1985a; Eales & Shostak, 1985b).

It is likely that changes in TH levels in response to nutritional status are species-specific and might depend on the metabolism, feeding habits and reproductive seasonality of the animals. For example, whereas fasting reduces TH levels in rodents, in northern elephant seal (*Mirounga angustirostris*) pups, 1, 3 and 7 weeks of fasting does not decrease the concentrations of plasma tT_3, free T_3, tT_4 or free T_4 levels, but up-regulates peripheral deiodinase activation pathways (Martinez et al., 2013). This suggests that in mammals adapted to prolonged food deprivation, the thyroid axis might not be suppressed during fasting and this phenomenon may be specific to animals that rely heavily on lipid-based metabolism (Martinez et al., 2017), as it is the case in some fish [e.g., Polar cod (*Boreogadus saida*) (Hop & Gjøsæter, 2013)]. Goldfish have been shown to display seasonal variations in thyroid physiology/function. TH circulating levels increase during growth phases (summer) and decrease during spawning events (fall) (Sohn et al., 1999), and T_3 inhibits gonadotropin releasing hormone (GnRH)-induced growth hormone release in recrudescent but not regressed females (Ma, Ladisa, Chang, & Habibi, 2020a) and at all reproductive stages in males (Ma et al, 2020b). In our study, all fish were gonadal recrudescent, and it is unlikely that reproductive stage might have biased our results. Furthermore, there were no measured gender-specific differences in the concentrations of either circulating TH. It is possible that different results might have

been obtained had only one sex been used, or if the experiment had been conducted at a different reproductive season (i.e., spawning season). Likewise, it is also possible that the response of the thyroid to nutritional status is time-dependent, and a shorter or longer period of fasting might have allowed us to see changes in TH levels.

Overall, in our study, we found no correlations between the expressions of TRH and TSH, and serum TH levels. Despite an increase in TRH and TSH expressions in overfed fish, no changes in TH levels were observed. In rainbow trout, TRH injections elevate plasma T_4 but not T_3 levels compared to saline injected fish (Eales & Himick, 1988) and TSH injections increase plasma T_4 but not T_3 (Milne & Leatherland, 1978). Similarly, intramuscular TSH injections increase plasma T_4 in brook trout (*Salvelinus fontinalis*) (Chan & Eales, 1976). However, in longchin goby (*Chasmichthys dolichognathus*) and hagfish (*Eptatretus burger*), IP injections of TRH do not affect serum T_4 levels (Tsuneki & Fernholm, 1975).

3.4.2.3. Central and hepatic thyroid hormone deiodination

THs can be metabolized by deiodination, sulfation and glucuronidation. DIO1 and DIO2 are the major activating enzymes, as they convert T_4 into the active T_3. DIO3 is the major TH-inactivating enzyme as it catalyzes inner-ring deiodination of both T_4 and T_3, to produce biologically inactive reverse T_3 (rT_3) and diiodothyronine (T_2). In our study, we chose not to measure DIO1 expression, as changes in its expression can be associated with either activation and/or deactivation of THs (Kelly, 2000; P. R. Larsen & Zavacki, 2012). In addition to DIO3, UGT enhances T_3 and T_4 removal by increasing the affinity

for DIO1 and by stimulating the clearance of TH through the bile and urine (de Vries et al., 2020). In fish, as in mammals, the liver is the main site of TH metabolism, though expression/activity of DIOs also occurs in the head kidney, pharyngeal region and gonads (Orozco & Valverde-R, 2005), as well as the brain [e.g., zebrafish (Parsons et al., 2020); parrotfish (Johnson & Lema, 2011)].

In our study, fasting induced time-dependent decreases in hepatic DIO3 and UGT but had no effect on DIO2 expression (hypothalamus and liver). In contrast to our results, in rodents, fasting increases the hepatic activities of DIO3 and UGT, which contributes to the inactivation of T_4 and T_3 and the decrease in serum T_3 levels (de Vries et al., 2020; Galton, Hernandez, & St Germain, 2014) and results in increased hypothalamic DIO2 mRNA content and enzyme activity (Diano et al., 1998). In hamsters, hypothalamic DIO2 expression is decreased during fasting-induced torpor (Cubuk, Markowsky, & Herwig, 2017). Our results contrast with other studies in fish. In rainbow trout, fasting (3-7 weeks) increases rT_3 glucuronidation (Finnson & Eales, 1999) and overall UGT activity (Blom, Andersson, & Förlin, 2000), and in Arctic charr, food deprivation decreases brain DIO2 expression compared to fed fish (Striberny, Jørgensen, Klopp, & Magnanou, 2019). Conversely, in zebrafish fasted for 3 weeks, liver DIO2 increases and DIO1 decreases (Jia et al., 2019).

In our study, overfeeding decreased hypothalamic expression of DIO2 after 14 days compared to control fish. In DIO2 knockout mice, high fat feeding induces obesity due to inability of THs to oxidize peripheral fat (Castillo et al., 2011). To our knowledge, there are only three other studies in fish reporting the effects of possible nutrient excess

on DIO metabolism: (1) common carp fed *ad libitum* for 6 weeks, show a non-significant trend for up-regulation of liver DIO2 expression (Geven, Huising, Flik, & Klaren, 2008), (2) brook trout fed a high caloric diet have higher conversion of T_4 to T_3 than a low caloric diet (Higgs & Eales, 1979), and (3) Arctic charr have a decreased T_4 to T_3 conversion when fed a 4 % BW ration (Eales & Shostak, 1985a).

Overall, our results point to a retention of THs during long-term fasting, shown by decreased DIO3 and UGT levels over time, leading to suppressed metabolism in times of prolonged food scarcity. The suppression of DIO2 during overfeeding in the hypothalamus might indicate a decrease in TH bioactivation, perhaps limiting negative feedback of the thyroid axis to increase TRH secretion (due to an increase in TRH expression seen at 14 days). However, the existence of a feedback of THs to the release of TRH by the hypothalamus in fish has never been demonstrated. For example T_4 injections in common carp have no effect on TRH mRNA levels (Geven, Verkaar, Flik, & Klaren, 2006). It is noteworthy that transcript expression of deiodinase enzymes may not necessarily reflect TH metabolism, as transcript expression is not always correlated to protein abundance or activity (Stitt & Gibon, 2014).

3.4.3. Response of appetite-regulating peptides

3.4.3.1 Response under fasting conditions

Our results show that fasting increases the expression of the orexigenic neuropeptide AgRP, has little effects on the anorexigenic neuropeptide POMC and has no effect on the expression of NPY or intestinal CCK expression.

In our study, hypothalamic POMC expression displayed a small but significant increase after 7 days but not 14 days of fasting compared to controls and was significantly downregulated after 14 days of fasting compared to 7 days. The response of POMC expression to fasting appears to be species- and time-specific, based on results from previous studies. Similar to our results, in rainbow trout, POMC-A1 and POMC-B mRNA expression increases after 4 months of fasting (Jørgensen, Bernier, Maule, & Vijayan, 2016). However, fasting does not affect POMC-A1 expression in rainbow trout after 28 days (Leder & Silverstein)], POMC expression after 1, 3, 5 or 7 days in goldfish (Cerdá-Reverter, Schiöth, & Peter, 2003) or after 4 days in Atlantic salmon (Kalananthan, Lai, et al., 2020). In contrast, other studies show that fasting decreases hypothalamic POMC expression [POMC-A in larval zebrafish after 2 days (Shanshan, Cuizhen, & Gang, 2016), and Atlantic salmon after 6 days (Valen, Jordal, Murashita, & Rønnestad, 2011)], similar to what is seen in rodents (Ahima, Kelly, Elmquist, & Flier, 1999; Bertile, Oudart, Criscuolo, Maho, & Raclot, 2003; Mizuno et al., 1998). The increase in POMC expression in fasted fish seen in our study at 7 days might be indicative of an inhibition of feeding/searching behaviour to limit energy expenditure, as suggested for rainbow trout (Jørgensen et al., 2016). The decrease in POMC mRNA expression after 14 days is in line with the anorexigenic role of this peptide, and possibly switches from a "protective" mechanism at 7 days (to avoid food searching) to active food seeking behaviour at 14 days.

Fasting increased AgRP expression at both time points, which is consistent with its orexigenic role previously shown in fish [e.g., goldfish (Cerdá-Reverter & Peter,

2003); Atlantic salmon (Kalananthan, Murashita, et al., 2020); European sea bass (Agulleiro et al., 2014); zebrafish (Song, Golling, Thacker, & Cone, 2003); (*Schizothorax prenanti*) (Wei et al., 2013); transgenic coho (J.-H. Kim, Leggatt, Chan, Volkoff, & Devlin, 2015)] and mammals [e.g., rats (Mizuno & Mobbs, 1999); Siberian hamsters (Day & Bartness, 2004)].

In our study, NPY expression was not affected by fasting, which contrasts with other studies in mammals and fish. In rats, food deprivation leads to an increase in the expression of NPY (Palou et al., 2009). In goldfish, fasting for 72 hours (Narnaware & Peter, 2001) or 4 days (Volkoff, Joy Eykelbosh, & Peter, 2003) increases hypothalamic and telencephalic NPY expressions compared to fed fish. However, similar to our results, fasting of 1, 3, 5, and 7 days in Mozambique tilapia (*Oreochromis mossambicus*) does not affect hypothalamic expression (Riley et al., 2008) and in winter skate, 2 weeks of fasting increases telencephalic but not hypothalamic NPY expression (MacDonald & Volkoff, 2009b). These results suggest that the fasting-induced changes in NPY expression vary with the species, time of fasting and brain regions considered.

CCK intestinal expression was not affected by fasting. Similar to our results, fasting induces no change in expression of gut CCK in Atlantic salmon (Murashita, Kurokawa, Nilsen, & Rønnestad, 2009) or in hypothalamus of goldfish (Volkoff et al., 2003). However, fasting decreases CCK mRNA levels in intestine of winter flounder (MacDonald & Volkoff, 2009a) and gilthead sea bream (*Sparus aurata*) (Babaei et al., 2017) and intestine and hypothalamus of grass carp (*Ctenopharyngodon idellus*) (up to 17 days) (Feng et al., 2012).

3.4.3.2. Response under overfeeding conditions

Overfeeding did not affect the expression of any of the appetite regulators examined compared to controls. The effects of overfeeding on the expressions of appetite regulators are unclear, as very few studies have been performed. In rodents, overfeeding increases POMC (Hagan et al., 1999) and AgRP expression (Cains, Blomeley, Kollo, Rácz, & Burdakov, 2017; Stofkova et al., 2009), decreases NPY mRNA expression (Ferretti, Fornari, Pedrazzi, Pellegrini, & Zoli, 2011; Plagemann et al., 1999), and diet-induced obesity (Kuhne & Stengel, 2019) or over-nourishment (Enes-Marques et al., 2020) decreases CCK levels and signalling. In zebrafish, long-term caloric excess diets do not alter AgRP (Löhr et al., 2018) or POMC (Montalbano et al., 2019) transcripts but increase NPY mRNA (Montalbano et al., 2019) compared to controls. Interestingly in our study, POMC expression was lower in overfed fish at 14 days compared to 7 days. Similar to the POMC increase seen in fasting, the downregulation of POMC under abundant food may be an energy expending mechanism as an attempt to increase food-seeking behaviour.

In zebrafish, overfeeding does not alter either AgRP (Löhr et al., 2018) or POMC (Montalbano et al., 2019) mRNA expression, but increases NPY expression (Montalbano et al., 2019) compared to control fish. Surprisingly, in our study, POMC expression was lower in overfed fish at 14 days compared to 7 days, similar what was observed for fasting fish. The time-dependent reduction in POMC expression may be the result of impaired signalling/resistance to a peripheral satiation signal. In mammals, diet-induced obesity is characterized by high levels of leptin (an adipose derived hormone that signals

the brain to decrease food intake), resulting in desensitization of leptin receptors in the brain (Sáinz, Barrenetxe, Moreno-Aliaga, & Martínez, 2015) and impairment of the leptin-mediated increases in POMC expression (Elmquist, 2001; Korner et al., 1999; S. Lin, Storlien, & Huang, 2000). Similarly, Japanese medaka with a leptin receptor knockout show suppressed POMC and increased AgRP and NPY (Chisada et al., 2014), and a strain of high fat rainbow trout become leptin impaired if fed for 4 weeks (Gong, Johansson, & Björnsson, 2016). Although we did not measure leptin levels in our study, it could be hypothesized that overfed fish might have impaired leptin (or another satiety signal) signalling (seen by decreased POMC and an increasing trend in AgRP).

3.5. Conclusion

In this study, we show that the thyroid axis is responsive to food rations, and different levels of the axis (central versus peripheral) show differences in a ration-dependent response. Our results suggest that, in goldfish, prolonged overfeeding may induce thyroid axis activation, possibly through a coordinated mechanism of (1) limited feedback by THs causing downregulation of DIO2 expression and upregulating TRH or (2) impaired signalling by an unknown satiety factor. In fasted fish, downregulated expression of TH removal enzymes (DIO3, UGT) from the liver might help to maintain a proper thyroid set-point during food deprivation. This partially supports our hypothesis that the thyroid axis is upregulated under conditions of food abundance but not that fasting downregulates thyroid function. As well, our results reject the hypothesis that different food rations would induce changes in circulating TH levels, suggesting that

these levels are not good indicators/proxies for energy balance. We did not find any clear correlation between changes in the thyroid axis and changes in appetite regulators, and further studies are required to determine the nature/existence of these interactions.

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Figures

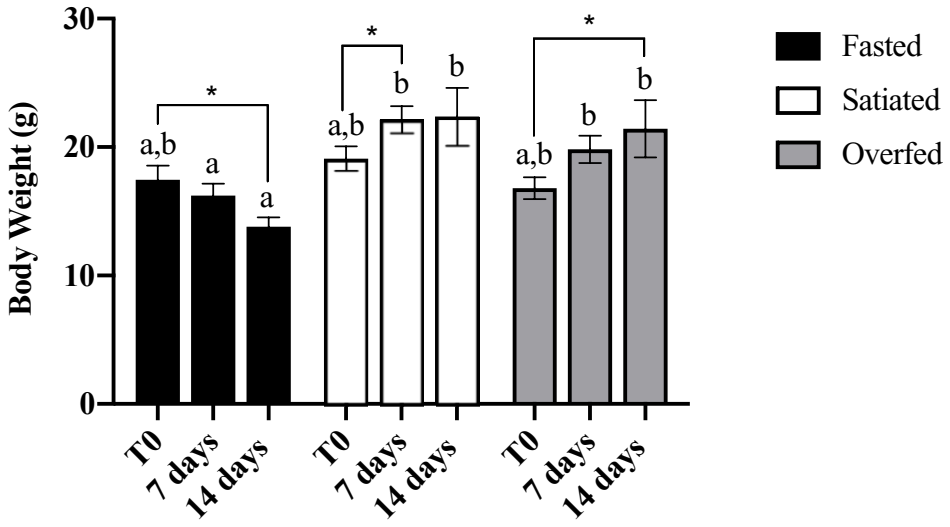

Figure 3.1. Body weight data for fasted, satiated (control) and overfed fish at experiment start (T0, n = 60), 7 (n = 30) and 14 days (n = 30). Data is presented as mean ± SEM. Bars with dissimilar superscripts indicate significant differences between groups (Mann-Whitney t-test, $p < 0.05$), and stars indicate significant differences within groups (Kruskal-Wallis t-test, $p < 0.05$).

