ABSTRACT

CHEUNG, EUGENE. Leptin, Appetite, and Reproduction: Novel Insights from a Teleost Fish. (Under the direction of Dr. Russell J. Borski).

Reproduction is a physiologically demanding process that relies on energy sufficiency, the lack of which can result in delayed puberty and suppression of reproductive function. The pleiotropic cytokine hormone leptin is a key signal in energy homeostasis that relays information on peripheral energy availability to central circuits. In mammals, leptin suppresses appetite and has been found to serve as a critical permissive signal in the activation and sustained integrity of the gonadotropic axis and onset of puberty. Although leptin effects on reproduction have been explored extensively in mammals, its actions and regulatory properties on reproduction in teleost fishes remain unclear. In the present study, we used the comparative teleost model – the Mozambique tilapia *Oreochromis mossambicus* – to investigate three primary research objectives: (1) Evaluation of leptin and cortisol effects on behavioral food intake and brain appetite-regulatory neuropeptides, and accordingly, whether the vertebrate model for leptin regulation of appetite is reflected in tilapia. Here, we found that treatment with recombinant tilapia leptin A (rtLepA), the dominant paralog of leptin in teleosts, decreased food intake, promoted anorexigenic proopiomelanocortin (*pomc*) and cocaine-and-amphetamine-regulated transcript (*cart*), and inhibited orexigenic neuropeptide Y (*npya*) and agouti-related peptide (*agrp2*). Cortisol initially decreased food intake, but substantially increased food consumption 6-hours post-injection and sharply elevated *npya* and *agrp2*, marginally increased corticotropin-releasing factor (*crf*), and exerted no discernable effect on *pomc* or *cart*; (2) Assessment of a role for leptin in pubertal onset and modulation of hypothalamic factors and gonadal sex steroids. In this second research objective, we found that transcript levels of *lepa* in the liver, the primary source of circulating leptin, increased with expression of kisspeptin (*kiss2*) and gonadotropin-

releasing hormone (*gnrh1*), gonadosomatic index, and sexual maturation of male and female tilapia. Intraperitoneal injection and 6-hour treatment with rtLepA in adult male fish increased brain *kiss2* and *gnrh1* transcript levels, while significantly reducing circulating sex steroid levels. In testicular explant culture, rtLepA suppressed sex steroid production similarly to our *in vivo* evidence; and (3) Examination of leptin interactions with hepatic vitellogenins (*vtg)*, gonadal sex steroids, and a possible role for the hormone in maternal mouthbrooding. Here, we found that in primary hepatocyte culture, rtLepA suppressed *vtgab* and *vtgc* transcript levels, while it stimulated insulin-like growth factor (*igf1*). Testosterone and estradiol reduced *lepa* levels, but disparately regulated *igf1* whereby testosterone increased and estradiol decreased *igf1* at 10 and 100 nM. The non-aromatizable androgen 11-ketotestosterone increased hepatocyte *lepa* and *igf1* transcript levels at 100 nM. Injection of estradiol into male fish resulted in a downregulation of hepatic *lepa* and *igf1* while promoting vitellogenins *vtgaa* and *vtgab*. While *lepa* transcript levels were sharply elevated in fasted females, leptin profiles of mouthbrooding females were similar to those of gravid females. These data suggest that appetite suppression associated with forced starvation during maternal mouthbrooding may not be regulated by leptin. Taken together, these studies suggest that while the appetite-regulatory actions of leptin are conserved among vertebrate classes, the hormone's reproductive actions appear divergent and exert inhibitory effects in teleost fishes. Here, we provide the first evidence for multimodal influence of leptin on the reproductive axis in a teleost fish through enhancement of key hypothalamic gonadotropic targets concurrent with suppression of peripheral factors. The hormone's inhibitory actions are most evident here in its suppression of hepatic vitellogenins and gonadal sex steroids. As leptin promotes somatotropic factors like IGF1 while limiting elements of the gonadotropic axis, we postulate that this effect may be associated with limiting energy partitioning to reproduction in

instances of negative energy balance when leptin has been shown to increase. Collectively, the current studies suggest a potential role for leptin in the onset of puberty in the tilapia, an overall inhibitory effect at peripheral targets including the liver and gonads, and critically, provide a fundamental advancement in our understanding of the role and function of leptin in vertebrates.

Leptin, Appetite, and Reproduction: Novel Insights from a Teleost Fish

by
Eugene Cheung

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Biology

Raleigh, North Carolina
2020

APPROVED BY:

Dr. Russell J. Borski Committee Chair	Dr. Heather Patisaul
Dr. John Godwin	Dr. Benjamin J. Reading

DEDICATION

To the Cheung family cats: Pepper, Pico, and 野-meow

BIOGRAPHY

Eugene Cheung was born in Hong Kong on October 31, 1990 to Edmond and Melody Cheung. He was raised in Richmond Hill, Ontario, Canada. Eugene developed an immense passion for aquaria at a young age and since 2002, he has cultivated countless species from common goldfish to shell-dwelling cichlids to marine butterflyfishes. It was his passion for fish and aquatic life that spurred his interest in scientific research. During his senior year at the University of Toronto, Eugene joined the laboratory of Dr. Robert Gerlai where he studied zebrafish behavior. After graduating from the University of Toronto with a H.B.Sc. in Environmental Sciences, Eugene went on to complete a M.Sc. at the University of Windsor with Dr. Dennis Higgs on the hormonal correlates of dominance in anemonefishes. In the summer of 2015, Eugene moved south of the border to join Dr. Russell Borski's laboratory at North Carolina State University in pursuit of his Ph.D. where he studied the role of leptin in the reproductive biology of teleost fishes. Eugene looks forward to a fruitful life-long career in the study of endocrinology.

Outside of research and his aquarium hobby, Eugene finds joy in wine-tasting and the company of good friends.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Dr. Russell Borski. You have fostered my growth as a scientist and critical thinker. I am immensely thankful to you for giving someone with little bench experience beforehand the chance to prove myself. I am grateful for your patience, guidance, and the fantastic scientific discourse we've shared through the years. I will always remember what you told me during my written preliminary exam: "Look beyond the little leaf and see the entire tree". I promise to answer the phone more readily from now on.

To my committee: I thank Dr. Benjamin Reading for his amazing mentorship. Through the years, you have always made time to discuss all of the things that have come along in my work, be it strange vitellogenin responses to the particularities of multimerization-bound cysteine-rich molecules. I was so lucky to have you on my committee. I thank Dr. Heather Patisaul for her equally amazing mentorship and fostering of my professional development. Your excitement and passion for science is infectious. Thank you for taking me to PANS with you in 2019 (and for convincing Russell to let me go!). I will miss your high fives. I thank Dr. John Godwin for encouraging me to pursue my doctorate and for his invaluable input into all of the experiments that have come to mind along the way. I had become familiar with your work since my time in Robert Gerlai's lab and when you came to speak at Windsor, I was admittedly starstruck. I am glad I had the chance to share fruitful scientific discussions with you for the past five years.

I thank the many members of the Borski lab who have helped make this doctorate possible. Of note: Michael Tada, you were always there when I needed help with my experiments and I'm glad for your friendship. Erika Atkins, you brought joy and sufficient hepatocytes to each cell culture experiment. Jamie Honeycutt, you taught me all of my basic lab

techniques and showed me the importance of theme songs from the 1980s. Scott Salger, you guided me through my first two years in the lab and taught me the tenets of good mentorship. And to all of the others without whom this would not have been doable: Hannah Reynolds, Jordan Taylor, Alexander Murr, Madeline Severance, Ryan Ezell, and William Lee.

I thank our collaborators at Monash University Malaysia: Dr. Ishwar Parhar, Dr. Satoshi Ogawa, Dr. Tomoko Soga, Dr. Takayoshi Ubuka, Dr. Andrew Walker, Kai Wee Ng, Indah Maisarah, Rachel Shalini, Shingo Nakajima, Kai Lin Cham, Victoria Tan, Siow Li Fong, and Serena Chow.

I thank the fantastic graduate services coordinator Freha Legoas for her tireless work in keeping everything in order for all of the graduate students. I thank Susan Marschalk, the Keeper of David Clark. I thank all of the wonderful staff at Thomas Hall: Kathleen Wilson, Kate Thompson, Suprava Misra, Denise Lambs, and Lara Mekeel. I thank all of the amazing staff at David Clark: Michelle Jewell, Wall Crumpler, David Newkirk, Delia, Milagro, and the Lobby Yurt. I thank the teaching faculty with whom I had the chance to work with during my teaching assistantships: Dr. Bill Grant, Dr. Miriam Ferzli, Dr. Benoit Jacquet, Dr. Lisa Parks, Dr. Jason Flores. I thank the Office of International Services – especially Sheila O'Reare – for making sure my visa was valid throughout my time here. I thank the Irwin lab for being the best lab neighbors anyone could ask for – Laura Hamon, Jonathan Giacomini, Victoria Amaral/Barnette.

I thank the Meitzen lab for their warm friendship and community. To Dr. John Meitzen, I express my endless appreciation for your mentorship and for welcoming me as an honorary member of your lab. To Dr. Amanda Krentzel, Dr. Jinyan Cao, Stephanie Proano, Christiana Miller, and Dave Dorris, thank you for your kindness and the snacks.

I thank all of my office-mates in Room 208. May Shrimp Taco bless us in all of our endeavors. I thank the 2015 cohort for their support and camaraderie throughout our time together in this program.

I thank my kind friends and pillars of support: Dr. Elsita Kiekebusch, I am glad I had the blessing of such a wonderful companion throughout this rigorous journey and I hope we will always tell each other our gossip du jour back to each other; Patty Aune, thank you for being my family away from home. You have always been my anchor of support in the many triumphs and trials that I have come across and words are not enough to express the gratitude I feel; Matthew Browning, you have been a brother to me. I am so thankful that our shared joy and passion for aquaria made us friends. Among many others: Steven Lombardo, Molly Harris, Jacob Dums, Dr. Christina Garcia, Dr. Stefanie Chen, Chelsea Phillips, Stephanie Johnstone, Connor Neagle, Dr. Marla Sokolowski, Candy Donaldson, Felicia Vincelli, Christopher Moser-Purdy, Sagi Gillera, Billy Marinello, Megan Serr, Magdalena Sorger, and many more.

To Mitch's Tavern, I thank you for the food and libations. To wine, I thank Shiraz, Cabernet Sauvignon, Pinot Noir, and sometimes Merlot. To Beth Honeycutt, I thank you for making me beautiful. To Kelly McConkey, I thank you for your endless patience and guiding me through the rough patches of the past five years.

To Derek Detweiler, I thank you for your love, kindness, and support. I'm so glad to be able to share this accomplishment with you.

To my cats, Pepper, Pico, and 野-meow, I thank you for your unconditional love, meows, and for not forgetting who I am when I visit Canada. Meow meow meow meow. Meow.

To the fish who have lived with me in Raleigh since 2015, I thank you for keeping me company.

I thank my parents, Edmond and Melody, for their constant support throughout my life.

To everyone, I thank you for your kindness and support through the years.

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1

LIST OF FIGURES

Chapter 1

Chapter 2

CHAPTER 1

Comparative insights on a role for leptin in the metabolic control of reproduction in vertebrates

Abstract

The activation and maintenance of reproductive systems is an energetically expensive venture. Hence, reproductive physiology is sensitive to an array of endogenous signals that provide information on metabolic and nutritional sufficiency. Although metabolic gating of reproductive function in mammals, as evidenced by studies demonstrating delayed puberty and perturbed fertility, has long been understood to be a function of energy sufficiency, an understanding of the endocrine regulators of this relationship have emerged only within recent decades. Peripheral signals have long been implicated in the physiological integration of metabolism and reproduction. Recent studies have begun to explore possible roles for the cytokine hormone leptin in the regulation of reproduction in teleost fishes, as well as a role for leptin as a catabolic stress hormone. In this review, we briefly explore the reproductive actions of leptin in mammals and teleost fishes and its possible role as a putative modulator of the reproductive axis during stress events, and the intended investigation of the reproductive actions of leptin in the comparative teleost model, Mozambique tilapia *Oreochromis mossambicus*.

Introduction

Vertebrate reproductive physiology is controlled by complex regulatory networks that integrate endogenous and environmental signals. These networks interact with the hypothalamic-pituitary-gonadal (HPG or gonadotropic) axis to regulate comprehensive aspects of reproductive function from the onset of pubertal maturation to the sustained integrity and function of the gonadotropic axis. The HPG axis is formed by hypothalamic gonadotropin-releasing hormone (GnRH); the pituitary gonadotropins (GTHs), luteinizing hormone (LH) and follicle stimulating hormone (FSH); and steroidal or peptidergic gonadal signals. These components are tightly interconnected via regulatory feedback loops that control the expression and secretion of upstream and downstream elements for maintenance of reproductive function (reviewed in Manfredi-Lozano *et al*., 2018).

While reproduction is a critical component of an organism's lifespan/biological fitness and key to the perpetuation of species, the energetic costliness of reproduction necessitates its dispensability in instances of disruption to energy/metabolic homeostasis. Hence, the HPG axis is sensitive to an array of endogenous signals that provide information on metabolic and nutritional sufficiency (Roa *et al*., 2010; Navarro & Tena-Sempere, 2012). Within this premise, the onset of puberty in mammals is known to be permitted by the acquisition of an optimal threshold of adiposity to maintain reproductive capacity, while conditions of metabolic stress and energy insufficiency can delay pubertal onset and gonadal function/development (Parent *et al*., 2003; Fernandez-Fernandez *et al*., 2006; Martos-Moreno *et al*., 2010). Although metabolic gating of reproductive function, as evidenced by studies demonstrating delayed puberty and perturbed fertility (Frisch 1984; Schwartz & Seeley, 1997; Gill *et al*., 1999), has long been understood to be a function of energy sufficiency (as in adiposity for mammals), elucidation and

understanding of the putative endocrine regulators of this relationship have emerged only within recent decades.

The identification of leptin (Zhang *et al*., 1994), an adipose hormone essential in the control of appetite and body weight, was followed by wide-ranging studies that established the hormone as an essential signal of energy sufficiency to a variety of mammalian neuroendocrine systems including the HPG axis. Of note, experimental evidence has demonstrated that leptin plays a key role in the regulation of pubertal onset and fertility, as instances of irregular metabolic state (i.e., obesity, energy insufficiency, under-/overnutrition) can disrupt the integrity of the HPG axis (Parent *et al*., 2003; Fernandez-Fernandez *et al*., 2006; Martos-Moreno *et al*., 2010; reviewed in Manfredi-Lozano *et al*., 2018). While a role for leptin in the reproductive biology of mammals has been established and continues to be the subject of comprehensive study (Sanchez-Garrido & Tena-Sempere, 2013; Manfredi-Lozano *et al*., 2016, 2018), the metabolic control of reproduction by leptin in other vertebrates remains poorly understood (Table 1).

Accounting for more than half of all extant vertebrates, teleost fishes offer practical and valuable systems for the comparative study of the endocrine regulation of reproduction. Despite this, endocrine regulation of teleost reproduction by peripheral signaling of metabolic status remains poorly understood when compared to their mammalian counterparts. Teleostean leptin orthologs were not discovered until nearly a decade following the 1994 identification of mammalian leptin (in *Fugu*, Kurokawa *et al*., 2005). While demonstrating some of the prototypical actions of mammalian leptin (i.e., appetite regulation – Won *et al*., 2012; Liu *et al*., 2018), teleostean leptin shows fundamental divergences from mammalian leptin including primary site of production and response to metabolic challenge (Kling *et al*., 2009; Li *et al*.,

2010; Trombley *et al*., 2012; Fuentes *et al*., 2012; Zhang *et al*., 2013; Baltzegar *et al*., 2014; Chen *et al*., 2016; Yan *et al*., 2016; Douros *et al*., 2017). Further, its reproductive properties remain largely unexplored. Here, I review the current state of knowledge in mammals and teleost fishes on leptin's interactions with the reproductive brain and integration of metabolic state via appetite-regulatory nuclei. I also discuss the importance of studying the hormone's reproductive actions in a comparative context, and present the basis and structure of this dissertation.

Leptin and the reproductive brain

The activity of the pulsatile and stimulatory GnRH, the major and terminal output for hypothalamic and central regulation of gonadal activity via the pituitary, is dependent on neuronal circuits sensitive to metabolic state and energy availability (Gamba & Pralong, 2006; Leshan *et al*., 2009). Investigations of these neuroendocrine pathways in mammals have yielded distinct interfacing networks that control GnRH both in and beyond the hypothalamus, including but not limited to the kisspeptin-neurokinin B-dynorphin (KNDy) neuronal family, metabolic neuropeptides including proopiomelanocortin (POMC), neuropeptide Y (NPY), and agouti-related peptide (AgRP), and other neuromodulators such as glutamate and gamma-aminobutyric acid (GABA) (Manfredi-Lozano *et al*., 2016).

The regulation of GnRH neurons is understood to result from conveyance of information on metabolic status via indirect signaling from peripheral hormones. Soon after its initial discovery, leptin was recognized as a key permissive signal for onset of puberty and reproductive function after impairment of gonadotropic signaling and gonadal function was observed in mammals demonstrating leptin or leptin receptor deficient mutations (Yu *et al*., 1997; Watanobe 2002; Sanchez-Garrido & Tena-Sempere, 2013). Beyond its absence due to genomic mutation,

the adipostatic properties of leptin indicated a key role for the hormone in relaying energy availability to the HPG axis. Accordingly, instances of metabolic strain and energy insufficiency have been shown to elicit similar pathologies (i.e., hypogonadism, delayed onset of puberty, and reduced fertility) to those observed in leptin deficient models (Roa & Tena-Sempere, 2014). While noted for its indispensability for pubertal onset and sustained fertility, the hormone's active effects on the HPG axis beyond signaling of energy sufficiency availability to central circuits to initiate the onset of puberty was subject to debate. Early pharmacological studies involving the application of the hormone to leptin-deficient human patients and rodent models failed to trigger puberty (True *et al*., 2011; Navarro & Tena-Sempere 2012; Elias & Purohit, 2013), indicating that leptin on its own is not sufficient to stimulate puberty. Instead this suggests a likely permissive role for leptin in the metabolically-gated onset of puberty.

Experimental evidence has demonstrated that leptin exerts its reproductive (and some metabolic) actions primarily at hypothalamic nuclei (Figure 1). However, the hormone's specific targets remain a subject of debate. The leptin receptor is expressed in key hypothalamic centers involved in the regulation of energy homeostasis and reproduction (Yu *et al.*, 1997; Watanobe 2002; Sanchez-Garrido & Tena-Sempere, 2013). The hypothalamic arcuate nucleus (ARC) contains populations attributed to control of the HPG axis. Of note are kisspeptin neurons that respond to changes in metabolic state and leptin treatment. Suppression of hypothalamic Kiss has been observed in instances of negative energy balance induced by feed restriction or deprivation (Navarro & Tena-Sempere, 2012), a condition which is also associated with leptin deficiency in mammals. Leptin treatment *in vivo* during metabolic strain has been shown to restore Kiss expression and stimulate Kiss neuron activity (Smith *et al.*, 2006; Castellano *et al.*, 2006; Backholer *et al.*, 2010; Qiu *et al.*, 2011), further corroborated with evidence for leptin

stimulation of Kiss *in vitro* in human and murine neuronal cell lines (Morelli *et al.*, 2005; Luque *et al.*, 2007). Interestingly, only approximately 15% of ARC Kiss neurons demonstrate leptin sensitivity/express the leptin receptor (Cravo *et al.*, 2011) and ablation of Kiss neuron-specific leptin receptors does not inhibit puberty nor fertility in mice (Donato *et al.*, 2011), suggesting the actions of other neuroendocrine pathways may be relaying leptin's reproductive actions through indirect targeting of the gonadotropic axis.

Metabolic neuropeptides at the arcuate nucleus

The indirect actions of peripheral metabolic hormones on the HPG axis are exerted upon central circuits via metabolic neuromodulators (Figure 1). Leptin's classical first order target neurons are localized to the arcuate nucleus of the hypothalamus (ARC) including agouti-related peptide/neuropeptide Y (AgRP/NPY) (Mercer *et al*., 1996) and proopiomelanocortin (POMC) (Figure 1; Cheung *et al*., 1997). These neurons have key roles in appetite regulation and energy homeostasis and have been identified as key candidates for the integration of metabolism and reproduction at the brain.

AgRP and NPY

The neuropeptides AgRP and NPY are leptin-sensitive signals coexpressed in neuronal populations at the ARC (Chronwall *et al*., 1985; Broberger *et al*., 1998) that respond to changes in metabolic state and energy availability. Both neuropeptides act as orexigenic signals to stimulate appetite and food intake (Clark *et al*., 1984; Horvath *et al*., 1992). In instances of nutritional restriction (i.e., fasting), expression of AgRP/NPY are increased at the ARC and act to inhibit anorexigenic pathways (Parker & Bloom, 2012). Importantly, AgRP/NPY neurons

express the leptin receptor, with experimental evidence indicating suppression of AgRP/NPY by leptin thereby reducing appetite (Korner *et al*., 2001). Neuroanatomically, projections from AgRP/NPY neurons extend to GnRH neurons (Turi *et al*., 2003). This putative regulatory dynamic has been corroborated with evidence for AgRP/NPY regulation of the GnRH pulse generator (Roa & Herbison, 2012). In addition, increased expression of AgRP/NPY as a consequence of nutritional deprivation is correlated with decreased circulating LH levels in rodent models (Brady *et al*., 1990) and experimental work demonstrates that administration of either neuropeptide can inhibit GnRH and GTH activity (Mcshane *et al*., 1992; Pierroz *et al*., 1996; Schioth *et al*., 2001; Vulliemoz *et al*., 2005). This has been shown to be the consequence of direct actions onto GnRH neurons and indirect inhibition through suppression of the POMC derivative aMSH (Turi *et al*., 2003; Roa & Herbison, 2012). Further, NPY knockout in a rodent model blunts LH suppression induced by fasting (Hill & Levine, 2003), therein suggesting a key role for the neuropeptide in regulation of the HPG axis during adverse metabolic circumstances. The relationship between leptin and central integration of metabolic state by these neuropeptides suggests that AgRP/NPY neuronal populations invoke an adaptive response to conditions of energetic and metabolic strain to allocate energy resources away from the costly HPG axis to other life-sustaining physiological systems, therein highlighting the context-dependent dispensability of reproduction.

POMC

ARC-localized POMC neurons have been described as major mediators in the central regulation of metabolism and energy homeostasis. POMC neurons express a variety of neuropeptides and neurotransmitters including β-endorphin, melanocortins, cocaine and

amphetamine-regulated transcript (CART), corticotropin-like intermediate peptide (CLIP), glutamate, and GABA (Solomon 1999). These neurons also express receptors for leptin, insulin, NPY and AgRP, indicating an endogenous capacity to sense metabolic signals from peripheral and central sources (Fuxe *et al*., 1997; Havel *et al*., 2000; Cowley *et al*., 2001; Balthasar *et al*., 2004; Konner *et al*., 2007; Quennell *et al*., 2009; Williams *et al*., 2010). The role of POMC neurons as a key central element in the regulation of energy homeostasis has been evidenced by proxy in *ob*/*ob* mice, in which obese mice showed reduced ARC POMC mRNA levels (Thornton *et al*., 1997; Balthasar *et al*., 2004). Further, the generation of POMC-null mice yielded an obese phenotype (Yaswen *et al*., 1999; Raffan *et al*., 2016), indicating an impairment of energy homeostasis through loss of function of this central regulating signal. Generally, this evidence suggests that ARC POMC neurons act as a key integrating nexus for the transmission of information on metabolic status and energy homeostasis to different neuroendocrine systems, with continuing indications of its regulation of the reproductive axis. Investigations into ARC POMC-derived neuropeptides have yielded a role for αMSH in the regulation of the reproductive axis. αMSH has also been shown to stimulate LH secretion at the pituitary in a variety of mammalian species (Reid *et al*., 1981; Reid *et al*., 1984; Celis *et al*., 1985; Durando *et al*., 1989; Backholer *et al*., 2009). Further, central leptin administration has been found to induce αMSH-dependent stimulation of GnRH and LH secretion. At the brain, αMSH exerts its metabolic and reproductive actions through melanocortin receptors 3 and 4 (MC3/4R), with evidence for a role of MC3/4R in the control of gonadotropins and pubertal onset (Schioth & Watanobe, 2002; Israel *et al*., 2012). Generally, the current evidence provides an indication for ARC POMC neurons, particularly its product αMSH, to be critical in the mediation of the central effects of metabolically-relevant signals such as leptin in the control of pubertal onset and reproduction.