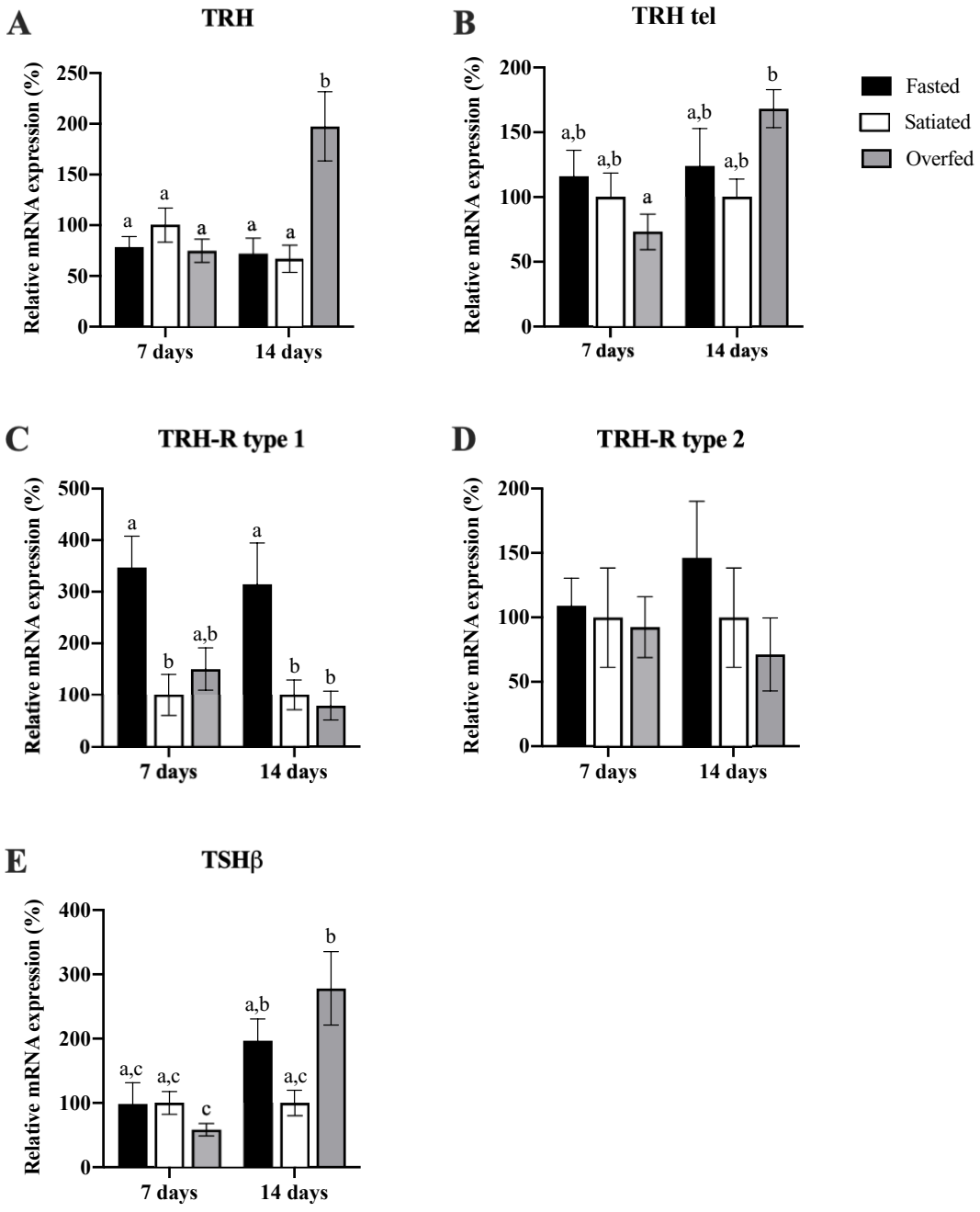

Figure 3.2. Relative mRNA expression of hypothalamic TRH (A), telencephalic TRH (B), pituitary TRH receptors type 1 (C) and type 2 (D), and pituitary TSHβ (E) for fasted, satiated (control) and overfed fish at 7 (n = 8 fish per group) and 14 days (n = 8 fish per group). Data is expressed as mean ± SEM and satiated fish data at 7 days is normalized to 100 %. Dissimilar superscripts within and between groups indicate significant differences (two-way ANOVA, $p < 0.05$).

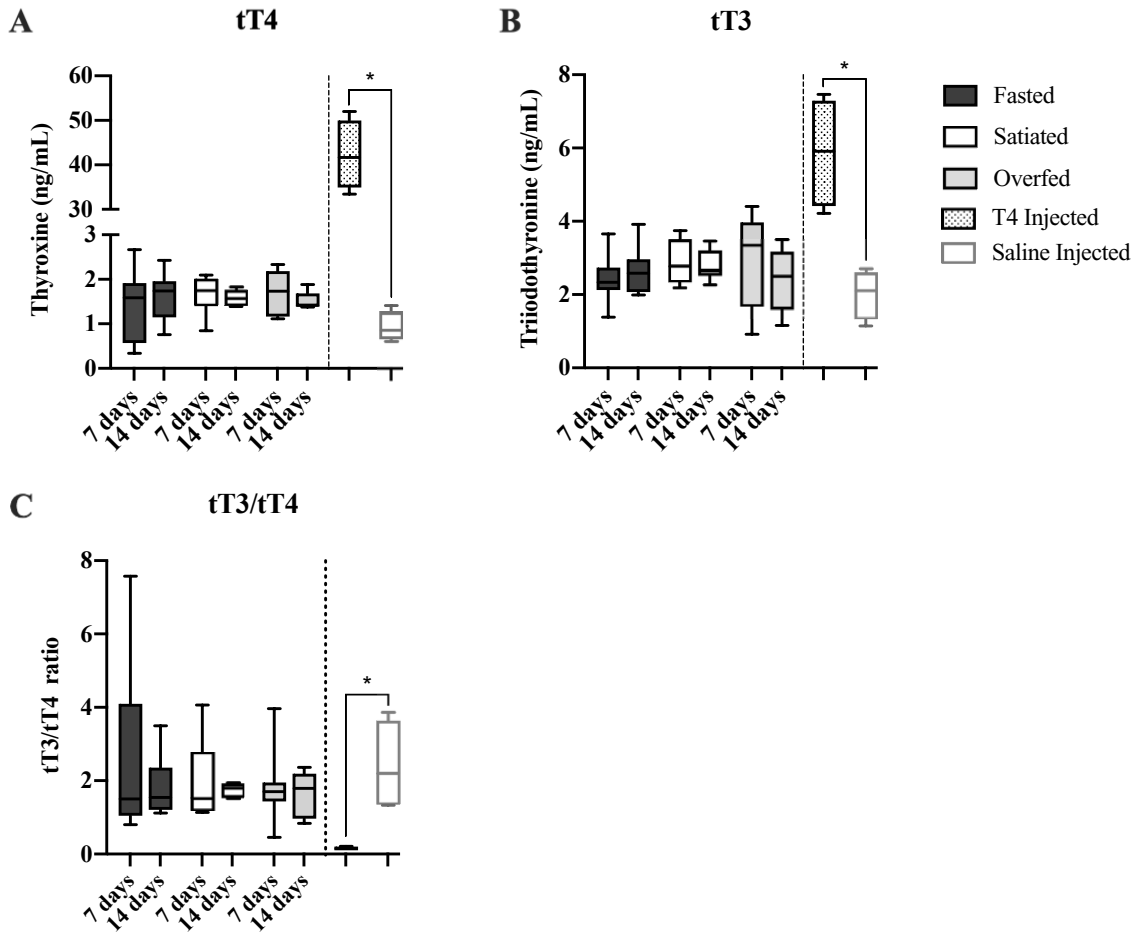

Figure 3.3. Box and whisker plots of serum concentrations (ng/mL) of total T_4 (tT_4, A), total T_3 (tT_3, B) and the ratio of tT_3 to tT_4 (C) for fasted [7 days (n = 10); 14 days (n = 6)], satiated (control) [7 days (n = 6); 14 days (n = 8)], overfed [7 days (n = 7); 14 days (n = 5)] and T_4 injected (n = 4) fish at 7 and 14 days. Boxes represent 25th, 50th and 75th percentiles of the dataset and whiskers represent minimum and maximum values of the dataset. The dashed line represents separation from experimental fish and T_4 and saline injected fish (ELISA control). Stars indicate significance from the ELISA control (unpaired t-test, $p < 0.05$).

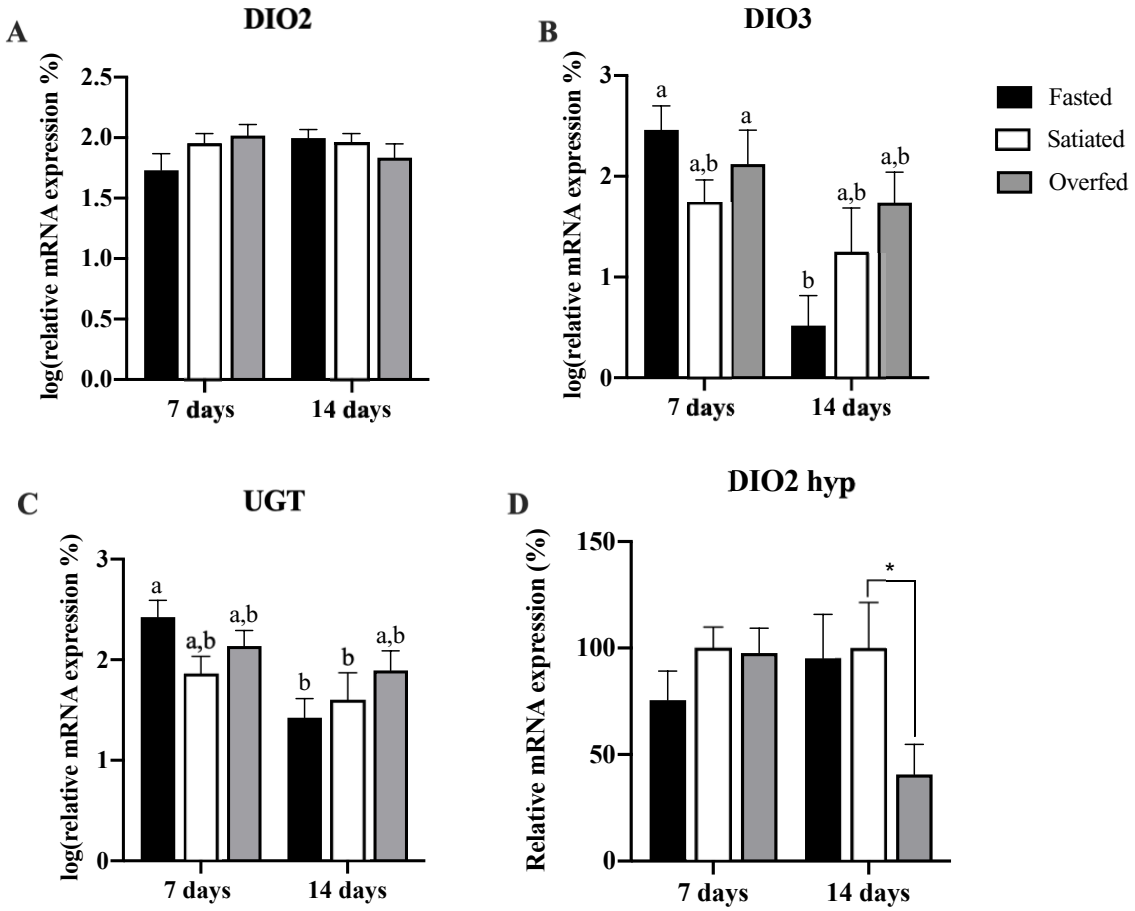

Figure 3.4. Relative mRNA expression of liver DIO2 (A), DIO3 (B) and UGT (C), and brain DIO2 (D) for fasted, satiated (control) and overfed fish (n = 8 per group) at 7 and 14 days. Data is expressed as mean ± SEM and satiated fish data are normalized to 100 %. Dissimilar superscripts within and between groups indicate significant differences (two-way ANOVA, $p < 0.05$). Stars indicate significance between groups (unpaired t-test, $p < 0.05$).