Leptin in teleost fishes

In fishes, as in mammals, reproduction is regulated by the HPG axis (Peter & Crim, 1979; Gopurrappilly *et al*., 2013; Juntti & Fernald, 2016). Generally, the fundamental mechanisms and pathways of the HPG axis have been well conserved in vertebrates, with some distinctions in organization of the hypothalamic-pituitary complex and the existence of several isoforms of key hormones in fishes relative to mammals. While the hypothalamus and pituitary are separated by the median eminence and hypophyseal portal system in mammals, no such connection exists in teleost fishes and hence hypothalamic neurons directly innervate the pituitary (Figure 2; Peter *et al.*, 1990). Further, ongoing studies indicate that genome duplication events have led to the production of paralogs (Taylor *et al.*, 2003), some of which may have undergone neofunctionalization (e.g., GnRH1, GnRH2, GnRH3) (Weber *et al.*, 1997; Steven *et al.*, 2003; reviewed in Somoza *et al.*, 2002, Lethimonier *et al.*, 2004, and Zohar *et al.*, 2010). Albeit fishes demonstrate some distinctive differences in the organization, structure, and genomic duplicity of the HPG axis, the overall neuroendocrinology of reproduction in fishes has been the subject of exhaustive on-going study and the core components are fairly well-understood. By contrast, the integration of information on metabolic status and energy sufficiency for the activation and function of the HPG axis has largely been ignored. Virtually nothing is known on the function of leptin in the reproductive biology of teleost fishes. The current vertebrate paradigm for metabolic control of reproduction places leptin as a key permissive signal for the HPG axis and likely required for its sustained function, but whether this model applies to teleosts remains to be elucidated.

The identification and cloning of an orthologous leptin gene in teleosts did not occur until eleven years following its initial identification in the *ob*/*ob* mouse (Zhang *et al*., 1994). The

seminal discovery of a teleostean leptin via gene synteny (in Fugu, Kurokawa *et al.*, 2005) revealed an approximate 13% conservation of primary structure to human and murine leptins. By contrast, sequential studies revealed that teleostean leptin retains similar tertiary structure to mammalian leptin (Kurokawa *et al.*, 2005; Huising *et al.*, 2006; Gorissen *et al.*, 2009). Studies show that diploid species produce two leptins – LepA and LepB – as a consequence of at least one genome duplication event (Gorissen et al, 2009; Zhang *et al.*, 2013; Shpilman *et al.*, 2013; Tang *et al.*, 2013; Douros *et al*., 2014; Ohga *et al.*, 2015; Chen *et al.*, 2016). Generally, it appears that LepA is the primary endocrine form of the hormone as transcript abundance is 10-100 times higher than LepB across various tissues (Douros *et al.*, 2014). Further, the primary site of expression and synthesis for teleostean leptin is at the liver (Kurokawa *et al.*, 2005; Huising *et al.*, 2006; Murashita *et al.*, 2008; Kurokawa & Murashita, 2009; Douros *et al.*, 2014), in contrast to mammals that produce leptin primarily in adipose tissue.

Leptin and teleost appetite

In teleosts studied to date, injection of heterologous and homologous species-specific leptin decreases appetite (e.g., *Carassius auratus*: Volkoff *et al*., 2003; *Oncorhynchus mykiss*: Murashita *et al*., 2008 *Morone saxatilis*: Won *et al*., 2012). Concurrent evidence suggests that the hormone exerts its appetite-decreasing effects by targeting its prototypical first order anorexigenic targets at the brain including POMC and CART while suppressing orexigens NPY and AgRP (e.g., *O. mykiss*: Gong *et al*., 2015; *C. auratus*: Volkoff *et al.*, 2003; Yan *et al*., 2016). Leptin expression and secretion may also increase postprandially, in some, but not all fish (e.g., *Cyprinus carpio*: Huising *et al*., 2006; *C. auratus*: Tinoco *et al*., 2012; *Piaractus mesopotamicus*: Volkoff *et al*., 2017). In the majority of teleosts examined, leptin increases with fasting in

contrast with the classically described leptin decline seen in mammals (e.g., *Salmo salar*: Ronnestad *et al*., 2010; *Epinephelus coioides*: Zhang *et al*., 2013; *Tanichthys albonubes*: Chen *et al*., 2016; *O. mossambicus*: Douros *et al*., 2017). Hence, despite the conserved appetite-regulatory actions of the hormone, leptin may have a number of divergent actions and properties in teleosts – as evidenced by its disparate response to fasting – not reflected in the current vertebrate paradigm.

Teleost leptin and central signaling

The lateral tuberal nucleus of the hypothalamus (Figure 2; nLT) is considered as the teleost homolog of the mammalian ARC through similarities in neuronal function and circuitry (Ogawa & Parhar, 2013; Soengas *et al*., 2018). The nLT has been shown to produce neuropeptides associated with energy homeostasis akin to the mammalian ARC. Albeit that comprehensive investigation into the localization of neuronal populations in fishes is scarce, studies have provided evidence that some teleosts express and produce NPY, AgRP, POMC, and CART at the nLT (Vallarino *et al.*, 1988; Cerda-Reverter *et al.*, 2000; Cerda-Reverter *et al.*, 2003; Cerda-Reverter & Peter, 2003; Forlano & Cone, 2007; Le *et al.*, 2016; Cerda-Reverter *et al.*, 2011). Further, these neuronal populations respond to metabolic challenges (Volkoff & Peter, 2001; Cerda-Reverter & Peter, 2003; Murashita *et al.*, 2009; Wan *et al.*, 2012; Agulleiro *et al.*, 2014) and hence, provide tandem phenotypic and functional evidence for homology to the mammalian ARC. In the context of neuronal circuitry and projections, POMC and NPY/AgRP neurons were shown to project to regions where MC4R is abundantly expressed, such as the preoptic area (POA) (Forlano & Cone, 2007; Agulleiro *et al.*, 2014). Neuroanatomical evidence

has also demonstrated the expression and localization of reproductive kisspeptins (Kiss1 and Kiss2) and GnRH1 to the nLT, as well as the POA (Servili *et al.*, 2011; Ogawa & Parhar, 2013).

Generally, evidence for nLT-ARC homology suggests that the nLT is similarly important in the central integration of information on energy homeostasis and metabolism. In mammals, the role of the ARC in sensing peripheral signals such as leptin is key to the sustained function of a variety of physiological axes including reproduction (Hill *et al.*, 2008). In fishes, the leptin receptor has been detected in the brain (Ohga *et al.*, 2015; Escobar *et al.*, 2016) and is notably localized to major regions of the hypothalamus of the European sea bass including the nLT (*Dicentrarchus labrax*: Escobar *et al.*, 2016). Additionally, whether teleost leptin acts similarly to mammalian leptin remains unclear. While mammalian leptin exerts a clear lipostatic role in its actions relevant to energy homeostasis (e.g., sensing of energy availability, stimulation of energy expenditure, reduction of food intake via POMC) and a permissive role in the onset of puberty and sustained reproductive function, evidence in teleost fishes thus far is unclear on a similar role for leptin. Several studies indicate leptin does not serve as an adipostat as its expression and secretion increases during catabolic conditions incurred by fasting (De Pedro *et al.*, 2006; Kling *et al.*, 2009; Fuentes *et al.*, 2012; Trombley *et al.*, Yuan *et al.*, 2016; Douros *et al.*, 2017; reviewed in Deck *et al.*, 2017), opposite to that which occurs in mammals (Ahima *et al*., 1996; Boden *et al*., 1996). Given the functional divergences reported in response to metabolic challenges, poor conservation of nucleotide and amino acid sequence between mammalian and teleost leptins, and the existence of multiple paralogs in fishes which may each carry distinctive functions, whether the hormone plays a reproductive role via actions on key regions like the nLT remains to be seen.

Leptin and teleost reproduction

The current state of knowledge on a possible role for leptin in the reproductive biology of fishes is limited almost exclusively to correlative studies in salmonids and sparing experimental analyses on leptin effects on pituitary gonadotropins (Figure 3, Table 1). Elevated leptin during sexual maturation – either hepatic gene expression, circulating levels, or both – has been reported in male Atlantic salmon (*Salmo salar*: Trombley & Schmitz, 2013; Trombley *et al.*, 2014), female chum salmon (*Oncorhynchus keta*: Choi *et al.*, 2014), Arctic charr (*Salvelinus alpinus*: Froiland *et al.*, 2010), ayu (*Plecoglossus altivelis*: Nagasaka *et al.*, 2006), and male chub mackerel (*Scomber japonicus*: Ohga *et al.*, 2017). Additionally, *in vitro* studies show increased LH and FSH release from common carp (*Cyprinus carpio*: Chen *et al.*, 2018), European sea bass (*D. labrax*: Peyon *et al*., 2001), and rainbow trout (*Oncorhynchus mykiss*: Weil *et al.*, 2003) pituitaries when treated with the hormone. To date, no studies have investigated a concurrent reproductive function for leptin at the brain and liver (location of vitellogenesis), both of which are key organs in the reproductive axis of fishes. Further, outside of a singular study published in the last year that used homologous leptin to investigate effects on pituitary gonadotropin expression in common carp (*C. carpio*: Chen *et al.*, 2018), the other studies to assess leptin effects on the reproductive biology of a fish used heterologous mammalian leptin, which may not be representative of endogenous leptin actions given that the primary structure between mammalian and teleostean leptins demonstrates only 13% conservation. Moreover, it is possible that homologous LepA may have overlapping as well as some distinct functions from heterologous hormone, indicating the importance of studying effects of the endogenous hormone. In tandem, these present a key gap in knowledge on our understanding of a function for leptin in the reproductive biology of fishes.

Leptin and the Endocrine Stress Axis

Leptin's metabolic actions in enhancing energy mobilization position the hormone as a possible key component of the endocrine stress response (see Deck *et al.*, 2017 for review). In vertebrate studies to date, leptin has been reported to interact with major facets of the endocrine stress axis. In mammals, cortisol treatment increases leptin expression and secretion both *in vivo* and in cultured adipocytes (de Vos *et al.*, 1995; Wabitsch *et al.*, 1996; Newcomer *et al.*, 1998). In rainbow trout, cortisol elevates hepatic leptin expression *in vivo* (Madison *et al.*, 2015). By contrast, leptin appears to exert inhibitory actions on the endocrine stress axis (Leal-Cerro *et al.*, 2001). In mammals, leptin inhibits hypothalamic CRF and adrenal cortisol secretion (Heiman *et al.*, 1997). Leptin suppression of the stress axis has also been reported in fishes in which leptin treatment decreases pituitary ACTH, attenuates CRF-induced ACTH activity, and decreases cortisol secretion (Gorissen *et al.*, 2012).

In teleost fishes, leptin has been evidenced to respond to stressors. While metabolic challenges associated with fasting, hyperosmotic stress, and hypoxia are understood to result in heightened HPI activity and circulating cortisol levels, these stressors have also been reported to coincide with increases to leptin expression and/or secretion (De Pedro *et al.*, 2006; Kling *et al.*, 2009; Lays *et al.*, 2009; Chu *et al.*, 2010; Cao *et al.*, 2011; Bernier *et al.*, 2012; Fuentes *et al.*, 2012; Trombley *et al.*, 2012; Baltzegar *et al.*, 2014; Douros *et al.*, 2014; Douros *et al.*, 2017). Some authors postulate that leptin may work as a catabolic signal in concert with glucocorticoids and catecholamines during instances of stress to mobilize energy substrates in order to meet the increased energetic demands incurred by the stressor (Deck *et al.*, 2017). Recent work from our group has evidenced a stimulatory effect of cortisol and the catecholamine epinephrine on hepatic leptin expression and secretion both *in vivo* and in primary tilapia hepatocytes

(Honeycutt, 2018). Cortisol and epinephrine mobilize energy reserves to increase glucose, an effect that may be enhanced through a stimulation of leptin, which induces hyperglycemia in tilapia (Baltzegar *et al.*, 2014).