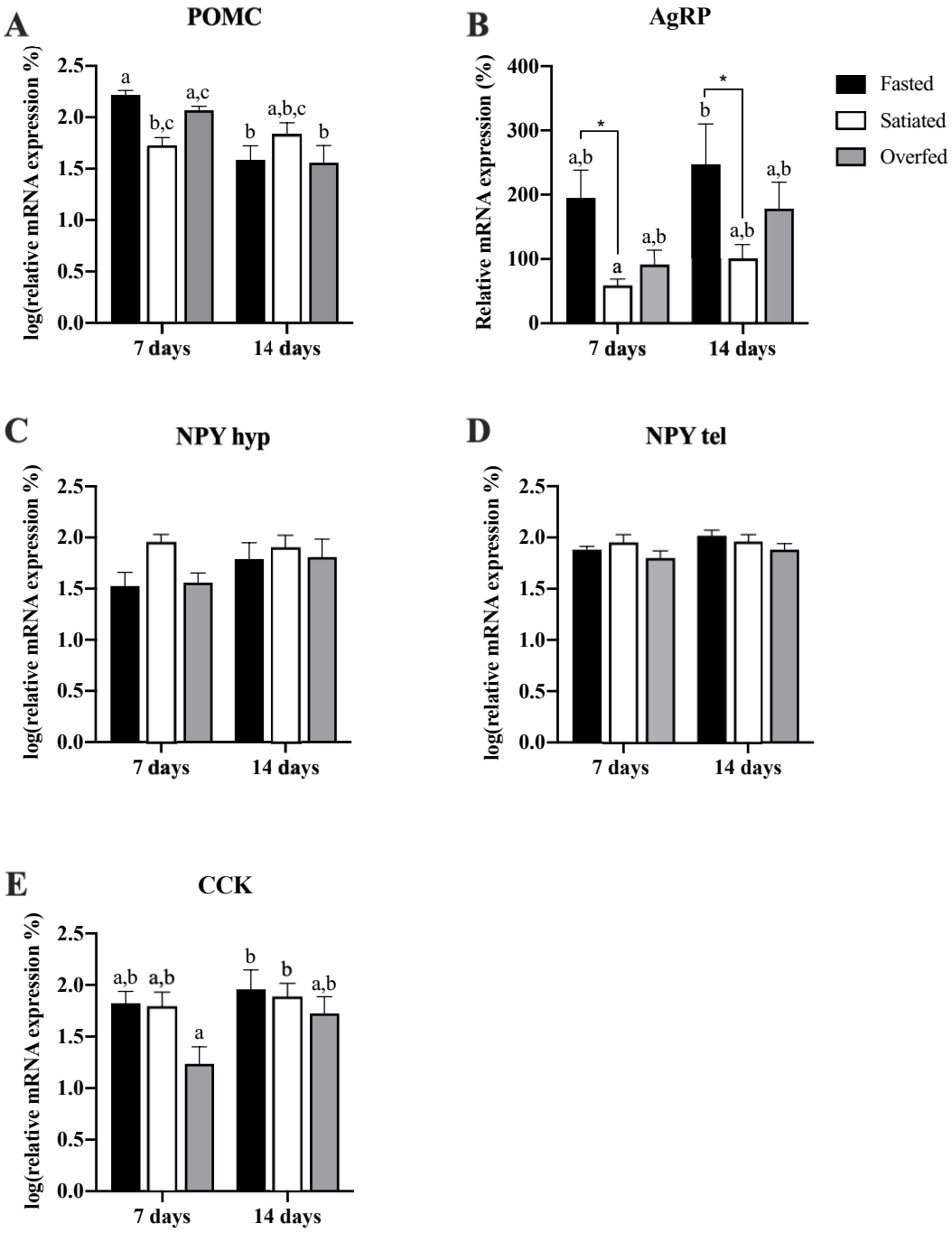

Figure 3.5. Relative mRNA expression of hypothalamic POMC (A), AgRP (B), NPY (C), telencephalic NPY (D) and intestinal CCK (E) (n = 8 each). Data are expressed as mean ± SEM and satiated fish data is normalized to 100 %. Dissimilar superscripts within and between groups indicate significant differences (two-way ANOVA, $p < 0.05$). Stars indicate significant difference relative to the control (unpaired t-test, $p < 0.05$).

Tables

Table 3.1. Sequences of primers used in study with GenBank Accession number.

Gene	Direction	5' – 3' Sequence	GenBank Accession #
TRH	Forward	AGACGGAGGACGAGAACCAC	AB179819.1
	Reverse	CGTCTTCGTAGTCGGTGTCC	
TRHR type 1	Forward	TGCTTCTCGGAGACAGGTGA	XM_026283702.1
	Reverse	GGTTGATGGCGCTGTTCAAG	
TRHR type 2	Forward	CAGGAGGAGCTGCAAAGAAC	XM_026227551.1
	Reverse	CAGGGTTGATCGCACTGTTA	
TSHβ	Forward	CTGTCAACACCACCATCTGC	AB003584.1
	Reverse	GGCACATTCATCACTGTTGG	
NPY	Forward	GCCTTCCTCTTGTTCGTCTG	M87297.1
	Reverse	TGGACCTTTTGCCATACCTC	
AgRP	Forward	ATGGCATCACATCCAAACC	AJ555492.1
	Reverse	GCTTTACCCAGATCCTCATCA	
POMC	Forward	CTGTGTGCGGGGTGGATCTGA	AJ431209.1
	Reverse	AATGGCTTTCTCCAGGGTAGACAG	
CCK	Forward	GAGGATGATGAAGAGCCCCG	U70865.1
	Reverse	TGTTGCCCATGGACTTGCTT	
DIO2	Forward	TGTCACTCCTGAGCTGTTCG	EU313786.1
	Reverse	GGAGACTCGAAGTCCAGCAG	
DIO3	Forward	TCTGCGTGTCAGACTCCAAC	EU313787.1
	Reverse	CTCCCGAAGTTGAGGATCAG	
UGT	Forward	GACAGAACTGGCCCAGAGAG	XM_026272069.1
	Reverse	CGCATCCTTCCACCTGTATT	
β-actin	Forward	ACTACTGGTATTGTGATGGACTCC	LC382464.1
	Reverse	CGGTCAGGATCTTCATCAGGTAG	
Elongation Factor 1-α	Forward	CTGAACCACCCTGGTCAGAT	AB056104.1
	Reverse	CGGTCGATCTTCTCCTTGAG	

Table 3.2. Overall effects of fasting and overfeeding on goldfish relative to controls, and differences between overfed and fasted fish. Up arrows (↑) indicate a significant increase in expression, levels or metrics, and down arrows (↓) indicate a significant decrease in expression, levels or metrics. No effect indicates no significant change. Hypo: hypothalamus; tel: telencephalon; pit: pituitary; orexigenic: appetite-stimulating; anorexigenic: appetite-inhibiting

	Effects of fasting	**Effects of overfeeding**	**Overfeeding versus fasting**
Body Weight	↓ after 7 and 14 days	No change	↑ after 7 and 14 days overfed compared to 7 and 14 days fasting
TH levels	No effect	No effect	No effect
Thyroid axis			
TRH (hypo)	No effect	↑ expression after 14 days	↑ after 14 days overfed compared to 7 and 14 days fasting
TRH (tel)	No effect	No effect	No effect
TRHR1 (pit)	↑ at 7 and 14 days	No effect	↓ at 14 days overfed compared to 7 and 14 days fasting
TRHR2 (pit)	No effect	No effect	No effect
TSHβ (pit)	No effect	↑ at 14 days	↓ at 7 overfed compared to 14 days fasting, and ↑ at 14 days overfed compared to 7 days fasting
DIO2 (liver)	No effect	No effect	No effect
DIO2 (hypo)	No effect	↓ at 14 days	No effect
DIO3 (liver)	No effect	No effect	↑ at 7 compared overfed to 14 days fasting
UGT (liver)	↑ at 7 days compared to 14 days	No effect	No effect
Appetite regulators			
POMC (anorexigenic)	↑ at 7 days	No effect	↑ at 7 overfed compared to 14 days fasting and ↓ at 14 overfed compared to 7 days fasting
AgRP (orexigenic)	↑ at 7 and 14 days	No effect	No effect

NPY (orexigenic)	No effect	No effect	No effect
CCK (anorexigenic)	No effect	No effect	↓ at 7 overfed compared to 14 days fasting

Chapter 4. Effects of Thyroxine and Propylthiouracil on Feeding Behaviour and the Expression of Genes Related to Appetite and Thyroid Function in Goldfish (*Carassius auratus*)

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Abstract

There is poor evidence for an association between thyroidal state, feeding and appetite regulation in fish. We assessed how an altered thyroid state influences feeding behaviour, food intake and expression of hypothalamic appetite-regulating peptides (Klotho-α and Klotho-β; orexin, OX; cholecystokinin, CCK; agouti-related peptide, AgRP; cannabinoid receptor 1, CB1) in goldfish. We also measured the expressions of hypothalamic, pituitary and liver transcripts that regulate the thyroid [thyrotropin-releasing hormone (TRH), thyrotropin-releasing hormone receptor (TRH-R) type 1, thyroid stimulating hormone beta (TSHβ), deiodinases (DIO2, DIO3), UDP-glucuronosyltransferase (UGT1A1), thyroid receptor alpha and beta (TRα, TRβ)], and circulating levels of total thyroxine (tT_4) and total triiodothyronine (tT_3). To achieve contrasting thyroidal conditions, we implanted goldfish with propylthiouracil (PTU) or T_4 osmotic pumps and administered these continuously over 12 days. T_4-implanted fish showed increased feeding behaviour but not food intake, while PTU did not alter either. We provide evidence for a negative feedback of T_4 at the pituitary, but not the hypothalamus, with downregulation of TSHβ and DIO2 transcript expression and increased DIO3 mRNA in hyperthyroid conditions. In hepatic tissues, DIO2 transcripts were suppressed under T_4 treatment, with no effect on thyroid receptors. There was a poor association between an altered thyroid state and appetite regulators. We show a novel role for the Klotho protein in the hypothalamus, as its expression is downregulated under a high thyroid load, indicative of a increased metabolic state. CCK expression was downregulated when peripheral THs were increased, suggesting a blunted hypothalamic response to regulate

energy balance. AgRP, OX or CB1 were not affected by thyroidal state relative to controls. In consensus with other studies, PTU does not appear to be a sensitive thyroid inhibitor to create hypothyroid conditions in fish. Overall, we show that unlike in mammals, hyperthyroid conditions in goldfish do not lead to an increased desire or need to consume food, furthering evidence for a weak link between the thyroid and appetite.

4.1. Introduction

The thyroid gland acts as an important metabolic regulator in vertebrates. In mammals, thyroid hormones (THs) are essential for development/function of the central nervous (Chan & Kilby, 2000), respiratory (Sadek, Khalifa, & Azoz, 2017), musculoskeletal systems (Salvatore, Simonides, Dentice, Zavacki, & Larsen, 2014) and have a major role in controlling energy expenditure in homeotherms that need to maintain a set body temperature (Danforth & Burger, 1984; Yavuz, Salgado Nunez del Prado, & Celi, 2019). The secretion and circulating levels of THs are regulated by the hypothalamus-pituitary-thyroid (HPT) axis, hereafter referred to as the thyroid axis. Thyrotropin releasing hormone (TRH) produced by the hypothalamus stimulates the secretion of thyroid stimulating hormone (TSH) by the pituitary (Ortiga-Carvalho, Chiamolera, Pazos-Moura, & Wondisford, 2011), which in turn stimulates the thyroid gland to synthesize and secrete THs. THs levels are controlled by a endocrine axis negative feedback loop, with circulating THs controlling production of TRH and TSH (Ortiga-Carvalho et al., 2011). High circulating levels of TH decreases production of TRH and TSH, thus returning TH to basal levels [see (Fekete & Lechan, 2014; Fliers, Kalsbeek, & Boelen, 2014; Roelfsema, Boelen, Kalsbeek, & Fliers, 2017; Zoeller, Tan, & Tyl, 2007)].

In fish, THs mediate developmental events related to metamorphosis, are essential ligands involved in control of life cycle events [e.g., flatfish body plan rearrangement (Marco António Campinho, 2019) and salmonid freshwater to seawater transition

(Dickhoff, Folmar, Mighell, & Mahnken, 1982)], and mediate thermal acclimation (Little, Kunisue, Kannan, & Seebacher, 2013; Little & Seebacher, 2014).

In fish as in mammals, THs consist of two major forms, thyroxine (T_4) and triiodothyronine (T_3), which are synthesized in thyroid follicular cells (Eales & Brown, 1993). TH synthesis is stimulated when TSH binds to its receptors on follicles and stimulates intracellular production of THs, involving the iodination of tyrosyl residues on the precursor protein thyroglobulin, a reaction catalyzed by thyroid peroxidases (TPO) (Rousset, Dupuy, Miot, & Dumont, 2000). T_4 is the major circulating form and converted in target cells to the more biologically active T_3 by activating deiodinase enzymes (DIO1, DIO2), while TH deactivation occurs by the inactivating deiodinase enzyme, DIO3, and conjugation enzyme, UDP-glucuronosyltransferase (UGT). T_3 exerts its effects within target cells by binding to TH receptors (TRs) to induce transcription of cell/target specific mRNAs (Kelly, 2000).

These regulatory pathways of the thyroid axis are well established in mammals and share common mechanisms/features with fish (Deal & Volkoff, 2020), however, there are still clear differences between the two groups. First and foremost, in fish, the nature of the major simulator of pituitary TSH release is unclear, with some evidence pointing to a hypothalamic corticotropin-releasing factor (De Groef, Van Der Geyten, Darras, & Kühn, 2006; D. A. Larsen, Swanson, Dickey, Rivier, & Dickhoff, 1998), while other studies support TRH (similar to mammals) (Chatterjee, Hsieh, & Yu, 2001). Second, while the existence of a pituitary negative feedback by THs to control TSH production has been shown in fish, there is little to no evidence of a feedback action of

THs to the hypothalamus (Deal & Volkoff, 2020; Geven, Verkaar, Flik, & Klaren, 2006). Lastly, there are major differences in the peripheral regulation of THs between mammals and fish, with variations in tissue distribution and efficiencies of deiodinase enzymes (Orozco & Valverde-R, 2005), and the presence of multiple isoforms of TRs in fish (Nelson & Habibi, 2009). While the role of the thyroid axis in fish has been well established in multiple physiological processes such as growth, development, and reproduction [reviewed by (Deal & Volkoff, 2020)], very few studies have focused on the role that thyroid hormones (and different thyroidal states) play in regulating feeding behaviour and appetite.

In mammals, the thyroid axis has been shown to regulate food intake and nutrient homeostasis, in part through interactions with appetite-regulating signals. For example, in rats, TRH decreases feeding in part via the inhibition of melanin-concentrating hormone (MCH, an orexigenic neuropeptide) and THs stimulate feeding via decreases in the expression of anorexigenic factors such as proopiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART). Conversely, the relationship between thyroid and appetite/feeding in fish is unclear (Deal & Volkoff, 2020, 2021). In coho salmon (*Oncorhynchus kisutch*), TH treatment increases food intake (Fagerlund, Higgs, McBride, Plotnikoff, & Dosanjh, 1980), and in goldfish (*Carassius auratus*), TRH injections increase food intake and the hypothalamic expression of orexin (OX) and CART (Abbott and Volkoff, 2011). Fasting induces increases in hypothalamic TRH expression in winter flounder (Buckley et al., 2010), but decreases in common carp

(*Cyprinus carpio*) (Huising et al., 2006), while goldfish show increased TRH and TSHβ expression after 14 days of overfeeding (Deal & Volkoff, 2021).

In order to shed some light on the mechanisms by which THs regulate the thyroid axis and feeding in fish, we examined the effects of thyroxine (T_4) and a thyroid inhibitor, propylthiouracil [PTU, an antithyroid thioamide drug that inhibits the actions of TPO, and used in the treatment of hyperthyroidism (Spaulding, 2007)] administered via osmotic pumps over 12 days on food intake. To determine how PTU or T_4 alter thyroid status we measured thyroid axis transcripts in central tissues (TRH, TRH-receptor 1, TSHβ), and hepatic tissue transcripts associated with TH conversion and action [TH deiodinases (DIO2, DIO3), UGT family 1 member A1 (UGT1A1) and TH receptors (TRα, TRβ)]. Moreover, we measured serum levels of total T_4 and total T_3 (which comprise both the bound and unbound forms of the hormone). To assess interactions between the thyroid axis and feeding, we assessed the mRNA expression of appetite-regulating peptides that stimulate (orexin, OX; agouti-related peptide, AgRP) or inhibit (cholecystokinin, CCK) food intake, as well as peptides that have recently shown to affect feeding behaviour in mammals that have not been thoroughly examined in fish (Klotho-α, KLα; Klotho-β, KLβ; cannabinoid receptor-1, CB1).