During stress events, the HPG axis is typically suppressed in favor of allocating energetic resources towards survival and catabolic processes. In teleosts, while leptin is understood to increase in response to stressors, there are currently no studies examining the relationship between stress, leptin, and the HPG axis of teleost fishes. This gap in our understanding of leptin biology in teleosts necessitates investigations into the hormone's role in reproductive function particularly within stress contexts and possible divergences from the currently understood model for leptin's permissive role in mammalian reproduction.

The teleost liver: Hepatic vitellogenesis, stress, and leptin

The physiological response to stress in teleosts incurs a stereotypic effect on reproductive function and the HPG axis similar to what is observed in other vertebrates. In response to the energetic costs imposed by a stressor, the hormonal cascade resulting from the endocrine stress response causes a shift in energy stores, metabolism, and allocation leading to a pronounced decline (or at least an alteration) in normal reproductive function including diminished HPG axis signaling and decreased gonadal mass (Schrek *et al*., 2010; Schrek 2010).

The liver is a critical component of the female teleost reproductive system. After the onset of puberty, ovarian growth and maturation are initiated by gonadotropin-induced gonadal estrogen signaling to the liver (Wallace 1985; Mommsen & Walsh, 1988; Arukwe & Goksoyr, 2003). Follicular development and oocyte growth are dependent on estrogen-responsive hepatic elements that generate vitellogenins (Vtgs) – egg yolk precursor proteins – that are transported

from the liver to the ovaries for deposition into oocytes and cleaved into derivative yolk proteins (Reading *et al*., 2011). As previously mentioned, the HPG axis is affected at all levels in response to stress. Kisspeptin and gonadotropin-releasing hormone neuronal activity and their respective neuropeptides at the preoptic area and hypothalamus are suppressed (Tena-Sempere *et al*., 2012; Mechaly *et al*., 2013; Ogawa & Parhar, 2013). As a consequence of the diminishment of the apex effectors of the HPG axis (as well as some direct glucocorticoid and catecholamine effects), pituitary gonadotropins and gonadal steroids are inhibited. This decline in overall HPG activity then causes a degradation in the terminal organs of the reproductive system: the gonads. In response to stress, hepatic vitellogenesis is disrupted resulting in a decline in ovarian mass and egg quality (Lahnsteiner *et al*., 2008; Reading *et al*., 2011; Li & Leatherland, 2012). Various studies using both *in vivo* and *in vitro* approaches have investigated glucocorticoid effects on Vtgs (e.g., Pankhurst & Van Der Kraak, 2000; Berg *et al*., 2004; Babin *et al*., 2007; Faught & Vijayan, 2018). Putative mechanisms regulating this dynamic indicate that glucocorticoid actions could be a result of direct effects on the estrogen-responsive hepatic induction elements (disruption of ER, Vtg transcripts) and/or indirect effects via reduction in estrogen secretion. In combination with leptin's response to various stressors and elements of the endocrine stress axis, as well as its predominant production site and colocalization with vitellogenins at the teleost liver, it is possible that leptin may modulate part of the hepatic response to stress to regulate vitellogenesis.

The liver is a major site for processing substrates and the mobilization of energy stores to enhance glucose production and release during energetically challenging events (Pilkis & Granner, 1992; Klover & Mooney, 2004; Polakof *et al*., 2012; reviewed in Fabbri & Moon, 2016). A study from our group on the Mozambique tilapia evidenced a role for glucose

mobilization during hyperosmotic stress, with declines in hepatic glycogen levels observed in response to osmotic challenge and leptin treatment (Baltzegar *et al*., 2014). A recent *in vitro* study suggests that leptin may suppress vitellogenins in rainbow trout liver slices (*O. mykiss*: Paolucci *et al.*, 2020). Whether this may occur in other teleosts or oviparous species remains to be determined. It is possible that leptin may contribute to mediating the inhibitory effects of stress on vitellogenesis in order to shunt energy substrates towards managing the energetic cost brought on by the stressor. Insulin-like growth factor 1 (IGF1) – that is produced predominantly in the liver in response to growth hormone and other factors – is central to promoting skeletal and somatic growth in vertebrates (Duan, 1997). Previous work from our group has evidenced leptin promotion of hepatic IGF1 (Won *et al*., 2012; Douros *et al*, 2017). A goal of this dissertation will be to elucidate a possible role for leptin in the allocation of resources between somatic and gonadal growth as evidenced by its actions on IGF1 and vitellogenesis.

Reproduction and leptin receptor mutants

Recent efforts in the genetic manipulation of the leptin receptor in zebrafish (*D. rerio*) and medaka (*Oryzias latipes*) have evidenced that leptin signaling may be dispensable for reproduction, as the absence of a functional leptin receptor does not inhibit fertility nor normal gonadal development (Chisada *et al*., 2014; Michel *et al*., 2016). At the same time, the leptin receptor-deficient medaka showed hyperphagia while the zebrafish mutants did not. The zebrafish leptin receptor mutant had increased insulin mRNA levels, which may suggest a compensatory or synergistic role for insulin in regulating numerous systems including the HPG axis. In mammals, the *ob/ob* and *db/db* mouse mutants, which lack functional leptin signaling, exhibit infertility, absent onset of puberty, and an overall disruption to gonadotropic function.

Administration of leptin to the leptin-deficient *ob/ob* mutant (Ahima *et al*., 1997) and reinsertion of the leptin receptor in the receptor-deficient *db/db* mutant (De Luca *et al*., 2005) have been found to induce the onset of puberty and rescue fertility and reproductive function. That said, recent studies employing the congenital elimination of the leptin receptor from hypothalamic nuclei critical to the Kiss-GnRH circuit and GnRH neurons specifically evidenced preserved pubertal onset and fertility (Quennell *et al*., 2009; Donato *et al*, 2011), suggesting that – as observed in the zebrafish and medaka leptin receptor mutants – leptin may be dispensable for reproduction. However, when considered in light of experimental evidence indicating leptin stimulation of Kiss, GnRH, and subsequent induction of puberty in leptin deficient rodent models (Castellano *et al*., 2006; Smith *et al*., 2006; Luque *et al*., 2007), it is possible that congenitally-manipulated leptin and leptin receptor-deficient mutants may simply be demonstrating developmental compensation and rewiring of central circuits. Overall, it is important to identify potential functions that leptin may have in regulating reproductive biology that may not be clearly identifiable in congenitally-manipulated models.

Dissertation objectives

This dissertation aims to explore the role of leptin in the reproductive biology of the Mozambique tilapia (*O. mossambicus*), a representative perciform fish, the largest order in the animal kingdom. I will address leptin function on reproduction through the course of three investigative research chapters:

1. Leptin and cortisol regulation of appetite and appetite-regulatory neuropeptides
2. A role for leptin in sexual maturation and the regulation of hypothalamic neuropeptides and sex steroids

3. Leptin regulation of vitellogenins, its response to sex steroids, and its possible role in mouthbrooding

These three chapters explore whether leptin exerts its prototypic appetite-regulatory function in a teleost model and – for the first time – how leptin modulates the key central and peripheral components of the teleost reproductive axis. Cumulatively, the current dissertation identifies a role for leptin in the regulation of teleost reproductive function and consequently, provides fundamental advancement in our understanding of reproductive biology.

The tilapia model

The Mozambique tilapia (Perciforme: Cichlidae: *O. mossambicus*) is a euryhaline cichlid native to southern Africa, with occurrences of global distribution due to anthropogenic dispersal. The combination of its high biological plasticity, suitability to captive rearing, indeterminate spawning, short generation time, robust behavioral repertoire, fully-sequenced genome, and complaisance to powerful research technologies establish the tilapia as an ideal candidate for scientific study. It has proven as an incredibly useful model organism in investigations of genetics, neurobiology, endocrinology, toxicology, physiology, and behavioral studies. Relative to other teleost fishes, considerable information is known about the metabolic effects of leptin and its interactions with various glucoregulatory hormones in tilapia. Additionally, we have developed and optimized technologies in our laboratory that will facilitate comprehensive study of leptin biology in tilapia, that includes availability of biologically active recombinant tilapia leptin A. From a comparative angle, the tilapia's relation to the vast species flocks of East African cichlids allows for studies of tilapiine fishes to fall within an ideal comparative framework due to the species-rich highly-proliferating lineages associated with the taxa, thereby

allowing for greater understanding of physiology in lower vertebrates and aquatic ectotherms. Notably, tilapia – including *O. mossambicus*, its congeners, and hybrids – are commercially important fishes, qualifying as the second most-farmed fish in the world and as a globally-significant protein source. Hence, investigations into tilapia reproductive physiology may provide applications in aquaculture to improve tilapia farming technologies and processes.

Despite comprehensive and continuing research on the reproductive physiology of teleost fishes, little is known on the function of endocrine signals -- like leptin -- that may play a key role in mediating energy allocation to and from reproductive processes. Investigation of leptin biology and its role in the reproduction of teleost fishes, comprising the largest taxa of vertebrates, may reveal the effects of divergent evolutionary histories (genome duplication events, ancestral separation from tetrapods) and differential approaches to energy homeostasis (ectothermy). The work described in the current dissertation describes – for the first time – a functional link between leptin and gonadotropic processes at the brain and liver in fishes, specifically the Mozambique tilapia (*O. mossambicus*) and through a comparative approach, discern the convergent and divergent properties of leptin biology among vertebrates.

REFERENCES

Agulleiro, M. J., Cortés, R., Leal, E., Ríos, D., Sánchez, E., & Cerdá-Reverter, J. M. (2014). Characterization, tissue distribution and regulation by fasting of the agouti family of peptides in the sea bass (Dicentrarchus labrax). General and comparative endocrinology, 205, 251-259.

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., & Flier, J. S. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature*, *382*(6588), 250-252.

Ahima, R. S., Saper, C. B., Flier, J. S., & Elmquist, J. K. (2000). Leptin regulation of neuroendocrine systems. Frontiers in neuroendocrinology, 21(3), 263-307.

Arukwe, A., & Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative hepatology*, *2*(1), 4.

Babin, P. J., Carnevali, O., Lubzens, E., & Schneider, W. J. (2007). Molecular aspects of oocyte vitellogenesis in fish. In *The fish oocyte* (pp. 39-76). Springer, Dordrecht.

Backholer, K., Bowden, M., Gamber, K., Bjørbæk, C., Iqbal, J., & Clarke, I. J. (2010). Melanocortins mimic the effects of leptin to restore reproductive function in lean hypogonadotropic ewes. Neuroendocrinology, 91(1), 27-40.

Backholer, K., Smith, J., & Clarke, I. J. (2009). Melanocortins may stimulate reproduction by activating orexin neurons in the dorsomedial hypothalamus and kisspeptin neurons in the preoptic area of the ewe. Endocrinology, 150(12), 5488-5497.

Baker, D. M., Larsen, D. A., Swanson, P., & Dickhoff, W. W. (2000). Long-term peripheral treatment of immature coho salmon (Oncorhynchus kisutch) with human leptin has no clear physiologic effect. General and comparative endocrinology, 118(1), 134-138.

Balthasar, N., Coppari, R., McMinn, J., Liu, S. M., Lee, C. E., Tang, V., & Lowell, B. B. (2004). Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. Neuron, 42(6), 983-991.

Baltzegar, D. A., Reading, B. J., Douros, J. D., & Borski, R. J. (2014). Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. J Endocrinol, 220(1), 61-72.

Barash, I. A., Cheung, C. C., Weigle, D. S., Ren, H. O. N. G. P. I. N. G., Kabigting, E. B., Kuijper, J. L., & Steiner, R. A. (1996). Leptin is a metabolic signal to the reproductive system. Endocrinology, 137(7), 3144-3147.

Berg, H., Modig, C. & Olsson, P.E. (2004). 17beta-estradiol induced vitellogenesis is inhibited by cortisol at the post-transcriptional level in Arctic char (Salvelinus alpinus). Reproductive Biology and Endocrinology, 2, 62.

Berg, A. H., Westerlund, L., & Olsson, P. E. (2004). Regulation of Arctic char (Salvelinus alpinus) egg shell proteins and vitellogenin during reproduction and in response to 17β-estradiol and cortisol. *General and Comparative Endocrinology*, *135*(3), 276-285.

Bernier, N. J., Gorissen, M., & Flik, G. (2012). Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp. Journal of Experimental Biology, 215(13), 2273-2282.

Boden, G., Chen, X., Mozzoli, M., & Ryan, I. (1996). Effect of fasting on serum leptin in normal human subjects. *The Journal of Clinical Endocrinology & Metabolism*, *81*(9), 3419-3423.

Brady, L. S., Smith, M. A., Gold, P. W., & Herkenham, M. (1990). Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. Neuroendocrinology, 52(5), 441-447.

Breen, K.M., Stackpole, C.A., Clarke, I.J., Pytiak, A.V., Tilbrook, A.J., Wagenmaker, E.R., Young, E.A., Karsch, F.J., 2004. Does the type II glucocorticoid receptor mediate cortisol-induced suppression in pituitary responsiveness to gonadotropin-releasing hormone?. Endocrinology. 145, 2739-2746.

Breen, K.M., Thackray, V.G., Hsu, T., Mak-McCully, R.A., Coss, D., Mellon, P.L., 2012. Stress levels of glucocorticoids inhibit LHβ-subunit gene expression in gonadotrope cells. Molecular Endocrinology. 26, 1716-1731.

Broberger, C., Johansen, J., Johansson, C., Schalling, M., & Hökfelt, T. (1998). The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proceedings of the National Academy of Sciences, 95(25), 15043-15048.

Bronson, F. H. (1987). Puberty in female rats: relative effect of exercise and food restriction. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 252(1), R140-R144.

Carruth, L.L., Jones, R.E. & Norris, D.O. (2000). Cell density and intracellular translocation of glucocorticoid receptor-immunoreactive neurons in the Kokanee salmon (Oncorhynchus nerka kennerlyi) brain, with an emphasis on the olfactory system. General and Comparative Endocrinology, 117, 66-76.

Castellano, J. M., Navarro, V. M., Fernández-Fernández, R., Roa, J., Vigo, E., Pineda, R., & Tena-Sempere, M. (2006). Expression of hypothalamic KiSS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. Diabetes, 55(9), 2602-2610.

Celis, M. E. (1985). Release of LH in response to alpha-MSH administration. Acta physiologica et pharmacologica latinoamericana: organo de la Asociacion Latinoamericana de Ciencias Fisiologicas y de la Asociacion Latinoamericana de Farmacologia, 35(3), 281-290.

Cerdá-Reverter, J. M., & Peter, R. E. (2003). Endogenous melanocortin antagonist in fish: structure, brain mapping, and regulation by fasting of the goldfish agouti-related protein gene. Endocrinology, 144(10), 4552-4561.

Cerdá-Reverter, J. M., Agulleiro, M. J., Guillot, R., Sánchez, E., Ceinos, R., & Rotllant, J. (2011). Fish melanocortin system. European journal of pharmacology, 660(1), 53-60.

Cerdá-Reverter, J. M., Anglade, I., Martınez-Rodrıguez, G., Mazurais, D., Muñoz-Cueto, J. A., Carrillo, M., & Zanuy, S. (2000). Characterization of neuropeptide Y expression in the brain of a perciform fish, the sea bass (Dicentrarchus labrax). Journal of chemical neuroanatomy, 19(4), 197-210.

Cerdá-Reverter, J. M., Ringholm, A., Schiöth, H. B., & Peter, R. E. (2003). Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. Endocrinology, 144(6), 2336-2349.

Chand, D. & Lovejoy, D.A. (2011). Stress and reproduction: controversies and challenges. General and Comparative Endocrinology, 171, 253-257.

Chen, J., Cao, M., Zhang, A., Shi, M., Tao, B., Li, Y., & Hu, W. (2018). Growth hormone overexpression disrupts reproductive status through actions on leptin. Frontiers in endocrinology, 9, 131.

Chen, T., Chen, S., Ren, C., Hu, C., Tang, D., & Yan, A. (2016). Two isoforms of leptin in the White-clouds Mountain minnow (Tanichthys albonubes): Differential regulation by estrogen despite similar response to fasting. General and comparative endocrinology, 225, 174-184.

Cheung, C. C., Clifton, D. K., & Steiner, R. A. (1997). Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology, 138(10), 4489-4492.

Cheung, C. C., Thornton, J. E., Kuijper, J. L., Weigle, D. S., Clifton, D. K., & Steiner, R. A. (1997). Leptin is a metabolic gate for the onset of puberty in the female rat. Endocrinology, 138(2), 855-858.

Chisada, S. I., Kurokawa, T., Murashita, K., Rønnestad, I., Taniguchi, Y., Toyoda, A., & Yoshiura, Y. (2014). Leptin receptor-deficient (knockout) medaka, Oryzias latipes, show chronical up-regulated levels of orexigenic neuropeptides, elevated food intake and stage specific effects on growth and fat allocation. *General and comparative endocrinology*, *195*, 9-20.

Choi, Y. J., Kim, N. N., Shin, H. S., & Choi, C. Y. (2014). The expression of leptin, estrogen receptors, and vitellogenin mRNAs in migrating female Chum Salmon, Oncorhynchus keta: the effects of hypo-osmotic environmental changes. Asian-Australasian journal of animal sciences, 27(4), 479.

Choi, Y.J, Habibi, H.R., Kil, G.S., Jung, M.M., Choi, C.Y., 2017. Effect of cortisol on gonadotropin inhibitory hormone (GnIH) in the cinnamon clownfish, Amphiprion melanopus. Biochemical and Biophysical Research Communications. 485, 342-348.

Chronwall, B. M., DiMaggio, D. A., Massari, V. J., Pickel, V. M., Ruggiero, D. A., & O'donohue, T. L. (1985). The anatomy of neuropeptide-Y-containing neurons in rat brain. Neuroscience, 15(4), 1159-1181.

Chu, D.L.H., Li, V.W.T. and Yu, R.M.K., (2010). Leptin: clue to poor appetite in oxygen-starved fish. Molecular and cellular endocrinology, 319(1), 143-146.

Clark, J. T., Kalra, P. S., Crowley, W. R., & Kalra, S. P. (1984). Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. *Endocrinology*, *115*(1), 427-429.

Consten D., Bogerd, J., Komen, J., Lambert, J.G.D., Goos, H.J.T., 2001a. Long-term cortisol treatment inhibits pubertal development in male common carp, Cyprinus carpioL. Biology of Reproduction. 64, 1063-1071.

Consten, D., Lambert, J.G.D., Goos, H.J.T., 2001b. Cortisol affects testicular development in male common carp, Cyprinus carpioL., but not via an effect on LH secretion. Comparative Biochemistry and Physiology, Part B. 129, 671-677.

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdán, M. G., Diano, S., Horvath, T. L., & Low, M. J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature, 411(6836), 480.

Cravo, R. M., Margatho, L. O., Osborne-Lawrence, S., Donato Jr, J., Atkin, S., Bookout, A. L., & Zigman, J. M. (2011). Characterization of Kiss1 neurons using transgenic mouse models. Neuroscience, 173, 37-56.

Davies, S., & Deviche, P. (2014). At the crossroads of physiology and ecology: food supply and the timing of avian reproduction. *Hormones and Behavior*, *66*(1), 41-55.

De Luca, C., Kowalski, T. J., Zhang, Y., Elmquist, J. K., Lee, C., Kilimann, M. W., ... & Chua, S. C. (2005). Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPR-B transgenes. *The Journal of clinical investigation*, *115*(12), 3484-3493.

De Pedro, N., Martinez-Alvarez, R., & Delgado, M. J. (2006). Acute and chronic leptin reduces food intake and body weight in goldfish (Carassius auratus). Journal of Endocrinology, 188(3), 513-520.

De Vos, P., Saladin, R., Auwerx, J., & Staels, B. (1995). Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. Journal of Biological Chemistry, 270(27), 15958-15961.

Donato, J., Cravo, R. M., Frazão, R., Gautron, L., Scott, M. M., Lachey, J., & Richardson, J. A. (2011). Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. The Journal of clinical investigation, 121(1), 355-368.

Douros, J. D., Baltzegar, D. A., Mankiewicz, J., Taylor, J., Yamaguchi, Y., Lerner, D. T., & Borski, R. J. (2017). Control of leptin by metabolic state and its regulatory interactions with pituitary growth hormone and hepatic growth hormone receptors and insulin like growth factors in the tilapia (Oreochromis mossambicus). General and comparative endocrinology, 240, 227-237.

Durando, P., Ferreira, A., & Celis, M. E. (1989). Acute administration of alpha-melanotropin exerts a stimulatory control on puberty. European Journal of Endocrinology, 120(5), 661-666. Ebling, F. J. (2005). The neuroendocrine timing of puberty. Reproduction, 129(6), 675-683.

Elias, C. F., & Purohit, D. (2013). Leptin signaling and circuits in puberty and fertility. Cellular and Molecular Life Sciences, 70(5), 841-862.

Escobar, S., Rocha, A., Felip, A., Carrillo, M., Zanuy, S., Kah, O., & Servili, A. (2016). Leptin receptor gene in the European sea bass (Dicentrarchus labrax): cloning, phylogeny, tissue distribution and neuroanatomical organization. General and comparative endocrinology, 229, 100-111.

Fabbri, E., & Moon, T. W. (2016). Adrenergic signaling in teleost fish liver, a challenging path. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *199*, 74-86.

Faught, E., & Vijayan, M. M. (2018). Maternal stress and fish reproduction: The role of cortisol revisited. *Fish and Fisheries*, *19*(6), 1016-1030.

Fernandez-Fernandez, R., Martini, A. C., Navarro, V. M., Castellano, J. M., Dieguez, C., Aguilar, E., & Tena-Sempere, M. (2006). Novel signals for the integration of energy balance and reproduction. *Molecular and cellular endocrinology*, *254*, 127-132.

Foo, J.T.W. & Lam, T.J. (1993). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, Oreochromis mossambicus. Aquaculture, 115, 145-158.

Forlano, P. M., & Cone, R. D. (2007). Conserved neurochemical pathways involved in hypothalamic control of energy homeostasis. Journal of Comparative Neurology, 505(3), 235-248.

Frisch, R. E., Revelle, R., & Cook, S. (1973). Components of weight at menarche and the initiation of the adolescent growth spurt in girls: estimated total water, lean body weight and fat. Human Biology, 469-483.

Frisch, R. E. (1984). Body fat, puberty and fertility. *Biological Reviews*, *59*(2), 161-188.

Friedman-Einat, M., & Seroussi, E. (2019). Avian Leptin: Bird's-Eye View of the Evolution of Vertebrate Energy-Balance Control. *Trends in Endocrinology & Metabolism*, *30*(11), 819-832.

Friedman-Einat, M., Cogburn, L. A., Yosefi, S., Hen, G., Shinder, D., Shirak, A., & Seroussi, E. (2014). Discovery and characterization of the first genuine avian leptin gene in the rock dove (Columba livia). *Endocrinology*, *155*(9), 3376-3384.

Frøiland, E., Murashita, K., Jørgensen, E. H., & Kurokawa, T. (2010). Leptin and ghrelin in anadromous Arctic charr: cloning and change in expressions during a seasonal feeding cycle. General and comparative endocrinology, 165(1), 136-143.

Fuentes, E. N., Kling, P., Einarsdottir, I. E., Alvarez, M., Valdés, J. A., Molina, A., & Björnsson, B. T. (2012). Plasma leptin and growth hormone levels in the fine flounder (Paralichthys adspersus) increase gradually during fasting and decline rapidly after refeeding. General and comparative endocrinology, 177(1), 120-127.

Fuxe, K., Tinner, B., Caberlotto, L., Bunnemann, B., & Agnati, L. F. (1997). NPY Y1 receptor like immunoreactivity exists in a subpopulation of β-endorphin immunoreactive nerve cells in the arcuate nucleus: a double immunolabelling analysis in the rat. Neuroscience letters, 225(1), 49-52.

Gamba, M., & Pralong, F. P. (2006). Control of GnRH neuronal activity by metabolic factors: the role of leptin and insulin. *Molecular and cellular endocrinology*, *254*, 133-139.

Gill, M. S., Hall, C. M., Tillmann, V., & Clayton, P. E. (1999). Constitutional delay in growth and puberty (CDGP) is associated with hypoleptinaemia. *Clinical endocrinology*, *50*(6), 721-726.