We hypothesized that, if fish display the same negative feedback loop as seen in mammals, increased TH levels would lead to an inhibited thyroid axis (e.g., decrease in TRH, TSHβ), with the opposite occurring following PTU thyroid inhibition. We also hypothesized that increased TH levels would lead to an increase in food intake and

feeding behaviour, while PTU treatment would have opposite effects, in part due to changes in the mRNA expression of appetite-regulating peptides.

This study extends our knowledge of thyroid regulation and action in lower vertebrates, as well as the thyroid-mediated control of feeding behaviour. This study is unique in that it analyzes both feeding behaviour and expression of genes associated with appetite control under a manipulated thyroidal state, furthering our understanding of how elevated TH levels might affect feeding/appetite in cold-blooded organisms. Furthermore, it deepens our understanding of the evolutionary nature of thyroid regulation and action in ectotherms.

4.2. Methods

4.2.1. Experimental animals

Goldfish (n = 30; average weight = 16.14 ± 1.22 g; average fork length = 8.58 ± 0.25 cm), were acclimated to a 16 h light:8 h dark cycle at 20 ºC, being fed once a day (10:00) to satiation with a 2 % wet body weight ration [number of fish x average fish weight (g) x 0.02/tank] of 2 mm sinking pellets (35 % crude protein, 10 % crude fat, 3 % crude fibre, 8.5 % moisture, 8 % ash; Omega Sea, Sitka, AK, USA). Following an acclimation period of 2 weeks, four experimental groups were divided in 10, 65-liter stock tanks (6 fish per group, 3 fish per tank). Two tanks were untreated (anesthesia), two were shams (anesthesia, surgical incision and sutures), two were implanted with pumps containing fish physiological saline [(0.11 M NaCl, 2.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM $NaHCO_3$, 0.5 mM NaH_2PO_4) (Burnstock, 1958)], two were implanted

with pumps containing PTU (12 µg/g) and two were implanted with pumps containing T_4 (5 µg/g). Both males and females were used, and all fish were reproductively regressed, based on their gonadosomatic index (GSI, gonad weight (g) / wet body weight (g) x 100; male GSI = 0.76 ± 0.09 %; female GSI = 1.46 ± 0.15 %) (Peng, Trudeau, Peter 1988)

4.2.2. Reagents and pump implantation

6-propyl-2-thiouracil (PTU) (CAS 0000051525) and L-thyroxine sodium salt pentahydrate (T_4) (CAS 6106-07-6) were purchased from Sigma Aldrich (Millipore Sigma, Oakville, ON, CA). PTU and T_4 stock solutions were made in 1.0 M NaOH and diluted to desired concentrations with fish physiological saline.

Doses of 12 µg/g of PTU and 5 µg/g of T_4 were chosen for pump implantation, based on their effectiveness following a single injection in previous studies in fish [PTU in freshwater tilapia (M. C. S. Peter & Peter, 2009) and coho salmon (Kang & Devlin, 2003), and T_4 in goldfish (Goodyear, 2012; Oshima & Gorbman, 1966)]. Prior to pump implantation, a few fish (n = 4) were submitted to single intraperitoneal (IP) injections (ventral side, anterior to the anus and posterior to the pelvic fins) of PTU (n = 3) and T_4 (n = 3) to verify that these doses affected feeding behaviour.

ALZET osmotic pumps (DURECT Corporation, Cupertino, CA, model #1002) with a volume of 100 µL and a constant delivery rate of 0.25 µL/hr were used (recommended by the manufacturer for animals 10-20 g). Drug concentrations were calculated so that pumps supplied 10 µg/hr (thus 12 µg/g/day) of PTU or 4.2 µg/hr (thus 5 µg/g/day) for T_4. Prior to insertion, pumps were filled with the PTU and T_4 solutions

and primed in saline solution at room temperature overnight (~18 hours) to ensure an accurate start-up gradient when surgically implanted. Pump administration for 12 days was chosen over 14 days (maximum time recommended by the manufacturer) to account for the pump priming period and reduce the possibility of pump infusion stopping at 14 days.

Prior to surgery, fish were fasted for 24 hours to avoid gut distention that might interfere with the surgical procedure. The implantation procedure was randomized in terms of treatment and tank. At the time of surgery (10:00), fish were deeply anaesthetized in a 0.5 mg/L solution of MS222. A 1 cm incision was made on the ventral side, posterior and lateral to the pelvic fins, and the osmotic pump was inserted into the IP cavity. The incision was closed with 5-0 non-absorbent monofilament sutures (Stoelting Company, Wood Dale, IL, USA) and tissue adhesive glue (Vetbond, 3M Animal Care Products, St. Paul, MN, USA), and treated with antibiotic solution (Melafix, Aquarium Pharmaceuticals, Mars Fishcare North America Inc., Chalfont, PA, USA) to prevent infection at suture sites. Fish recovered from surgery within 5 minutes, and none showed signs of stress (lowering of the dorsal fin, erratic locomotion).

4.2.3. Food intake and feeding behaviour

Food intake and food-seeking/locomotor behaviour were assessed daily during the experimental period. Given the large number of tanks to be assessed, observations were either done manually or through video recording. Each day, randomized sets of tanks were attributed an observation method. Observations were made for one hour and always

took place during the regular scheduled feeding time (10:00) to account for diurnal fluctuations in hormones, gene expression and any other physiological parameter. Fish were presented with an approximate 4 % body weight ration of pellets. Behaviour was quantified by counting the number of "complete acts" (complete consumption of a pellet) and "incomplete acts" (bumping a pellet with a closed mouth, engulfing of a pellet followed by spitting it out, or an attempt at engulfment without completion) for each individual in a tank based on methods by (Volkoff, 2013). The average daily food intake (mg food/g fish) per tank was calculated by dividing the average amount of food consumed [difference between weight of uneaten pellets left in the tank (~3 mg per pellet) from the initial weight of food offered) by the average weight of fish and the number of fish per tank (n = 3).

4.2.4. Tissue and serum sampling

After 12 days, fish were euthanized by immersion in MS222, measured and weighed, and sacrificed by spinal section and sampled for serum and tissues.

Blood was collected from the caudal peduncle with 27-gauge syringed needles, let clot for three hours at room temperature and centrifuged at 5000 rpm for 15 min. Serum was collected and stored at -80 °C until analysis. Whole brain, pituitary and liver were collected and stored in RNAlater (Qiagen, Mississauga, ON, Canada) at -20 °C until RNA extractions were performed. Hypothalami were dissected from whole brains at the time of RNA extraction.

All procedures followed the animal care protocols approved by Memorial University of Newfoundland Animal Care Committee following the guidelines of the Canadian Council on Animal Care guide to the care and use of experimental animals.

4.2.5. RNA extraction and cDNA synthesis

Hypothalamus, pituitary and liver samples were extracted for RNA using a GeneJET™ RNA Purification Kit (Fermentas, Burlington, ON, Canada) following the manufacturer's protocol. Using a NanoDrop ND-2000 (NanoDrop Technologies Inc., Wilmington, USA), final RNA concentrations were determined by optical density at 260 nm. Quality of RNA was assessed by measuring the ratio of the sample tissue at 260 and 280 nm, only samples with a ratio between 1.8 and 2.1 were used in subsequent quantification.

Total RNA was then reverse transcribed to cDNA using a SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK). Total RNA (500 ng) was mixed on ice with 5x TransAmp Buffer (4 μL), Reverse Transcriptase (1 μL) and RNase-free water for a reaction volume of 20 μL. In a Bio-Rad C-1000 Touch Thermal Cycler (Bio-Rad, Mississauga, ON, Canada), the following program was set: 25 °C for 10 min (annealing), 42 °C for 30 min (reverse transcription) and 85 °C for 5 min (inactivation). The cDNA product was diluted 1:10 with RNase-free water and frozen at -20 °C until quantitative polymerase chain reaction (qPCR) analysis.

4.2.6. Quantitative polymerase chain reaction (qPCR)

Using Primer 3 software (https://primer3.org/), three sets of primer pairs were designed for each of the transcripts of interest (TRH, TRH-R type 1, TSHβ, DIO2, DIO3, UGT1A1, TRα, TRβ, OX, CCK, AgRP, Klotho-α, Klotho-β and CB1), based on available sequences (see Table 1 for primer sequences and accession numbers) and synthesized by Integrated DNA technologies (IDT, Coralville, Iowa, USA). Primers were designed with a product size range of 150-250 base pairs and ensured the spanning of an exon-exon junction. Primer optimization was done to determine the primer pair with highest efficiency and correlation for a given tissue of interest (hypothalamus, pituitary or liver). Briefly, 5 μL SYBR Green (SensiFAST™ No-ROX Kit, Bioline, London, UK), each primer pair (10 μM, 0.4 μL forward and reverse) and 0.2 μL RNase free water was mixed with a series of diluted cDNA samples (4 μL) [1:2, 1:4, 1:8, 1:16, water (no template control)] in triplicate for a reaction volume of 10 μL and run using a Bio-Rad CFX96 Real-Time System on a C1000 Touch Thermal Cycler followed by melt-curve analysis. The primers pairs were considered adequate if they had efficiencies close to 100 % and correlation coefficients close to 1 ($0.9 < R^2 < 1.0$) (Table 1). Specificity of primer pairs was determined by a melt curve analysis to ensure one specific PCR product (one single amplicon peak). There was no amplification of the no template control (water instead of cDNA).

In order to determine the most stable reference gene for mRNA expression analysis, sampled tissues (hypothalamus, pituitary and liver) from treatments (sham, saline, PTU and T_4) were normalized against three housekeeping genes (elongation factor

1α, EF1α; β-actin; ribosomal 18S) to test stability. Normfinder software (Andersen, Jensen, & Ørntoft, 2004) in RStudio (V 1.2.5001, RStudio Team, Boston, MA) was run to determine the reference gene with lowest cycle variance (highest stability). For central tissues (hypothalamus and pituitary), β-actin was the most stable, with 18S having highest stability in liver tissues.

Relative mRNA expression analysis was carried out using a Bio-Rad CFX96 Real Time System on a C1000 Touch Thermal Cycler with the following program set: 95 ºC for 2 min followed by 40 cycles of 95 ºC for 5 s, 60 ºC for 10 s and 72 ºC for 20 s. On a 96-well plate, cDNA samples were run in duplicate, including a no template control (water instead of cDNA) for a reaction volume of 10 µL. Expression levels were compared using the relative Ct (ΔΔCT) method using the CFX Maestro Software (Bio-Rad, Mississauga, ON, Canada) and was calculated as follows: (1) The average Ct of the reference gene (β-Actin or 18S) was subtracted from the average Ct of the transcript of interest to determine the ΔCT for each sample, (2) the ΔCT of the calibrator (fish with saline pumps) was subtracted from the ΔCT of each of the samples to determine the ΔΔCT, and (3) this number was then used to determine the amount of mRNA relative to the calibrator and normalized by β-Actin or 18S. Control groups (saline pumps) relative mRNA expression was set at 100 % and other ration levels were displayed relative to this by the formula: [(100 x mean Ct of each sham or pump treatment) / average Ct of saline fish].

4.2.7. Total thyroxine and total triiodothyronine ELISA

Enzyme-linked immunosorbent assays (ELISAs) for total T_4 (tT_4) and total T_3 (tT_3) were purchased from Monobind Inc. (Lake Forest, CA, USA). Validation of the ELISA was done by running serial dilutions of serum samples adjacent to standards – a parallel relationship between samples and standards ensured kit validation. The kits were run following the manufacturer's protocol, with standard curve modifications made to the tT_4 assay. The 100 ng/mL T_4 standard was diluted 1:1.5 with PBS (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 8.0) to produce a standard curve with concentrations of 100 ng/mL – 0.19 ng/mL. This ensured greater accuracy when detecting both high and low serum concentrations for T_4 and PTU fish, respectively. A negative ELISA control consisted of ultrapure analytical grade distilled water (high background). Samples were run in duplicate and read at 450 nm using a Biotek Synergy Mx Fluorescence plate reader (BioTek Instruments Inc., Winooski, VT, USA).

4.2.8. Statistics

All data was analyzed in GraphPad Prism 9 (version 9.0.0) with a significance level set at $p < 0.05$. Data was assessed for normality using the Kolmogorov-Smirnov distance test. If data failed to meet normality, it was logarithmically transformed before analysis. Food intake, feeding behaviour, relative mRNA expression and hormone data were analyzed using an ordinary one-way ANOVA followed by Tukey's multiple comparison test. In some instances, a more robust unpaired t-test was run between treatments (PTU and T_4) and controls (saline). Data are expressed as mean ± SEM.

4.3. Results

4.3.1. Serum total thyroid hormones

Pump implantation had no effect on TH levels, as there were no differences in hormone levels between sham-operated and saline treated fish (Suppl. Fig. B1)

Fish implanted with pumps containing T_4 had significantly higher serum levels of tT_4 compared to PTU and controls (Fig. 4.1A, one-way ANOVA). Goldfish with PTU pumps had lower serum tT_3 levels compared to saline controls (Fig. 4.1B, unpaired t-test). A lower tT_3 to tT_4 ratio was seen in T_4-implanted fish compared to PTU-treated and control fish (Fig. 4.1C, one-way ANOVA).

4.3.2. Feeding behaviour and food intake

There were no significant differences in food intake (Suppl. Fig. B2) or feeding behaviour (data not shown) between saline-implanted fish, sham fish and untreated controls, suggesting the surgical procedure did not induce stress or alter behaviour.

When comparing between experimental treatments, goldfish implanted with T_4 pumps had a significantly higher number of feeding acts over 12 days compared to PTU- and saline-treated fish, while no differences were seen between PTU- and saline-treated fish (Fig. 4.2A, one-way ANOVA). There were no significant differences in daily food intake between treatments (Fig. 4.2B).

4.3.3. Central thyroid axis transcripts

Hypothalamic TRH expression was not altered in fish implanted with PTU or T_4 compared to controls (Fig. 4.3A). In the pituitary, transcripts of TRH-R type 1 were not affected by either PTU or T_4 (Fig. 4.3B), but TSHβ expression was significantly lower in fish implanted with T_4 pumps compared to saline-treated fish (Fig. 4.3C, one-way ANOVA).

4.3.4. Central deiodinase transcripts

We assessed the expressions of DIO2 and DIO3. DIO1 was not examined as, unlike DIO2, it can catalyze both activation and inactivation of T_4 (Zavacki et al., 2005).

In the hypothalamus, T_4 administration did not alter hypothalamic DIO2 expression (Fig, 4.4A), but increased DIO3 expression compared to controls (Fig. 4.4B, one-way ANOVA). In pituitaries, T_4 significantly downregulated DIO2 and upregulated DIO3 transcripts compared to controls (Fig 4.4C, 4.4D, one-way ANOVA). PTU had no effect on the expression of deiodinase enzymes in either tissue.

4.3.5. Deiodinase and thyroid receptor hepatic transcripts

In the liver, DIO2 was significantly downregulated by T_4, treatment but was not affected by PTU, compared to controls (Fig. 4.5A, one-way ANOVA). UGT1A1, TRα and TRβ expressions were not affected by either PTU or T_4 treatment (Fig. 4.5B-D).

4.3.6. Central appetite-regulating transcripts

KLα, KLβ, and CCK expressions were significantly downregulated following T_4 treatment compared to controls, but were not affected by PTU (Fig. 4.6A, 4.6B, 4.6D; unpaired t-test and one-way ANOVA). Orexin levels were higher in PTU-treated fish compared to T_4-treated fish, but neither T_4 nor PTU-treated fish differed from saline controls (Fig. 4.6C, one-way ANOVA). AgRP and CB1 transcripts were not affected by either treatment (Fig. 4.6E, 4.6F).

4.4. Discussion

4.4.1. Effects of experimental hyperthyroidism induced by T_4 treatment

4.4.1.1. Circulating total T_4 and T_3

Chronic T_4 treatment resulted in higher serum tT_4, but not tT_3, levels, leading to a decreased tT_3/tT_4 ratio.

The increase in tT_4 levels shows that chronic infusion of T_4 via osmatic pumps leads to a hyperthyroid state in goldfish. T_4 exposure/injection has previously been shown to increase plasma T_4 levels in other fish species, e.g., yearling coho salmon (Iwata, Nishioka, & Bern, 1987), amago salmon (*Oncorhynchus rhodurus*) (Miwa & Inui, 1985), rainbow trout (*Oncorhynchus mykiss*) (Madsen, 1990) and Mozambique tilapia (*Oreochromis mossambicus*) (Subburaju, Wan, & Lam, 1998). In female rabbitfish (*Siganus guttatus*), T_4 treatment increases levels of both T_4 and T_3 for up to 72 hours after a single injection (Ayson & Lam, 1993). In our study, the lack of increase in tT_3 levels might be due to the progressive stimulation of deactivation (DIO3) and reduction of

bioactivation (DIO2) pathways in response to persistently high TH levels in order to limit concentrations of the bioactive form. This might be a long-term response, and differences in T_3 levels might have been detected had we measured TH levels at several shorter time intervals.

4.4.1.2. Feeding behaviour and food intake

Chronic administration of T_4 increased feeding/searching behaviour, shown by an increase in total number of feeding acts, but did not affect food intake. T_4-treated fish displayed a high number of "incomplete feeding acts", i.e., spitting up of pellets or bumping or missing of pellets without actual ingestion.

Our results are in line with previous research in mammals [e.g., mice (Murphy & Nagy, 1976); rats (McEachron, Lauchlan, & Midgley, 1993)] and reptiles [e.g., tiger salamanders (*Ambystoma tigrinum*) (Duvall & Norris, 1980)] showing that T_4 administration increases locomotor/searching activity. In mammals and birds, along with increased locomotor activity, exposure to THs usually increases food intake [e.g., mice (Shinya Ishii et al., 2008; Kong et al., 2004), domestic fowl (Bermudez, Forbes, & Injidi, 1983) and red-winged blackbirds (*Agelaius phoeniceus*) (Robinzon & Rogers, 1979)].

Similarly, a number of studies in fish [e.g., Atlantic cod (*Gadus morhua*) (Castonguay & Cyr, 1998); firemouth cichlid (*Thorichthys meeki*) and Jack Dempsey (*Cichlasoma biocellatu*) (Spiliotis, 1973; Woodhead, 1970)] show similar increases in locomotor activity upon T_4 exposure. Whether this increased locomotor activity is mirrored by an increase in food consumption as seen in mammals is not clear, as to our knowledge no study has examined the two in conjunction.

Very few studies have been published on the effects of THs on feeding behaviour and food intake in fish. In both goldfish (single injection of 0.5 µg/g; Goodyear, 2012) and green sunfish (*Lepomis cyanellus*) (100 µg; (Goodyear, 2012; Gross, Fromm, & Roelofs, 1963), IP injections of T_4 increase food intake, and in juvenile coho salmon, fish fed T_4 and T_3 supplemented feed (20 ppm, but not 50-100 ppm) display increased food consumption (Higgs, Fagerlund, McBride, & Eales, 1979). The discrepancy in results between previous studies and our study could be due to different doses of THs or treatment methods (single vs. chronic treatment; oral administration vs. single injections).

4.4.1.3. Central thyroid transcripts

T_4 treatment had no effect of either hypothalamic TRH expression or pituitary TRH-R expression but induced a decrease in pituitary TSHβ expression.

In mammals, there is an inverse relationship between TH levels, and hypothalamic TRH, and pituitary TRH-R and TSH expression levels. Rats IP implanted for 7 days with osmotic pumps containing T_3 have suppressed TRH mRNA (Kakucska, Rand, & Lechan, 1992), while hypothalamic T_3 implants reduce both TRH immunoreactivity and mRNA expression (Dyess et al., 1988), demonstrating a direct negative feedback of THs on the hypothalamus. At the level of the pituitary in rats, both T_4 and T_3 reduce the number of TRH receptors *in vitro* (Perrone & Hinkle, 1978), and injections of T_3 *in vivo* reduce TRH-R transcript levels (Schomburg & Bauer, 1995). In both humans and rats, T_4 treatment decreases plasma TSH concentrations (P. R. Larsen & Frumess, 1977; Reichlin & Utiger, 1967), and T_3 injections represses TSH transcription after 5 days of daily treatment in mice (Shupnik, Chin, Habener, & Ridgway, 1985). It

has been shown in hyperthyroid rats that changes in TRH are small compared to alterations in TSH (De Greef, Rondeel, Van Haasteren, Klootwijk, & Visser, 1992), suggesting that in mammals, the feedback of THs is mainly exerted at the pituitary level.

In fish, TH appears to regulate TSHβ at the level of the pituitary, but there is little evidence for an involvement of THs in the regulation of hypothalamic TRH, and to our knowledge, there are no studies examining the effect of THs on TRH-Rs in fish.

Our results are consistent with previous studies in fish showing a lack of effects of THs on TRH levels. Injections of T_4 in common carp (Geven et al., 2006), injections of T_3 in Nile tilapia (*Oreochromis niloticus*) (Ogawa et al., 2013) and immersion of juvenile Senegalese sole (*Solea senegalensis*) in T_4 (Iziga et al., 2010) do not affect TRH expression. The reason for the lack of hypothalamic TRH response to THs is not known. In goldfish, it has been suggested that a feedback to the pituitary occurs through secretion of a thyrotrophin inhibitory factor (TIF; released from the hypothalamus to inhibit pituitary TSH release), as implantation of T_4 pellets in the hypothalamus decreases thyroid activity (radioiodine uptake into the thyroid) (R. E. Peter, 1971) and pituitary lesions induce hyperthyroidism (R. E. Peter, 1970). However, to date, this TIF has not yet been identified (Bromage, 1975).

Our results support the existence of a direct regulation of TSHβ by T_4 at the level of the pituitary. Similarly, injections with T_4 in common carp (Geven et al., 2006) and immersion in T_4 or T_3 in red drum (*Sciaenops ocellatus*) (Jones, Cohn, Wilkes, & MacKenzie, 2017), suppress the expression of pituitary TSHβ (and TSHα in red drum).

Our results suggest that THs (specifically T_4) negatively feed back to the thyroid axis but exert actions at the level of the pituitary, and not at the level of the hypothalamus.

4.4.1.4. Central deiodinases and hepatic deiodinases and TRs

In our study, T_4 treatment decreased the mRNA expression of pituitary DIO2 and increased the expression of DIO3 in both the hypothalamus and pituitary.

Similar to our results, in mice, daily T_3 IP injections lead to the upregulation of brain and pituitary DIO3 expression (Barca-Mayo et al., 2011), and subcutaneous T_3 injections decrease DIO2 activity in the pituitary (Croteau, Davey, Galton, & St Germain, 1996). Incubation of rat hypothalami with radioiodide labeled T_4 + T_3 results in non-detectable formation of labeled T_3, indicative of reduced bioactivation under increased T_4 levels (Kaplan & Yaskoski, 1981).

To our knowledge, there are only two other studies examining the effects of THs on DIO2 and DIO3 in central tissues in fish. In red drum, a combined T_4 and T_3 treatment decreases pituitary DIO2 and inhibits DIO3 expression (Jones et al., 2017), and in striped parrotfish (*Scarus iseri*), T_3 treatment (by immersion) upregulates DIO3 brain expression but does not affect brain DIO2 expression (Johnson & Lema, 2011). In our study, the 12-day period of T_4 administration likely caused a long-term response in central tissues, increasing central deactivation and reducing bioactivation. However, despite a pronounced decrease in pituitary DIO2 expression – suggesting feedback to inhibit TSHβ - there was only a weak effect of T_4 on hypothalamic DIO2.

It is possible that low DIO2 expression levels in goldfish hypothalamus [~10-fold compared to pituitary, similar to what is seen in parrotfish (Johnson & Lema, 2011)] might have prevented us from detecting major variations in transcript levels, even under conditions of increased circulating T_4 levels.

Similar to what occurs in central tissues, T_4 decreased hepatic DIO2 expression, suggesting reduced bioactivation of TH in the liver, but had no effect on hepatic UGT1A1 or TH receptor expression.

In mammals, DIO2 is absent from the liver and primarily expressed in brown adipose tissue and the CNS (Köhrle, 1999) whereas birds display high hepatic DIO2 expression levels (Gereben et al., 1999). In mammals, DIO1 is present in hepatic tissues and converts T_4 to T_3 (P. R. Larsen & Zavacki, 2012). In pigs, hyperthyroid induction by T_4 administration increases the activity and immunoblotting signal of hepatic DIO1 (Wassen et al., 2004), while mice show upregulated DIO1 mRNA expression and activity when injected IP with T_3 over 14 days (Jonas et al., 2015; Zavacki et al., 2005). Unlike mammals, birds display DIO2 expression in the liver (Gereben et al., 1999). Broiler chickens fed both T_4 or T_3 in the diet show a decrease in hepatic 5'-monodeiodination (Decuypere, Buyse, Scanes, Huybrechts, & Kuhn, 1987) and incubation of chicken liver homogenates in T_4 results in an increase of T_3 in a time-dependent manner (Lam & Harvey, 1986), suggesting increased hepatic T_4 to T_3 conversion by DIO2.

Our results of are in line with other studies in fish showing decreased hepatic DIO2 mRNA expression following TH exposure [larval sea lampreys (*Petromyzon marinus*) (Stilborn, Manzon, Schauenberg, & Manzon, 2013); killifish (*Fundulus*

heteroclitus) (García-G, Jeziorski, Valverde-R, & Orozco, 2004); Nile tilapia (Mol, Van der Geyten, Kühn, & Darras, 1999)], likely compensating for increased levels of circulating T_4.

Glucuronidation by hepatic UGTs (in particular the isozyme, UGT1A1) is one of the major degradation pathways of T_4 and the formation of TH glucuronides allow their biliary and fecal excretions (van der Spek, Fliers, & Boelen, 2017). Degradation pathways tend to be stimulated when animals are exposed to increased TH levels. For example, in mammals, *in vitro* treatment of liver microsomes with T_4 increases UGT activity and expression in a dose-dependent manner (Hong & Kim, 1997; Kato et al., 2008; Visser, Kaptein, & Harpur, 1991).

In our study, hepatic UGT1A1 expression was not affected by T_4 treatment. To our knowledge, the effects of THs on UGTs have not been assessed in fish. However, in zebrafish (*Danio rerio*), treatment with the pesticide pentachlorophenol (PCP) increases plasma T_4 levels, and decreases T_3 levels, and concurrently increases mRNA levels of hepatic UGT1A3 [another form of UGT1 encoded by a different exon (Wang, Huang, & Wu, 2014; Yu et al., 2014)], likely in order to increase biliary elimination of conjugated THs when T_4 is elevated, similar to what is seen in mammals.

In mammals, TRs are activated once T_3 enters a target cell and binds to TR domains (Chi, Chen, Tsai, Tsai, & Lin, 2013), however, TR expression levels show an inverse correlation to levels of TH. In rats, hepatic TRα and TRβ expressions are decreased when T_3 levels are high (Sadow et al., 2003), and in adult mice, chronic T_3 injections (for 14 days) decreases hepatic TRα and TRβ mRNA levels (Ohba et al.,

2017). In our study, T_4 had no effect on hepatic TR expression, which is in contrast with previous studies in fish. Hepatic TRα and TRβ expressions are both upregulated following T_3 exposure in male striped parrotfish (Johnson & Lema, 2011) and following T_4 exposure in conger eel (*Conger myriaster*) (Kawakami et al., 2006). In addition, treatment of Senegalese sole larvae previously exposed to thiourea (which prevents T_4 formation in thyroid follicles) with T_4 upregulates TRβ expression (Manchado, Infante, Rebordinos, & Cañavate, 2009), suggesting that THs activates the transcription of TRβ and in turn, downstream genes. The lack of expression changes under situations of high TH levels in our study is not clear but may be that due to decreased hepatic bioactivation and high circulating T_4, there was limited T_3 available within cells to elicit a change in the transcription of T_3 receptors.

4.4.1.5. Thyroxine downregulates orexigenic and anorexigenic pathways in the hypothalamus

In our study, T_4 treatment decreased hypothalamic Klotho-α, Klotho-β, OX and CCK expressions but had no effect on either AgRP or CB1.

Klotho proteins are encoded by the KL gene and consist of Klotho-α, Klotho-β or Klotho-γ, which have been shown to be involved in many physiological processes in mammals, including ageing and nutrient metabolism (Rao, Landry, et al., 2019). These proteins are essential co-receptors for the high-affinity binding of fibroblast growth factors (FGF) to their receptors (FGFRs) (Dolegowska, Marchelek-Mysliwiec, Nowosiad-Magda, Slawinski, & Dolegowska, 2019; Kuro-o, 2019). FGFs regulate metabolic processes in mammals. For example, FGF19 is a satiety hormone secreted

from the intestine following a meal and FGF21 is secreted by the liver during fasting (Kuro-o, 2019) and functions through Klotho-β to regulate glucose and lipid metabolism (Shi et al., 2018). Klotho gene knockout or knockdown mice are lean and have decreased white adipose tissue mass (Ohnishi, Kato, Akiyoshi, Atfi, & Razzaque, 2011; Ohnishi, Nakatani, Lanske, & Razzaque, 2009; Ohnishi & Razzaque, 2010), while overexpression of Klotho leads to suppression of insulin-like growth factor I (IGF-I) (Kurosu et al., 2005). Klotho proteins have also been shown to interact with appetite regulators and general metabolism. Overnutrition leads to the repression of Klotho (Martins, 2016) and ICV injections of α-Klotho in obese mice decrease AgRP and increases POMC neuron activity, mirrored by a reduction in food intake (Landry et al., 2020, 2021); aerobic exercise increases brain expression of Klotho in mice and rats (Ji et al., 2018; Rao, Zheng, Huang, Feng, & Shi, 2019) to eliminate reactive oxygen species produced from oxidative metabolism, suggesting Klotho regulates energy balance. Although the link between thyroid, Klotho and appetite is unclear, it has been shown that preadipocyte cells from obese mice display increases in Klotho mRNA when incubated with T_3 (Mizuno, Takahashi, Okimura, Kaji, & Chihara, 2001).