Godwin, J.R., Thomas, P., 1993. Sex change and steroid profiles in the protandrous anemonefish Amphiprion melanopus(Pomacentridae, Teleostei). General and Comparative Endocrinology. 91, 144-157.

Goikoetxea, A., Todd, E.V., Gemmell, N.J., 2017. Stress and sex: does cortisol mediate sex change in fish?. Reproduction. 154, R149-R160.

Gong, N., Jönsson, E., & Björnsson, B. T. (2015). Acute anorexigenic action of leptin in rainbow trout is mediated by the hypothalamic Pi3k pathway. *Journal of Molecular Endocrinology*, *56*(3), 227-238.

Gopurappilly, R., Ogawa, S., & Parhar, I. S. (2013). Functional significance of GnRH and kisspeptin, and their cognate receptors in teleost reproduction. *Frontiers in endocrinology*, *4*, 24.

Gore, A.C., Attardi, B., DeFranco, D.B., 2006. Glucocorticoid repression of the reproductive axis: effects on GnRH and gonadotropin subunit mRNA levels. Molecular and Cellular Endocrinology. 256, 40-48.

Gorissen, M., Bernier, N. J., Manuel, R., de Gelder, S., Metz, J. R., Huising, M. O., & Flik, G. (2012). Recombinant human leptin attenuates stress axis activity in common carp (Cyprinus carpio L.). General and comparative endocrinology, 178(1), 75-81.

Gorissen, M., Bernier, N. J., Nabuurs, S. B., Flik, G., & Huising, M. O. (2009). Two divergent leptin paralogues in zebrafish (Danio rerio) that originate early in teleostean evolution. Journal of Endocrinology, 201(3), 329-339.

Gutierrez, J., Parrizas, M., Carneiro, N., Maestro, J.L., Maestro, M.A. & Planas, J. (1993). Insulin and IGF-I receptors and tyrosine kinase activity in carp ovaries: changes with reproductive cycle. Fish Physiology and Biochemistry, 11, 247-254.

Havel, P. J., Hahn, T. M., Sindelar, D. K., Baskin, D. G., Dallman, M. F., Weigle, D. S., & Schwartz, M. W. (2000). Effects of streptozotocin-induced diabetes and insulin treatment on the hypothalamic melanocortin system and muscle uncoupling protein 3 expression in rats. Diabetes, 49(2), 244-252.

Heiman, M. L., Ahima, R. S., Craft, L. S., Schoner, B., Stephens, T. W., & Flier, J. S. (1997). Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. Endocrinology, 138(9), 3859-3863.

Hill, J. W., & Levine, J. E. (2003). Abnormal response of the neuropeptide Y-deficient mouse reproductive axis to food deprivation but not lactation. Endocrinology, 144(5), 1780-1786.

Hill, J. W., Elmquist, J. K., & Elias, C. F. (2008). Hypothalamic pathways linking energy balance and reproduction. American Journal of Physiology-Endocrinology and Metabolism, 294(5), E827-E832.

Horvath, T. L., Naftolin, F., Kalra, S. P., & Leranth, C. S. A. B. A. (1992). Neuropeptide-Y innervation of beta-endorphin-containing cells in the rat mediobasal hypothalamus: a light and electron microscopic double immunostaining analysis. *Endocrinology*, *131*(5), 2461-2467.

Hoskins, L.J., Xu, M. & Volkoff, H. (2008). Interactions between gondotropin-releasing hormone (GnRH) and orexin in the regulation of feeding and reproduction in goldfish (Carrasius auratus). Hormones and Behavior, 54, 379-385.

Huising, M. O., Geven, E. J., Kruiswijk, C. P., Nabuurs, S. B., Stolte, E. H., Spanings, F. T., & Flik, G. (2006). Increased leptin expression in common carp (Cyprinus carpio) after food intake but not after fasting or feeding to satiation. Endocrinology, 147(12), 5786-5797.

Israel, D. D., Sheffer-Babila, S., De Luca, C., Jo, Y. H., Liu, S. M., Xia, Q., & Chua Jr, S. C. (2012). Effects of leptin and melanocortin signaling interactions on pubertal development and reproduction. Endocrinology, 153(5), 2408-2419.

Juntti, S. A., & Fernald, R. D. (2016). Timing reproduction in teleost fish: cues and mechanisms. *Current opinion in neurobiology*, *38*, 57-62.

Kennedy, G. C., & Mitra, J. (1963). Body weight and food intake as initiating factors for puberty in the rat. The Journal of physiology, 166(2), 408-418.

Kikuchi, Y., Hosono, K., Yamashita, J., Kawabata, Y. & Okubo, K. (2015). Glucocorticoid receptor exhibits sexually dimorphic expression in the medaka brain. General and Comparative Endocrinology, 223, 47-53.

Kling, P., Rønnestad, I., Stefansson, S. O., Murashita, K., Kurokawa, T., & Björnsson, B. T. (2009). A homologous salmonid leptin radioimmunoassay indicates elevated plasma leptin levels during fasting of rainbow trout. General and comparative endocrinology, 162(3), 307-312.

Klover, P. J., & Mooney, R. A. (2004). Hepatocytes: critical for glucose homeostasis. *The international journal of biochemistry & cell biology*, *36*(5), 753-758.

Könner, A. C., Janoschek, R., Plum, L., Jordan, S. D., Rother, E., Ma, X., & Kahn, C. R. (2007). Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. Cell metabolism, 5(6), 438-449.

Korner, J., Savontaus, E., Chua Jr, S. C., Leibel, R. L., & Wardlaw, S. L. (2001). Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus. Journal of neuroendocrinology, 13(11), 959-966.

Kurokawa, T., Uji, S., & Suzuki, T. (2005). Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, Takifugu rubripes. Peptides, 26(5), 745-750.

Lahnsteiner, F., Soares, F., Ribeiro, L., & Dinis, M. T. (2008). -Egg quality determination in teleost fish. In *Methods in reproductive aquaculture* (pp. 171-202). CRC Press.

Lays, N., Iversen, M. M. T., Frantzen, M., & Jørgensen, E. H. (2009). Physiological stress responses in spotted wolffish (Anarhichas minor) subjected to acute disturbance and progressive hypoxia. Aquaculture, 295(1-2), 126-133.

Le, H. T., Angotzi, A. R., Ebbesson, L. O., Karlsen, Ø., & Rønnestad, I. (2016). The ontogeny and brain distribution dynamics of the appetite regulators NPY, CART and pOX in larval Atlantic cod (Gadus morhua L.). PLoS One, 11(4), e0153743.

Leal-Cerro, A., Soto, A., Martínez, M. A., Dieguez, C., & Casanueva, F. F. (2001). Influence of cortisol status on leptin secretion. Pituitary, 4(1-2), 111-116.

Lethimonier, C., Madigou, T., Muñoz-Cueto, J. A., Lareyre, J. J., & Kah, O. (2004). Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. General and comparative endocrinology, 135(1), 1-16.

Leshan, R. L., Louis, G. W., Jo, Y. H., Rhodes, C. J., Münzberg, H., & Myers, M. G. (2009). Direct innervation of GnRH neurons by metabolic-and sexual odorant-sensing leptin receptor neurons in the hypothalamic ventral premammillary nucleus. *Journal of Neuroscience*, *29*(10), 3138-3147.

Li, G. G., Liang, X. F., Xie, Q., Li, G., Yu, Y., & Lai, K. (2010). Gene structure, recombinant expression and functional characterization of grass carp leptin. General and comparative endocrinology, 166(1), 117-127.

Li, M., & Leatherland, J. F. (2012). The interaction between maternal stress and the ontogeny of the innate immune system during teleost embryogenesis: implications for aquaculture practice. *Journal of fish biology*, *81*(6), 1793-1814.

Liu, C. Z., He, A. Y., Ning, L. J., Luo, Y., Li, D. L., Zhang, M. L., & Du, Z. Y. (2018). Leptin selectively regulates nutrients metabolism in Nile tilapia fed on high carbohydrate or high fat diets. Frontiers in endocrinology, 9, 574.

Luque, R. M., Kineman, R. D., & Tena-Sempere, M. (2007). Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. Endocrinology, 148(10), 4601-4611.

Madison, B. N., Tavakoli, S., Kramer, S., & Bernier, N. J. (2015). Chronic cortisol and the regulation of food intake and the endocrine growth axis in rainbow trout. J Endocrinol, 226(2), 103-19.

Maestro, M.A., Planas, J.V., Moriyama, S., Gutierrez, J., Planas, J. & Swanson, P. (1997). Ovarian receptors for insulin and insulin-like growth factor I (IGF-I) and effects of IGF-I on steroid production by isolated follicular layers of the preovulatory coho salmon ovarian follicle. General and Comparative Endocrinology, 106, 189-201.

Mandiki, S.N.M., Milla, S., Nkogo Robles, S. & Kestemont, P. (2017). Corticosteroids deeply depress the in vitro steroidogenic capacity of Eurasian perch ovary at the end of the reproductive cycle. General and Comparative Endocrinology, 245, 44-54.

Manfredi-Lozano, M., Roa, J., Ruiz-Pino, F., Piet, R., Garcia-Galiano, D., Pineda, R., & Dieguez, C. (2016). Defining a novel leptin–melanocortin–kisspeptin pathway involved in the metabolic control of puberty. Molecular metabolism, 5(10), 844-857.

Manfredi-Lozano, M., Roa, J., & Tena-Sempere, M. (2018). Connecting metabolism and gonadal function: Novel central neuropeptide pathways involved in the metabolic control of puberty and fertility. *Frontiers in neuroendocrinology*, *48*, 37-49.

Martos-Moreno, G. A., Chowen, J. A., & Argente, J. (2010). Metabolic signals in human puberty: effects of over and undernutrition. *Molecular and cellular endocrinology*, *324*(1-2), 70-81.

Mcshane, T. M., May, T., Miner, J. L., & Keisler, D. H. (1992). Central actions of neuropeptide-Y may provide a neuromodulatory link between nutrition and reproduction. Biology of Reproduction, 46(6), 1151-1157.

Mechaly, A. S., Viñas, J., & Piferrer, F. (2013). The kisspeptin system genes in teleost fish, their structure and regulation, with particular attention to the situation in Pleuronectiformes. *General and comparative endocrinology*, *188*, 258-268.

Mercer, J. G., Hoggard, N., Williams, L. M., Lawrence, C. B., Hannah, L. T., Morgan, P. J., & Trayhurn, P. (1996). Coexpression of leptin receptor and preproneuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. Journal of neuroendocrinology, 8(10), 733-735.

Michel, M., Page-McCaw, P. S., Chen, W., & Cone, R. D. (2016). Leptin signaling regulates glucose homeostasis, but not adipostasis, in the zebrafish. *Proceedings of the National Academy of Sciences*, *113*(11), 3084-3089.

Milla, S., Wang, N., Mandiki, S.N.M., Kestemont, P., 2009. Corticosteroids: friends or foes of teleost reproduction?. Comparative Biochemistry and Physiology, Part A. 153, 242-251.

Mommsen, T. P., & Walsh, P. J. (1988). 5 Vitellogenesis and oocyte assembly. In *Fish physiology* (Vol. 11, pp. 347-406). Academic Press.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries. 9, 211-268.

Morelli, A., Marini, M., Mancina, R., Luconi, M., Vignozzi, L., Fibbi, B., & Maggi, M. (2005). Sex steroids and leptin regulate the “first Kiss”(KiSS 1/G-protein-coupled receptor 54 system) in human gonadotropin-releasing-hormone-secreting neuroblasts. The journal of sexual medicine, 5(5), 1097-1113.

Mukherjee, D., Majumder, S., Moulik, S.R., Pal, P., Gupta, S., Guha, P. & Kumar, D. (2017). Membrane receptor cross talk in gonadotropin-, IGF-I-, and insulin-mediated steroidogenesis in fish ovary: an overview. General and Comparative Endocrinology, 240, 10-18.

Murashita, K., Kurokawa, T., Ebbesson, L. O., Stefansson, S. O., & Rønnestad, I. (2009). Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (Salmo salar). General and Comparative endocrinology, 162(2), 160-171.

Murashita, K., Uji, S., Yamamoto, T., Rønnestad, I., & Kurokawa, T. (2008). Production of recombinant leptin and its effects on food intake in rainbow trout (Oncorhynchus mykiss).

Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 150(4), 377-384.

Nagasaka, R., Okamoto, N., & Ushio, H. (2006). Increased leptin may be involved in the short life span of ayu (Plecoglossus altivelis). Journal of Experimental Zoology Part A: Comparative Experimental Biology, 305(6), 507-512.

Navarro, V. M., & Tena-Sempere, M. (2012). Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. Nature Reviews Endocrinology, 8(1), 40.

Newcomer, J. W., Selke, G., Melson, A. K., Gross, J., Vogler, G. P., & Dagogo-Jack, S. (1998). Dose-dependent cortisol-induced increases in plasma leptin concentration in healthy humans. Archives of general psychiatry, 55(11), 995-1000.

Nozu, R., Nakamura, M., 2015. Cortisol administration induces sex change from ovary to testis in the protogynous wrasse, Halichoeres trimaculatus. Sexual Development. 9, 118-124.

Ogawa, S., & Parhar, I. S. (2013). Anatomy of the kisspeptin systems in teleosts. General and comparative endocrinology, 181, 169-174.

Ohga, H., Hirata, D., Matsumori, K., Kitano, H., Nagano, N., Yamaguchi, A., & Matsuyama, M. (2017). Possible role of the leptin system in controlling puberty in the male chub mackerel, Scomber japonicus. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 203, 159-166.

Ohga, H., Matsumori, K., Kodama, R., Kitano, H., Nagano, N., Yamaguchi, A., & Matsuyama, M. (2015). Two leptin genes and a leptin receptor gene of female chub mackerel (Scomber japonicus): molecular cloning, tissue distribution and expression in different obesity indices and pubertal stages. General and comparative endocrinology, 222, 88-98.

Pankhurst, N.W. & Van Der Kraak, G. (2000). Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout in vivo, through the action of cortisol. General and Comparative Endocrinology, 117, 225-237.

Parent, A. S., Teilmann, G., Juul, A., Skakkebaek, N. E., Toppari, J., & Bourguignon, J. P. (2003). The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. *Endocrine reviews*, *24*(5), 668-693.

Parker, J. A., & Bloom, S. R. (2012). Hypothalamic neuropeptides and the regulation of appetite. Neuropharmacology, 63(1), 18-30.

Peter, R. E., & Crim, L. W. (1979). Reproductive endocrinology of fishes: gonadal cycles and gonadotropin in teleosts. *Annual Review of Physiology*, *41*(1), 323-335.

Peter, R. E., Yu, K. L., Marchant, T. A., & Rosenblum, P. M. (1990). Direct neural regulation of the teleost adenohypophysis. Journal of Experimental Zoology, 256(S4), 84-89.

Peyon, P., de Celis, S. V. R., Gómez-Requeni, P., Zanuy, S., Pérez-Sánchez, J., & Carrillo, M. (2003). In vitro effect of leptin on somatolactin release in the European sea bass (Dicentrarchus labrax): dependence on the reproductive status and interaction with NPY and GnRH. General and comparative endocrinology, 132(2), 284-292.

Peyon, P., Zanuy, S., & Carrillo, M. (2001). Action of leptin on in vitro luteinizing hormone release in the European sea bass (Dicentrarchus labrax). Biology of reproduction, 65(5), 1573-1578.

Pierroz, D. D., Catzeflis, C. H. A. N. T. A. L., Aebi, A. C., Rivier, J. E., & Aubert, M. L. (1996). Chronic administration of neuropeptide Y into the lateral ventricle inhibits both the pituitary-testicular axis and growth hormone and insulin-like growth factor I secretion in intact adult male rats. Endocrinology, 137(1), 3-12.

Pilkis, S. J., & Granner, D. K. (1992). Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. *Annual review of physiology*, *54*(1), 885-909.

Polakof, S., Panserat, S., Soengas, J. L., & Moon, T. W. (2012). Glucose metabolism in fish: a review. *Journal of Comparative Physiology B*, *182*(8), 1015-1045.

Putti, R., Varricchio, E., Gay, F., Elena, C., & Paolucci, M. (2009). Leptin effects on testis and epididymis in the lizard Podarcis sicula, during summer regression. *General and comparative endocrinology*, *160*(2), 168-175.

Qin, C., Wang, B., Sun, C., Jia, J. & Li, W. (2014). Orange-spotted grouper (Epinephelus coioides) adiponectin receptors: molecular characterization, mRNA expression, and subcellular location. General and Comparative Endocrinology, 198, 47-58.

Qiu, J., Fang, Y., Bosch, M. A., Rønnekleiv, O. K., & Kelly, M. J. (2011). Guinea pig kisspeptin neurons are depolarized by leptin via activation of TRPC channels. Endocrinology, 152(4), 1503-1514.

Quennell, J. H., Mulligan, A. C., Tups, A., Liu, X., Phipps, S. J., Kemp, C. J., & Anderson, G. M. (2009). Leptin indirectly regulates gonadotropin-releasing hormone neuronal function. Endocrinology, 150(6), 2805-2812.

Raffan, E., Dennis, R. J., O'Donovan, C. J., Becker, J. M., Scott, R. A., Smith, S. P., & Summers, K. M. (2016). A deletion in the canine POMC gene is associated with weight and appetite in obesity-prone labrador retriever dogs. Cell metabolism, 23(5), 893-900.

Rak, A, Mellouk, N., Froment, P. & Dupont, J. (2017). Adiponectin and resistin: potential metabolic signals affecting hypothalamo-pituitary gonadal axis in females and males of different species. Reproduction, 153, R215-R226.

Rastegar, S., Parimisetty, A., Sulliman, N.C., Narra, S.S., Weber, S., Rastegar, M., Viranaicken, W., Couret, D., Planesse, C., Strahle, U., Meilhac, O., Lefebvre d'Hellencourt, C. & Diotel, N.

(2019). Expression of adiponectin receptors in the brain of adult zebrafish and mouse: links with neurogenic niches and brain repair. Journal of Comparative Neurology, 527, 2317-2333.

Reading, B. J., Sullivan, C. V., & Schilling, J. (2011). Vitellogenesis in fishes. *Encyclopedia of fish physiology: from genome to environment*, *1*, 635-646.

Reid, R. L., Ling, N., & Yen, S. S. C. (1981). α Melanocyte stimulating hormone induces gonadotropin release. The Journal of Clinical Endocrinology & Metabolism, 52(1), 159-161.

Reid, R. L., Ling, N., & Yen, S. S. C. (1984). Gonadotropin-Releasing Activity of of α-Melanocyte-Stimulating Hormone in Normal Subjects and in Subjects with Hypothalamic-Pituitary Dysfunction. The Journal of Clinical Endocrinology & Metabolism, 58(5), 773-777.

Roa, J., & Herbison, A. E. (2012). Direct regulation of GnRH neuron excitability by arcuate nucleus POMC and NPY neuron neuropeptides in female mice. Endocrinology, 153(11), 5587-5599.

Roa, J., & Tena-Sempere, M. (2014). Connecting metabolism and reproduction: roles of central energy sensors and key molecular mediators. Molecular and cellular endocrinology, 397(1-2), 4-14.

Roa, J., García-Galiano, D., Castellano, J. M., Gaytan, F., Pinilla, L., & Tena-Sempere, M. (2010). Metabolic control of puberty onset: new players, new mechanisms. Molecular and cellular endocrinology, 324(1-2), 87-94.

Rønnestad, I., Nilsen, T. O., Murashita, K., Angotzi, A. R., Moen, A. G. G., Stefansson, S. O., & Kurokawa, T. (2010). Leptin and leptin receptor genes in Atlantic salmon: cloning, phylogeny, tissue distribution and expression correlated to long-term feeding status. *General and comparative endocrinology*, *168*(1), 55-70.

Sanchez-Garrido, M. A., & Tena-Sempere, M. (2013). Metabolic control of puberty: roles of leptin and kisspeptins. Hormones and behavior, 64(2), 187-194.

Sanchez-Gurmaches, J., Cruz-Garcia, L., Gutierrez, J. & Navarro, I. (2012). Adiponectin effects and gene expression in rainbow trout: an in vivo and in vitro approach. Journal of Experimental Biology, 215, 1373-1383.

Schreck, C. B. (2010). Stress and fish reproduction: the roles of allostasis and hormesis. *General and comparative endocrinology*, *165*(3), 549-556.

Schreck, C. B., Contreras-Sanchez, W., & Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. In *Reproductive Biotechnology in Finfish Aquaculture* (pp. 3-24). Elsevier.

Schiöth, H. B., & Watanobe, H. (2002). Melanocortins and reproduction. Brain Research Reviews, 38(3), 340-350.

Schioth, H. B., Kakizaki, Y., Kohsaka, A., Suda, T., & Watanobe, H. (2001). Agouti-related peptide prevents steroid-induced luteinizing hormone and prolactin surges in female rats. Neuroreport, 12(4), 687-690.

Schwartz, M. W., & Seeley, R. J. (1997). Neuroendocrine responses to starvation and weight loss. *New England Journal of Medicine*, *336*(25), 1802-1811.

Servili, A., Le Page, Y., Leprince, J., Caraty, A., Escobar, S., Parhar, I. S., & Kah, O. (2011). Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. Endocrinology, 152(4), 1527-1540.

Shpilman, M., Hollander-Cohen, L., Ventura, T., Gertler, A., & Levavi-Sivan, B. (2014). Production, gene structure and characterization of two orthologs of leptin and a leptin receptor in tilapia. General and comparative endocrinology, 207, 74-85.

Smith, J. T., Clifton, D. K., & Steiner, R. A. (2006). Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction, 131(4), 623-630.
Solomon, S. (1999). POMC-derived peptides and their biological action. Annals of the New York Academy of Sciences, 885(1), 22-40.

Soengas, J. L., Cerdá-Reverter, J. M., & Delgado, M. J. (2018). Central regulation of food intake in fish: an evolutionary perspective. *Journal of molecular endocrinology*, *60*(4), R171-R199.

Solomon-Lane, T.K., Crespi, E.J., Grober, M.S., (2013). Stress and serial adult metamorphosis: multiple roles for the stress axis in socially regulated sex change. Frontiers in Neuroscience. 7.

Somoza, G. M., Miranda, L. A., Strobl-Mazzulla, P., & Guilgur, L. G. (2002). Gonadotropin-releasing hormone (GnRH): from fish to mammalian brains. Cellular and molecular neurobiology, 22(5-6), 589-609.

Steven, C., Lehnen, N., Kight, K., Ijiri, S., Klenke, U., Harris, W. A., & Zohar, Y. (2003). Molecular characterization of the GnRH system in zebrafish (Danio rerio): cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. General and comparative endocrinology, 133(1), 27-37.

Tang, Y., Yu, J., Li, H., Xu, P., Li, J., & Ren, H. (2013). Molecular cloning, characterization and expression analysis of multiple leptin genes in Jian carp (Cyprinus carpio var. Jian). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 166(2), 133-140.

Taylor, J. S., Braasch, I., Frickey, T., Meyer, A., & Van de Peer, Y. (2003). Genome duplication, a trait shared by 22,000 species of ray-finned fish. Genome research, 13(3), 382-390.

Teitsma, C.A., Anglade, I., Bailhache, T., Tujague, M., Toutirais, G., Ducouret, B. & Kah, O. (1998a). Distribution of glucocorticoid receptors in the brain of rainbow trout (Oncorhynchus mykiss): possible relationships with the neuroendocrine systems controlling reproduction. Bull. Fr. Pêche Piscic., 350-351, 597-607.

Teitsma, C.A., Anglade, I., Toutirais, G., Munoz-Cueto, J.A., Saligaut, D., Ducouret, B. & Kah, O. (1998b). Immunohistochemical localization of glucocorticoid receptors in the forebrain of the rainbow trout (Oncorhynchus mykiss). Journal of Comparative Neurology, 401, 395-410.

Teitsma, C.A., Anglade, I., Lethimonier, C., Dréan, G.L., Saligaut, D., Ducouret, B., Kah, O., 1999. Glucocorticoid receptor immunoreactivity in neurons and pituitary cells implicated in reproductive functions in rainbow trout: a double immunohistochemical study. Biology of Reproduction. 60, 642-650.