Very little is known about the functions of Klotho in fish. In zebrafish, Klotho has been shown to be involved in organogenesis (Mangos et al., 2012), ageing and calcification (Singh et al., 2019), but there is no information about its potential role in the regulation of feeding and metabolism. In our study, we show a downregulation in the expression of both hypothalamic Klotho forms under conditions of elevated serum tT_4

levels. While this result is not clear, it may indicate our fish were under an increased metabolic load.

Orexin (OX, also known as hypocretin), is an orexigenic peptide that increases both food consumption and behaviour when injected in fish [e.g., goldfish (Volkoff, Bjorklund, & Peter, 1999)] and mammals [e.g., rats (Sakurai et al., 1998)]. In our study, T_4 did not alter OX expression relative to controls, although hyperthyroid fish had lower OX expression compared to PTU fish. Our results are in line with evidence in rats, in which IP injections of T_4 and T_3 do not affect central OX mRNA levels (S Ishii et al., 2003; López, Seoane, Señarís, & Diéguez, 2001) or the expression of OX receptors (López, Tena-Sempere, & Diéguez, 2010).

In mammals and fish, CCK is a satiation factor secreted by the brain and intestine (Vigna, 1985). Little is known with regard to the relationship between thyroid status and CCK. In rats, neonatal hyperthyroidism result in increased CCK protein levels in the cingulate cortex and hippocampus (Woodhams et al., 1983) but IP injections of T_4 in adult females does not alter hypothalamic CCK mRNA (Holland, Norell, & Micevych, 1998). In our study, downregulation of hypothalamic CCK expression under hyperthyroid conditions might contribute to an increase in energy expenditure in order to counterbalance the energy expending effects of increased circulating T_4.

We observed no effect of T_4 administration on the mRNA expression of AgRP. To our knowledge, there has been no study analyzing the expression of AgRP following thyroid status manipulation in fish. In mammals, AgRP knockout mice are lean and have high circulating TH levels (Flier, 2006) and hyperthyroidism-induced hyperphagia in rats

is associated with upregulated AgRP mRNA (López, Alvarez, Nogueiras, & Diéguez, 2013). However, in humans, similar AgRP levels are seen between hyperthyroid and euthyroid patients (Tohma et al., 2015).

There is emerging evidence on the role of CB1 receptors in regulating food intake. In rodents, CB1 mRNA is co-expressed with other appetite-regulating peptides, e.g., OX and cocaine- and amphetamine related transcript (CART) and knocking out CB1 decreases CART mRNA expression and leads to leanness (Cota et al., 2003). In rats, CB1 mRNA expression in the striatum increases after IP T_3 injections and is associated with increased locomotor activity (Asúa et al., 2008; Diez et al., 2008), suggesting an association between THs and CB1 in this brain region. The lack of change in hypothalamic CB1 expression in our study indicates that the endocannabinoid system might not be sensitive to changes in thyroid status in fish, and other brain regions need be analyzed to determine effects, if any.

4.4.2 Effects of PTU implantation

Overall, we saw no major effect of PTU on TH levels, food intake or on the gene expression of thyroid axis components and appetite-regulating peptides.

Mammals (Cooper et al., 1983; Kundu et al., 2006) and birds (Bachman & Mashaly, 1987; Leung, Taylor, & Van Iderstine, 1985) respond to PTU treatment by clear decreases in TH levels. In our study, PTU treatment had no effect on tT_4 levels, but resulted in a small but significant decrease in serum tT_3 levels. Our results are in line with other studies is fish showing PTU-induced decreases in T_3 levels, e.g., in coho salmon

(Ebbesson, Björnsson, Stefansson, & Ekström, 1998), Pacific hagfish (*Eptatretus stoutii*) (Kerkof, Boschwitz, & Gorbman, 1973; Plisetskaya & Gorbman, 1982) and climbing perch (*Anabas testudineus*) (Varghese & Oommen, 1999). However, other studies show no effect of PTU on circulating T_3 levels [e.g., coho salmon (Sullivan, Darling, & Dickhoff, 1987), rainbow trout (Eales, 1981; Milne & Leatherland, 1978) and steelhead trout (*O. mykiss*) (Allen & Cristy, 1978)]. Differences between studies are likely due to differences in administration mode [i.e., immersion (Ebbesson et al., 1998) versus supplemented feed (Sullivan et al., 1987)], in doses or in duration of treatment. It seems that either low- or high-concentrations of PTU administered affects the T_3 to T_4 ratio. In Mozambique tilapia, feeding low dose (5 µg/g) of PTU increases plasma T_4 levels but both T_3 and T_4 levels decrease after feeding a high dose (20 µg/g) of PTU for 15 days (M. C. S. Peter & Peter, 2009); In zebrafish, PTU treatment (100 mg/L in water) decreases circulating levels of both T_3 and T_4 (Van Der Ven, Van Den Brandhof, Vos, Power, & Wester, 2006).

With a lack of major decreased circulating THs in our study, we also see a lack of an effect by PTU on food intake and behaviour. In mammals, the effects of PTU treatment on feeding are contradictory and unclear. For example, food intake decreases in rats fed a diet supplemented with PTU after 30 days (Hood, Liu, & Klaassen, 1999), and during long-term (6 months) PTU treatment (given in water) (Alva-Sánchez, Pacheco-Rosado, Fregoso-Aguilar, & Villanueva, 2012). Conversely, another study shows mice fed PTU display increases in food intake and body weight compared to controls (Johannessen, 1966). Behavioural consequences of PTU administration are less known,

with some rats showing a reduction in food intake also mirrored in behavioural changes, i.e., reduced locomotor activity when PTU exceeds 20 mg/kg/day administered via oral gavage over 24 days (Nambiar et al., 2013).

In our study, PTU administration did not alter either feeding behaviour or food intake. In fish, there is no clear evidence of an effect of PTU on feeding. In coho salmon, diets containing PTU reduce food consumption in wild [(6.0 mg/g) (Sullivan et al., 1987)] and growth hormone (GH) transgenic coho salmon [(20 ug/g) (Kang & Devlin, 2003)]. Discrepancies between studies may be due to a different PTU exposure times [12-days in our study compared to 79- (Sullivan et al., 1987) and 84-days (Kang & Devlin, 2003)], doses administered, and delivery methods (implantation versus feed supplemented). Therefore, the 12-day exposure in our experiment may not have depressed TH levels enough to reduce the metabolic actions at target tissues to reduce feeding behaviour and appetite. A reason for this may be due to the diffuse nature of thyroid follicles in fish (Chanet & Meunier, 2014), where PTU did not have sufficient time to reduce TH production in follicles and diminish TH output. Unlike mammals (where PTU also affects deiodinases), in fish, PTU is thought to exert its effect primarily at the thyroid follicles by inhibition of the enzyme TPO – although no TPO homologue has currently been discovered in fish (Klaren, Geven, & Flik, 2007).

A lack of change in feeding behaviour in our study is likely due to the lack of effect of PTU on circulating TH levels. To our knowledge, there has been no studies examining the effects of PTU of feeding behaviour in fish, however, in mummichog (*F. heteroclitus*), individuals from a polluted river site have enlarged thyroid follicles,

elevated T_4 levels but normal T_3 levels, and display slow, sluggish behaviour and poor success in prey capture (Zhou, John-Alder, Weis, & Weis, 2000), possibly a result of contaminant-induced inhibition of TH receptors, which would in turn block the negative feedback in the hypothalamus–pituitary–thyroid axis.

As PTU had no clear effects on TH levels, it is not surprising that no significant expression changes were seen in central and hepatic transcripts of thyroid axis-related genes.

In rodents, PTU-induced hypothyroidism induces decreases in hypothalamic TRH levels/expression (Perello & Nillni, 2007; Segerson et al., 1987), pituitary TSH levels/expression (Männistö, Ranta, & Leppäluoto, 1979; Perello & Nillni, 2007) and a decrease in the number (de Lean A., Ferland, Drouin, Kelly, & Labrie, 1977) and mRNA expression (Mori, Yamada, & Kobayashi, 1988; Schomburg & Bauer, 1995) of pituitary TRH receptors. PTU also inhibits hepatic DIO1 (Mandel et al., 1992) and upregulates hepatic TRα and TRβ mRNA expression (Zandieh-Doulabi, Platvoet-ter Schiphorst, Kalsbeek, Wiersinga, & Bakker, 2004). Central tissues also show increased DIO2 activity during PTU induced hypothyroid conditions in mammals (Bates, St. Germain, & Galton, 1999; Serrano-Lozano, Montiel, Morell, & Morata, 1993) and birds [chicken (Gereben et al., 1999)].

In our study, it is possible that PTU administered for a longer period or at higher concentrations, might have induced hypothyroid conditions and led to changes in the expressions of hypothalamic TRH and pituitary TSHβ centrally, as seen in zebrafish (Liu et al., 2011; Schmidt & Braunbeck, 2011)]. To our knowledge, in fish, direct effects of

PTU on thyroid follicles have only been shown in zebrafish (Van Der Ven et al., 2006) and sea bream (*Sparus aurata*) (Campinho, Morgado, Pinto, Silva, & Power, 2012), where PTU immersion leads to activation of thyroid follicles (i.e., increased cell size), a response mediated increased TSH binding at follicles, the levels of which increase owing to low circulating TH levels.

Moreover, PTU did not affect hepatic DIOs, UGT1A1 or TR expression in our study. In fish, treatment with antithyroid agents generally results in increased expression of hepatic DIO2 [methimazole (MMI) in male striped parrotfish (Johnson & Lema, 2011) and Nile tilapia (Mol et al., 1999); PTU in sea bream (Morgado, Campinho, Costa, Jacinto, & Power, 2009)] and a decrease in TRs mRNA [MMI decreases TRα and TRβ in yellow catfish (P*elteobagrus fulvidraco*) (Chen et al., 2015)]. The lack of major PTU-induced tT3 reduction in our study is the likely reason why do not see an effect of PTU on TH activating and signalling pathways.

PTU treatment had has no effect on the expression of hypothalamic appetite-regulating peptides. There is limited evidence on how a hypothyroid state, or goitrogens, affect central neuropeptides in mammals. In rats, AgRP mRNA is not affected by hypothyroidism due to aminotriazole treatment (López, Seoane, Tovar, Señarís, & Diéguez, 2002), but decreases following MMI treatment (Herwig et al., 2014). In hypothyroid rats (via aminotriazole), OX hypothalamic mRNA expression is not affected (López et al., 2001), but CB1 is required to maintain locomotion in MMI induced hypothyroid mice (Giné et al., 2017). These results suggest poor association between a

lowered thyroidal state and appetite-regulating neuropeptides, even though a reduction in food intake and increase in body weight is generally seen.

In our study, PTU had no effect on any of the peptides examined. It is possible that the dosage used was too high. PTU inhibits the TPO-catalyzed iodination of tyrosyl residues in thyroid follicles, and it has been shown in *in vitro* studies that if iodine remains at adequate concentrations, it can block the inhibitory effect of PTU (Taurog, 1976), therefore, PTU would lose its effectiveness and not result in lowered circulating THs. The lack of effect could also be due to a relatively short experimental period, which would not have provided enough time for a widespread action of PTU, as thyroid follicles are largely dispersed throughout goldfish body (Richard E Peter, 1970). Finally, as PTU has been shown to have species-specific effects in mammals (Paul et al., 2013), it is possible that PTU does not induce TPO inhibition in fish thyroid follicles.

4.5. Conclusion

In this study, we show that a hyperthyroid state was induced by T_4 administration over 12 days, but implantation with PTU did not create hypothyroidism. The elevated TH load caused an increase in the number of total feeding acts, indicating a behaviour-mediated response, however with no change in food consumption. In goldfish, according to our results, the pituitary, but not the hypothalamus, is sensitive to increased levels of T_4 and displays patterns consistent with the negative feedback seen in fish and mammals. Hepatic tissues show a long-term response that tends to reduce bioactivation of THs, with no changes in receptor expression, likely due to a limited amount of circulating tT_3 after

12 days. The expression of genes associated with appetite does not indicate an association between altered thyroid status and appetite regulation. The responsiveness of the Klotho protein suggests a very strong association between circulating THs and this gene and indicates it may play a metabolic role similar to what is seen in mammals. Our hypotheses are partially supported, in that (1) negative feedback similar to that of mammals was seen under increased THs, but only in the pituitary, while PTU did not elicit a response, and (2) an increase in feeding behaviour, but not in food intake, was observed, and these changes do not seem to be due to changes in the expression of central appetite regulating peptides.

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Figures

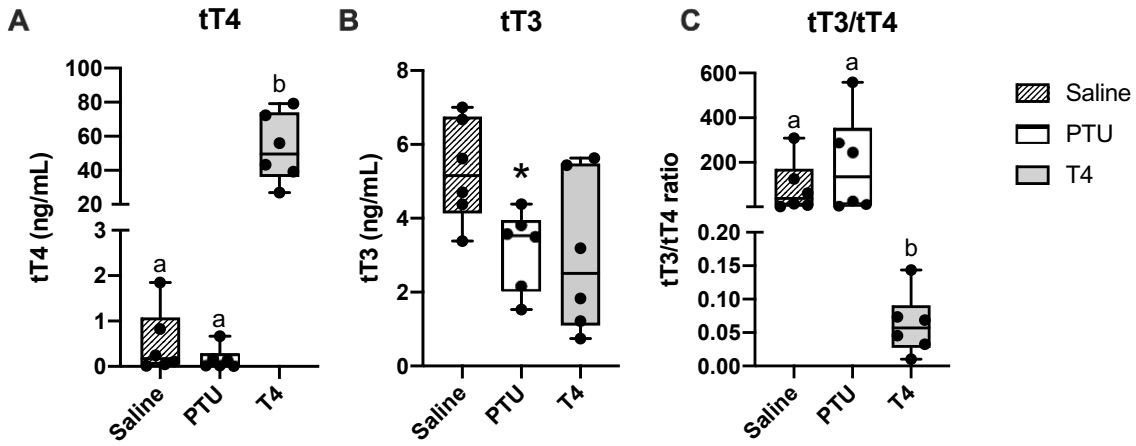

Figure 4.1. Serum concentrations (ng/mL) of circulating total (tT4, A), total T3 (tT3, B) and the ratio of total T3 to T4 (tT3/tT4, C) for fish implanted with saline (control, n = 6), PTU (n = 6) and T_4 (n = 5). Boxes represent 25th, 50th and 75th percentiles of the dataset and whiskers represent minimum and maximum values of the dataset. Dissimilar superscripts indicate significant differences between all groups (one-way ANOVA, $p < 0.05$), and stars (*) indicate groups significantly different from the control (unpaired t-test, $p < 0.05$).

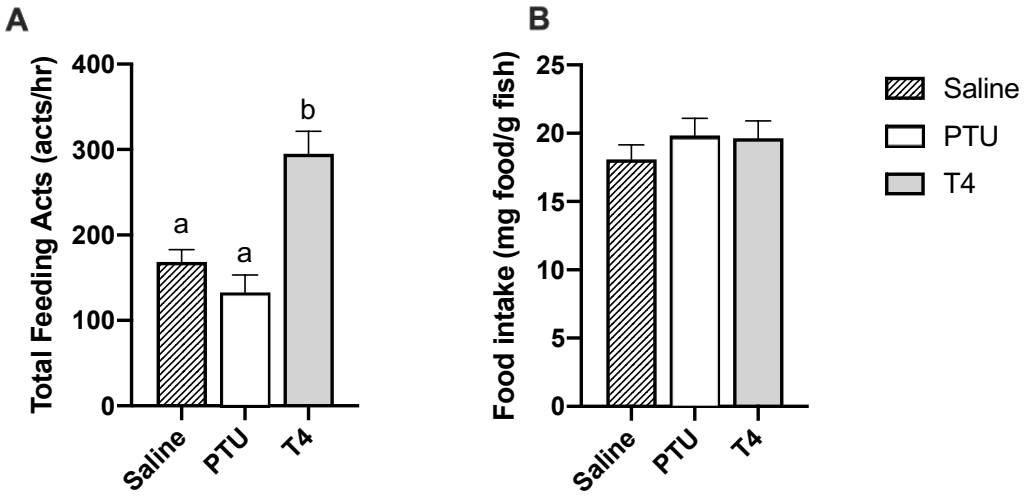

Figure 4.2. Total feeding acts (A) and food intake (B) averaged over 12 days for fish implanted with osmotic pumps containing saline (control, n = 12 observations), PTU (n = 6 observations) and T_4 (n = 7 observations). Total feeding acts is expressed as the total number of acts per hour, and food intake is measured as milligrams of food per gram body weight of fish. Dissimilar superscripts between groups indicate significant differences (two-way ANOVA, $p < 0.05$).

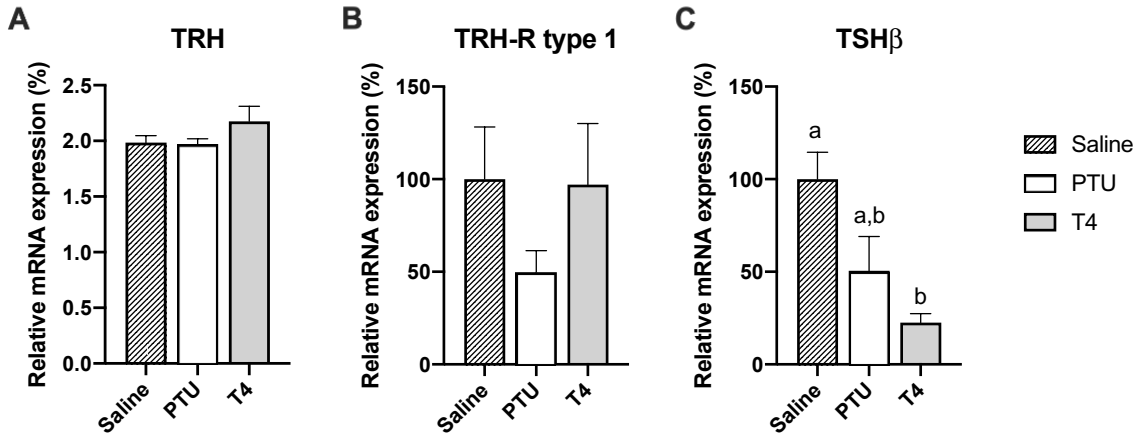

Figure 4.3. Relative mRNA expression of hypothalamic TRH (A), pituitary TRH-R type 1 (B) and TSHβ (C) for fish implanted with osmotic pumps containing saline (control, n = 6), PTU (n = 6) and T_4 (n = 6). Data is expressed as mean ± SEM and control fish data are normalized to 100 %. Dissimilar superscripts between groups indicate significant differences (one-way ANOVA, $p < 0.05$).

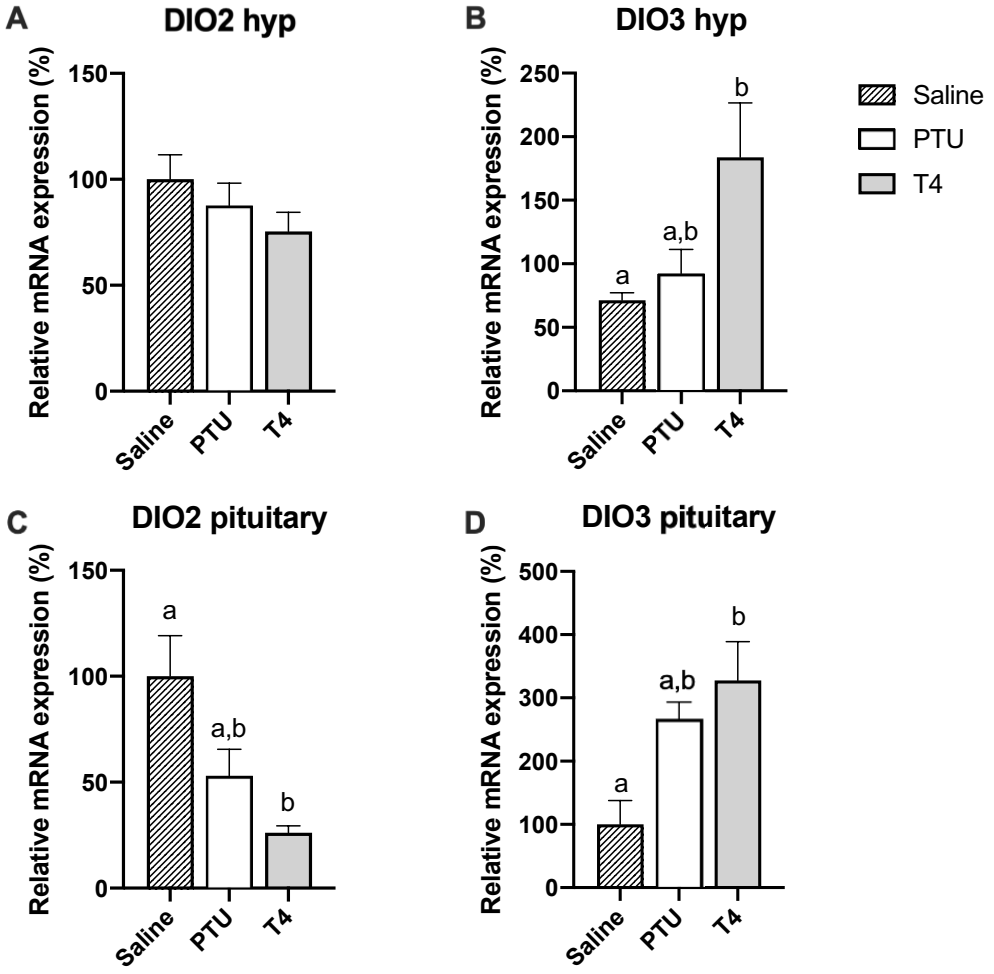

Figure 4.4. Relative mRNA expression of hypothalamic (hyp) DIO2 (A) and DIO3 (B), and pituitary DIO2 (C) and DIO3 (D) for fish implanted with osmotic pumps containing saline (control, n = 6), PTU (n = 6) and T_4 (n = 6). Data is expressed as mean ± SEM and control fish data are normalized to 100 %. Dissimilar superscripts between groups indicate significant differences (one-way ANOVA, $p < 0.05$).

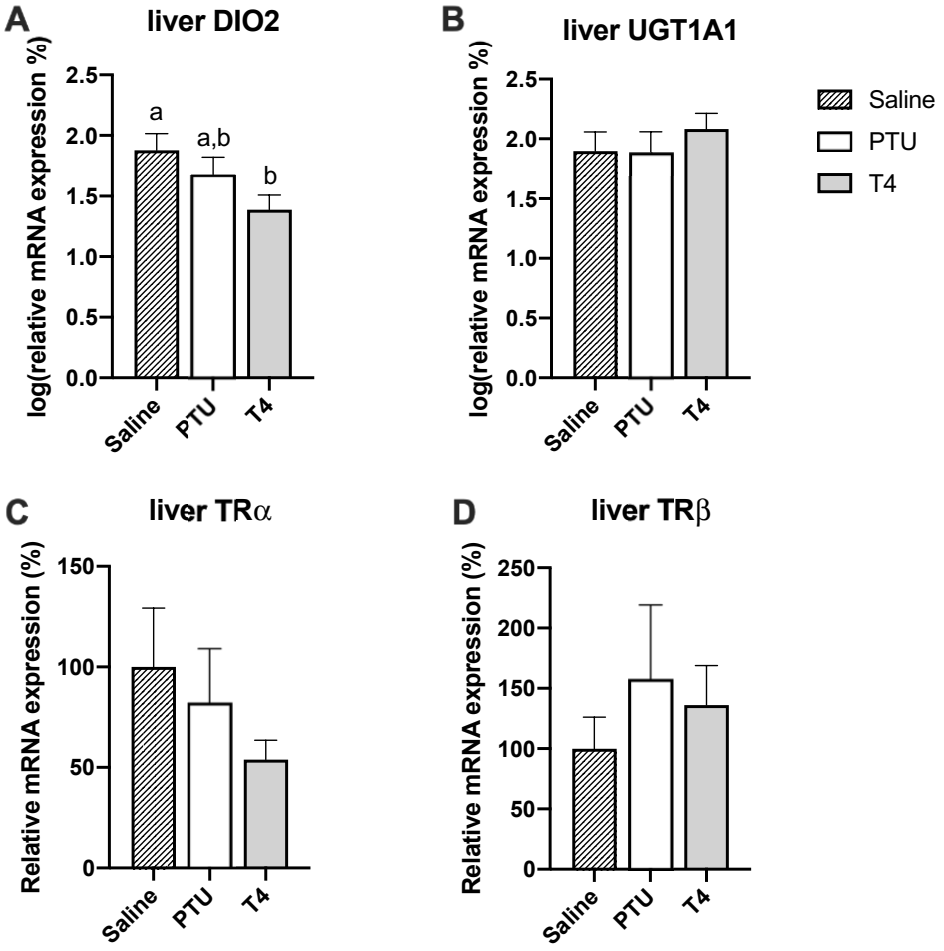

Figure 4.5. Relative mRNA expression of liver DIO2 (A), UGT1A1 (B), TRα (C) and TRβ (D) for fish implanted with osmotic pumps containing saline (control, n = 6), PTU (n = 6) and T_4 (n = 6). Data is expressed as mean ± SEM and control fish data are normalized to 100 %. Dissimilar superscripts between groups indicate significant differences (one-way ANOVA, $p < 0.05$).

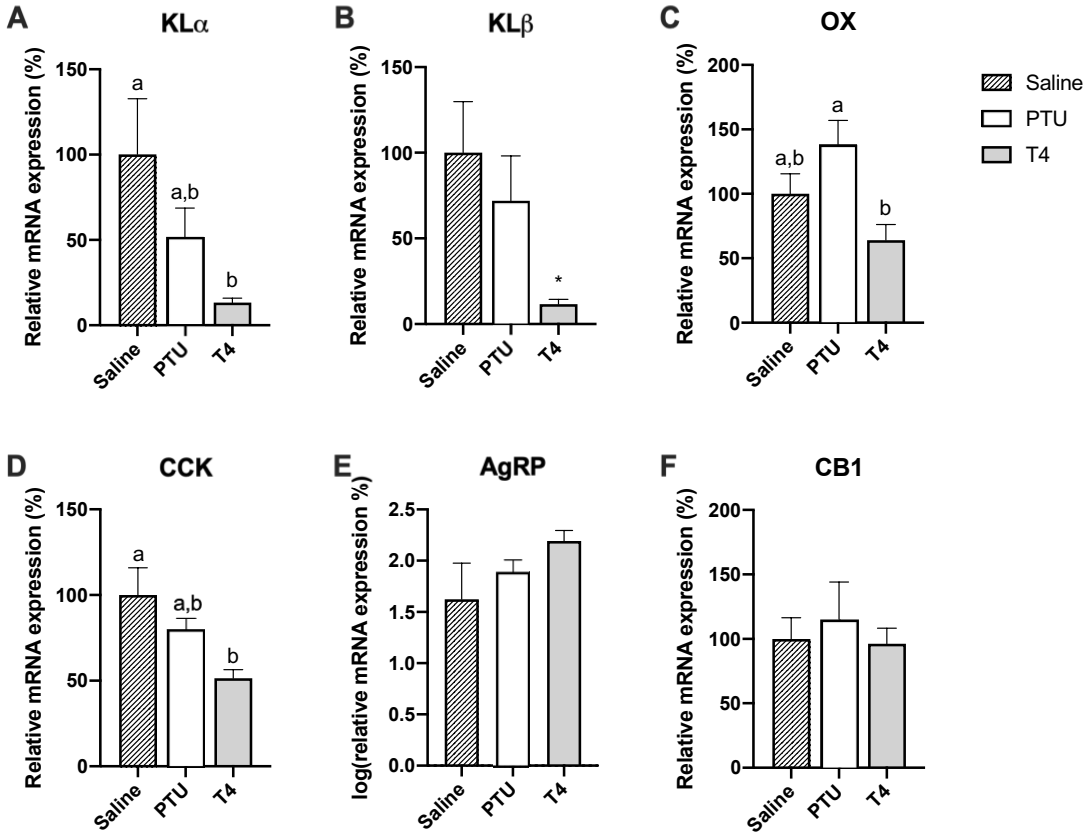

Figure 4.6. Relative mRNA expression of hypothalamic KLα (A), KLβ (B), OX (C), CCK (D), AgRP (E) and CB1 (F) for fish implanted with osmotic pumps containing saline (control, n = 6), PTU (n = 6) and T_4 (n = 6). Data is expressed as mean ± SEM and control fish data are normalized to 100 %. Dissimilar superscripts indicate significant differences between all groups (one-way ANOVA, $p < 0.05$). Stars (*) indicate significant differences compared to controls only (unpaired t-test, $p < 0.05$).

Tables

Table 4.1. Sequences of primers used in the study with GenBank Accession number, efficiency (%) and correlations (R^2).

Primer	Direction	5' – 3' Sequence	GenBank Accession #	Efficiency (%)	R^2
TRH	Forward	AGACGGAGGACGAGAACCAC	AB179819.1	97 %	0.98
	Reverse	CGTCTTCGTAGTCGGTGTCC			
TRHR type 1	Forward	TGCTTCTCGGAGACAGGTGA	XM_026283702.1	98 %	0.99
	Reverse	GGTTGATGGCGCTGTTCAAG			
TSHβ	Forward	CTGTCAACACCACCATCTGC	AB003584.1	102 %	0.98
	Reverse	GGCACATTCATCACTGTTGG			
AgRP	Forward	ATGGCATCACATCCAAACC	AJ555492.1	98 %	0.98
	Reverse	GCTTTACCCAGATCCTCATCA			
CCK	Forward	GAGGATGATGAAGAGCCCCG	U70865.1	97 %	0.98
	Reverse	TGTTGCCCATGGACTTGCTT			
KL-α	Forward	TGTGGCACCTGGTATCAAAA	XM_026220001.1	105 %	0.98
	Reverse	GGCTTTGCTGTTCTCCTGTC			
KL-β	Forward	TCGGTGTGTCCGAGTCAGTA	GBZM01008473.1	101 %	0.97
	Reverse	TGTGAAGCAGGACTCCAGTG			
CB1	Forward	GCAGCGTCATCTTCGTCTAC	XM_026194969.1	102 %	0.99
	Reverse	GCGCTCCTAACTTGAACAGA			
OX	Forward	GAGTTCAGCTGCTCCTCTTCA	DQ923590.1	99 %	0.98
	Reverse	ACTGCCGCGTCGTTATTAAA			
TRα	Forward	CCATCACACCAGTTGTGGAC	AY973629.1	99 %	0.94
	Reverse	CCTCCATTCTTCAGCTGCTC			
TRβ	Forward	GTGTCTCGCTGTCCTCCTTC	AY973630.1	96 %	0.91
	Reverse	CTTGTGCTTGCGGTAGTTGA			
DIO2	Forward	TGTCACTCCTGAGCTGTTCG	EU313786.1	98 %	0.99
	Reverse	GGAGACTCGAAGTCCAGCAG			
DIO3	Forward	TCTGCGTGTCAGACTCCAAC	EU313787.1	91 %	0.98
	Reverse	CTCCCGAAGTTGAGGATCAG			
UGT1A1	Forward	GACAGAACTGGCCCAGAGAG	XM_026272069.1	104 %	0.98
	Reverse	CGCATCCTTCCACCTGTATT			
β-actin	Forward	ACTACTGGTATTGTGATGGACTCC	LC382464.1	98 %	0.97

	Reverse	CGGTCAGGATCTTCATCAGGTAG			
Ribosomal 18S	Forward	AAACGGCTACCACATCCAAG	XR_003291850.1	97 %	0.99
	Reverse	CACCAGACTTGCCCTCCA			

Chapter 5. Conclusion

The purpose of this thesis was to better understand the interplay between the thyroid axis and appetite regulation in goldfish. This was accomplished through altering both nutritional and thyroid status. By measuring circulating levels of thyroid hormone (TH), and the mRNA expression of genes associated with the thyroid axis and appetite-regulation in central and peripheral tissues, I was able to produce original results that fill a current gap in our knowledge of the relationships between central appetite regulation and thyroid function in fish. Using goldfish as a model, I have shown that (1) nutritional status affects the thyroid axis in a time-dependent manner with a weak central and peripheral response of appetite regulating peptides, and (2) thyroid status alteration leads to changes in feeding behaviour but not food intake, does not strongly associate with changes in orexigenic or anorexigenic transcripts but provides strong evidence for thyroid axis negative feedback regulation through the pituitary (and not the hypothalamus).

Nutritional status manipulation is a common tool to examine endocrinological changes in freshwater fish species (Bertucci et al., 2019), as periods of fasting or limited food are a common seasonal occurrence in wild populations. This offers the ability in a laboratory setting to submit experimental animals to “extreme” negative energy scenarios and analyze their response(s). However, the effects of overfeeding, or an abundance or excess of food to provide a positive energy scenario has not received much attention. In chapter 3, I examined how two nutritional statuses, i.e., fasting and overfeeding, affect the thyroid axis and the ability to regulate appetite, and whether there were any clear correlations between the two. I show for the first time that there is a poor temporal

association between fasting and thyroid physiology, while overfeeding leads to an apparent stimulation of the thyroid axis, as seen by upregulation of hypothalamic TRH and TSH. This indicates a central activation of this endocrine axis in order to offset the positive energy accrued when consuming food in excess. Despite an activation of the thyroid axis at the central level, peripheral levels of THs were not responsive to changes in food abundance, regardless of a decrease in central bioactivation (DIO2) and a time-dependent decrease in hepatic deactivation. I uncovered an apparent inhibition of central appetite-regulating circuits (i.e., POMC, AgRP) when food was over abundant, supporting the notion that central thyroid axis activation may have occurred to expend the energy brought about by an increase in caloric consumption.

Creation of hyper- or hypothyroid conditions is a common method in mammalian experimental thyroidal research (Atici, Menevse, Baltaci, & Mogulkoc, 2018; Jouda, Alsamawi, & Qasim Ali, 2017). It is still unclear whether different thyroidal states alter food consumption in fish, as differences in administration methods and species used between studies have led to contradictory results making it difficult to draw clear conclusions. In chapter 4, I created hyperthyroid conditions by 12 days of constant infusion of thyroxine (T_4) and demonstrated that hyperthyroid fish had food intake similar to controls, but displayed more pronounced feeding behaviour, as seen by an increase in number of total feeding acts (specifically more incomplete versus complete). Moreover, the T_4 treatment allowed me to provide evidence for a direct negative feedback of T_4 to the pituitary (downregulating TSH and DIO2, upregulating DIO3). The hyperthyroid state also resulted in responses from appetite-regulating peptides in the

hypothalamus, through a downregulation of anorexigenic peptides (Klotho, CCK), suggesting a stimulation of feeding, which is consistent with the increase in feeding behaviour. Administration of propylthiouracil (PTU) did not result in hypothyroid conditions, or significantly alter the mRNA expression of any gene examined, suggesting that PTU might not be a good thyroid inhibitor in fish.

This research has filled a gap, as past research in fish thyroid endocrinology has focused mainly on the development of methods to increase growth and condition by thyroid treatments on a large scale, with very few studies focused on the actual effect of food on thyroid physiology. To my knowledge no small-scale study has ever examined thyroid manipulation to understand how feeding behaviour is affected when food is present. Furthermore, this research represents the first study in fish to examine the expression of central appetite regulating peptides, when thyroid and energy statuses are altered.

It is noteworthy that the studies present some limitations. For both chapter 3 and 4, I caution that changes in the expression of mRNA do not always correlate to changes in protein levels, especially when measuring the expression of enzymes. This is in large part due to the amount of post-transcriptional and/or post-translational processing that can occur to mRNA and subsequent proteins (Hack, 2004). In chapter 4, although chronic administration was done, hypothyroidism was never established. The use of PTU as an effective goitrogen in fish seems to widely vary across the literature, regardless of concentration or administration type used. Therefore, it may be necessary to utilize more than one dosage per study, or perhaps the use of multiple goitrogens in a single study that

have a similar mechanism of action [similar to what was done by (Campinho, Morgado, Pinto, Silva, & Power, 2012)].

Future thyroid work should attempt to address either short-term (e.g., hours) or long-term (e.g., months) effects in fish, as we have shown that 2-4 weeks may not be a long enough time period to examine changes in expression of genes or levels of hormones. The use of *in situ hybridization* might also be a useful approach. These methods would allow us to examine the expression or protein content of thyroid components and neuropeptides in specific brain nuclei compartments, rather than in whole hypothalamus, and thus to better understand actual neural interactions between peptides.

I believe much can still be garnered from utilizing fish as a tool to study the thyroid axis. There is still plenty to uncover in terms of the functioning of this axis, as the evolution from basal to more derived vertebrates seems accompanied by a transition from hypothalamic independence to the use of TRH as a thyrotropic factor. Furthermore, the use of the thyroid system in fish as a tool to examine potential effects of climate related changes may provide a useful biomarker for wild populations – since I have shown that changes in TH levels mediate food seeking behaviour, then what impact do abiotic factors have on thyroid systems in fish, and will this affect the ability to find/consume food?

5.1. Literature Cited

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Appendix A: Chapter 3 Supplementary Figures

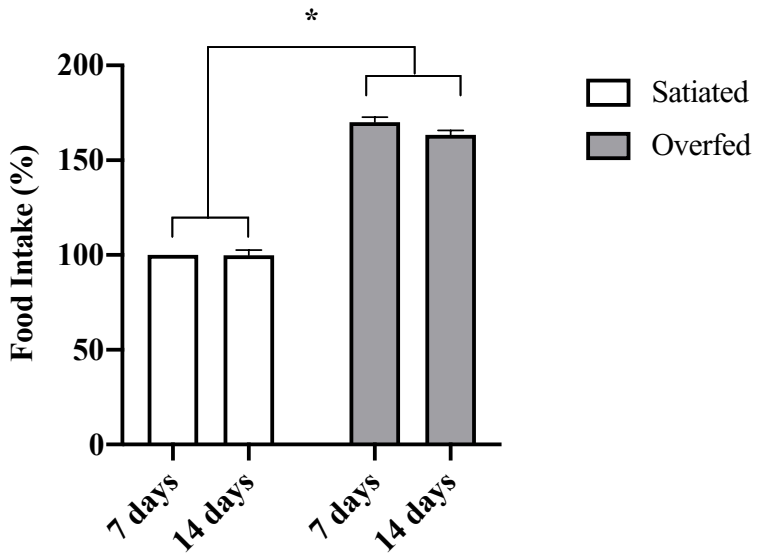

Figure A1. Mean daily food intake of satiated (control) and overfed fish at 7 (n = 40) and 14 (n = 20) days. Food intake of satiated fish at day 7 is normalized to 100 % and all data is presented as mean ± SEM. Stars and bars indicate significant differences between groups (Mann-Whitney t-test, $p < 0.05$).

Appendix B: Chapter 4 Supplementary Figures and Tables

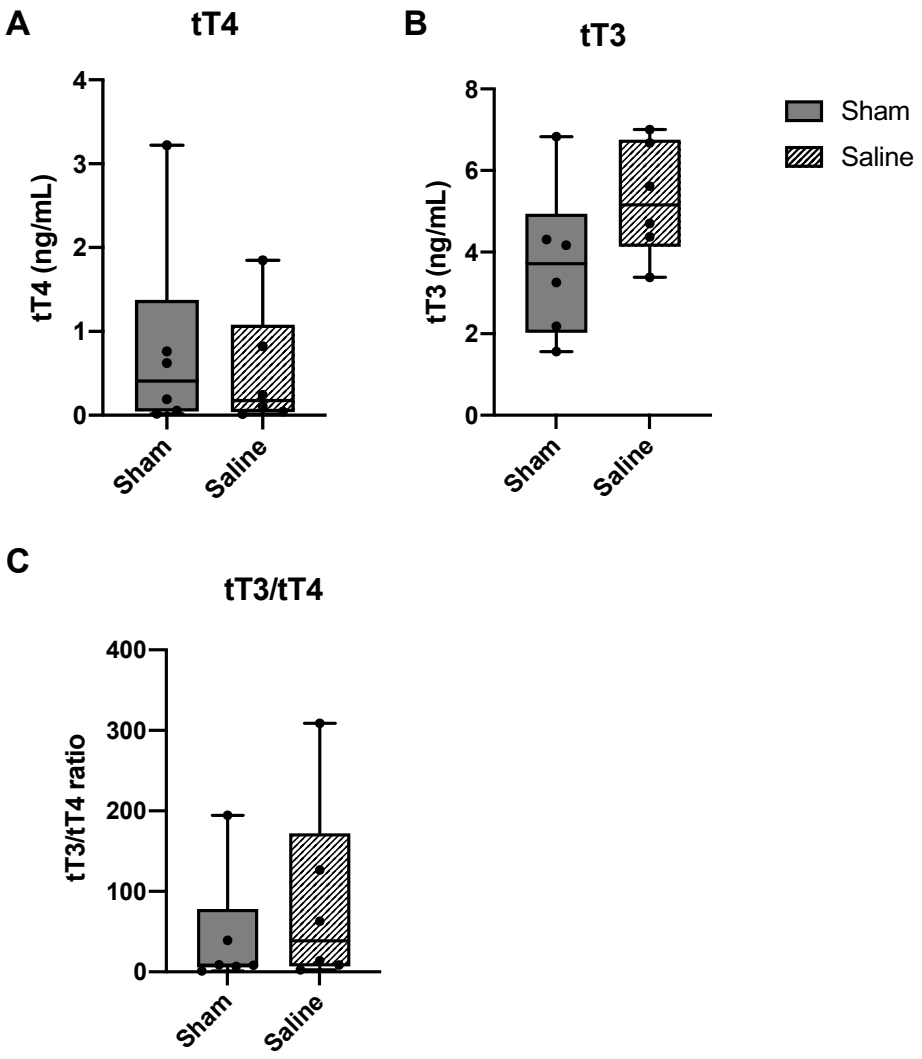

Figure B1. Serum concentrations (ng/mL) of circulating total (tT4, A), total T3 (tT3, B) and the ratio of total T3 to T4 (tT3/tT4, C) for sham fish (n = 4), and fish implanted with saline (control, n = 6). Boxes represent 25th, 50th and 75th percentiles of the dataset and whiskers represent minimum and maximum values of the dataset.

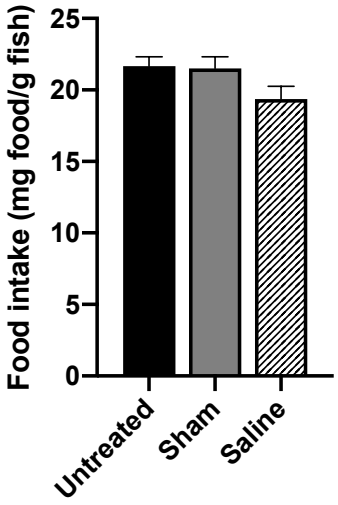

Figure B2. Food intake (mg food/g fish) after 12 days for untreated (n = 6) and sham (n = 6), and fish implanted with osmotic pumps containing saline (control, n = 6).