Teitsma, C.A., Bailhache, T., Tujague, M., Balment, R.J., Ducouret, B., Kah, O., 1997. Distribution and expression of glucocorticoid receptor mRNA in the forebrain of the rainbow trout. Neuroendocrinology. 66, 294-304.

Tena-Sempere, M. (2007). Roles of ghrelin and leptin in the control of reproductive function. Neuroendocrinology, 86(3), 229-241.

Tena-Sempere, M., Felip, A., Gómez, A., Zanuy, S., & Carrillo, M. (2012). Comparative insights of the kisspeptin/kisspeptin receptor system: lessons from non-mammalian vertebrates. *General and comparative endocrinology*, *175*(2), 234-243.

Thornton, J. E., Cheung, C. C., Clifton, D. K., & Steiner, R. A. (1997). Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. Endocrinology, 138(11), 5063-5066.

Tinoco, A. B., Nisembaum, L. G., Isorna, E., Delgado, M. J., & de Pedro, N. (2012). Leptins and leptin receptor expression in the goldfish (Carassius auratus). Regulation by food intake and fasting/overfeeding conditions. *Peptides*, *34*(2), 329-335.

Todd, E.V., Ortega-Recalde, O., Liu, H., Lamm, M.S., Rutherford, K.M., Cross, H., Black, M.A., Kardailsky, O., Marshall Graves, J.A., Hore, T.A., Godwin, J.R., Gemmell, N.J., 2019. Stress, novel sex genes, and epigenetic reprogramming orchestrate socially controlled sex change. Science Advances. 5(7).

Trombley, S., & Schmitz, M. (2013). Leptin in fish: possible role in sexual maturation in male Atlantic salmon. Fish physiology and biochemistry, 39(1), 103-106.

Trombley, S., Maugars, G., Kling, P., Björnsson, B. T., & Schmitz, M. (2012). Effects of long-term restricted feeding on plasma leptin, hepatic leptin expression and leptin receptor expression in juvenile Atlantic salmon (Salmo salar L.). General and comparative endocrinology, 175(1), 92-99.

Trombley, S., Mustafa, A., & Schmitz, M. (2014). Regulation of the seasonal leptin and leptin receptor expression profile during early sexual maturation and feed restriction in male Atlantic salmon, Salmo salar L., parr. General and comparative endocrinology, 204, 60-70.

True, C., Kirigiti, M. A., Kievit, P., Grove, K. L., & Smith, M. S. (2011). Leptin is not the critical signal for kisspeptin or luteinising hormone restoration during exit from negative energy balance. Journal of neuroendocrinology, 23(11), 1099-1112.

Tsatsanis, C., Dermitzaki, E., Avgoustinaki, P., Malliaraki, N., Mytaras, V. & Margoiris, A.N. (2015). The impact of adipose tissue-derived factors on the hypothalamic-pituitary-gonadal (HPG) axis. Hormones, 14, 549-562.

Turi, G. F., Liposits, Z., Moenter, S. M., Fekete, C., & Hrabovszky, E. (2003). Origin of neuropeptide Y-containing afferents to gonadotropin-releasing hormone neurons in male mice. Endocrinology, 144(11), 4967-4974.

Unniappan, S. (2010). Ghrelin: an emerging player in the regulation of reproduction in non-mammalian vertebrates. General and Comparative Endocrinology, 167, 340-343.

Vallarino, M., Danger, J. M., Fasolo, A., Pelletier, G., Saint-Pierre, S., & Vaudry, H. (1988). Distribution and characterization of neuropeptide Y in the brain of an elasmobranch fish. Brain Research, 448(1), 67-76.

von Krogh, K., Bjorndal, G.T., Nourizadeh-Lillabadi, R., Ropstad, E., Haug, T.M., Weltzien, F.A. (2019). Cortisol differentially affects cell viability and reproduction-related gene expression in Atlantic cod pituitary cultures dependent on stage of sexual maturation. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 236, 110517.

Volkoff, H., Eykelbosh, A. J., & Peter, R. E. (2003). Role of leptin in the control of feeding of goldfish Carassius auratus: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. *Brain research*, *972*(1-2), 90-109.

Volkoff, H., & Peter, R. E. (2001). Characterization of two forms of cocaine-and amphetamine-regulated transcript (CART) peptide precursors in goldfish: molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. Endocrinology, 142(12), 5076-5088.

Volkoff, H., Sabioni, R. E., Coutinho, L. L., & Cyrino, J. E. P. (2017). Appetite regulating factors in pacu (Piaractus mesopotamicus): tissue distribution and effects of food quantity and quality on gene expression. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *203*, 241-254.

Vulliémoz, N. R., Xiao, E., Xia-Zhang, L., Wardlaw, S. L., & Ferin, M. (2005). Central infusion of agouti-related peptide suppresses pulsatile luteinizing hormone release in the ovariectomized rhesus monkey. Endocrinology, 146(2), 784-789.

Wabitsch, M., Jensen, P. B., Blum, W. F., Christoffersen, C. T., Englaro, P., Heinze, E., & Hauner, H. (1996). Insulin and cortisol promote leptin production in cultured human fat cells. Diabetes, 45(10), 1435-1438.

Wallace, R. A. (1985). Vitellogenesis and oocyte growth in nonmammalian vertebrates. In *oogenesis* (pp. 127-177). Springer, Boston, MA.

Wan, Y., Zhang, Y., Ji, P., Li, Y., Xu, P., & Sun, X. (2012). Molecular characterization of CART, AgRP, and MC4R genes and their expression with fasting and re-feeding in common carp (Cyprinus carpio). Molecular biology reports, 39(3), 2215-2223.

Watanobe, H. (2002). Leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion in vivo in rats. The Journal of physiology, 545(1), 255-268.

Weber, G. M., Powell, J. F. F., Park, M., Fischer, W. H., Craig, A. G., Rivier, J. E., & Sherwood, N. M. (1997). Evidence that gonadotropin-releasing hormone (GnRH) functions as a prolactin-releasing factor in a teleost fish (Oreochromis mossambicus) and primary structures for three native GnRH molecules. Journal of Endocrinology, 155, 121-132.

Weil, C., Le Bail, P. Y., Sabin, N., & Le Gac, F. (2003). In vitro action of leptin on FSH and LH production in rainbow trout (Onchorynchus mykiss) at different stages of the sexual cycle. General and comparative endocrinology, 130(1), 2-12.

Wendelaar Bogna, S.E. (1997). The stress response in fish. Physiological Reviews, 77, 591-625.

Whirledge, S., Cidlowski, J.A., (2017). Glucocorticoids and reproduction: traffic control on the road to reproduction. Trends in Endocrinology & Metabolism. 28, 399-415.
Williams, K. W., Margatho, L. O., Lee, C. E., Choi, M., Lee, S., Scott, M. M., & Elmquist, J. K. (2010). Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. Journal of Neuroscience, 30(7), 2472-2479.

Won, E. T., Baltzegar, D. A., Picha, M. E., & Borski, R. J. (2012). Cloning and characterization of leptin in a Perciform fish, the striped bass (Morone saxatilis): control of feeding and regulation by nutritional state. General and comparative endocrinology, 178(1), 98-107.

Yan, A. F., Chen, T., Chen, S., Ren, C. H., Hu, C. Q., Cai, Y. M., & Tang, D. S. (2016). Goldfish leptin-AI and leptin-AII: function and central mechanism in feeding control. International journal of molecular sciences, 17(6), 783.

Yan, A., Chen, Y., Chen, S., Li, S., Zhang, Y., Jia, J., & Tang, D. (2017). Leptin stimulates prolactin mrna expression in the goldfish pituitary through a combination of the pi3k/akt/mtor, mkk3/6/p38mapk and mek1/2/erk1/2 signalling pathways. International journal of molecular sciences, 18(12), 2781.

Yang, G., Song, Q., Sun, S., Qin, J., Jia, J., Yuan, X., Zhang, Y. & Li, W. (2018). Ctrp9 and adiponectin receptors in Nile tilapia (Oreochromis niloticus): molecular cloning, tissue distribution and effects on reproductive genes. General and Comparative Endocrinology, 265, 160-173.

Yaswen, L., Diehl, N., Brennan, M. B., & Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nature medicine, 5(9), 1066.

Yu, W. H., Kimura, M., Walczewska, A., Karanth, S., & McCann, S. M. (1997). Role of leptin in hypothalamic–pituitary function. Proceedings of the National Academy of Sciences, 94(3), 1023-1028.

Yuan X, Li A, Liang XF, Huang W, Song Y, He S. Leptin expression in mandarin fish Siniperca chuatsi (Basilewsky): regulation by postprandial and short-term fasting treatment. Comp Biochem Physiol A Mol Integr Physiol (2016) 194:8–18.

Zhang, H., Chen, H., Zhang, Y., Li, S., Lu, D., Zhang, H., & Lin, H. (2013). Molecular cloning, characterization and expression profiles of multiple leptin genes and a leptin receptor gene in orange-spotted grouper (Epinephelus coioides). General and comparative endocrinology, 181, 295-305.

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., & Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. General and comparative endocrinology, 165(3), 438-455

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, *372*(6505), 425-432.

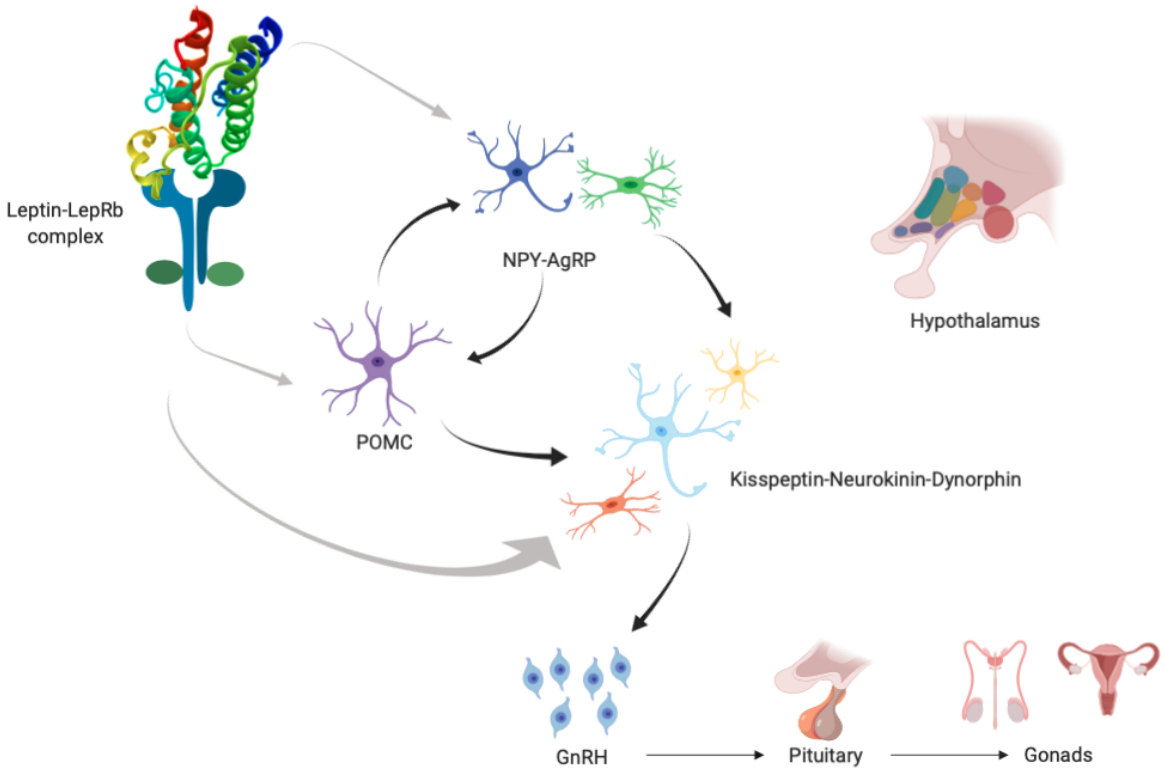

Figure 1. A simplified schematic representation of leptin inputs at the mammalian reproductive brain. Briefly, leptin exerts its reproductive actions via indirect regulation of GnRH by directly targeting its classical first order neuronal targets including NPY/AgRP and POMC, as well as the apex HPG axis Kiss1 neuronal population/KNDy pulse generator. Kiss1 then stimulates GnRH to regulate the overall function of the HPG axis.

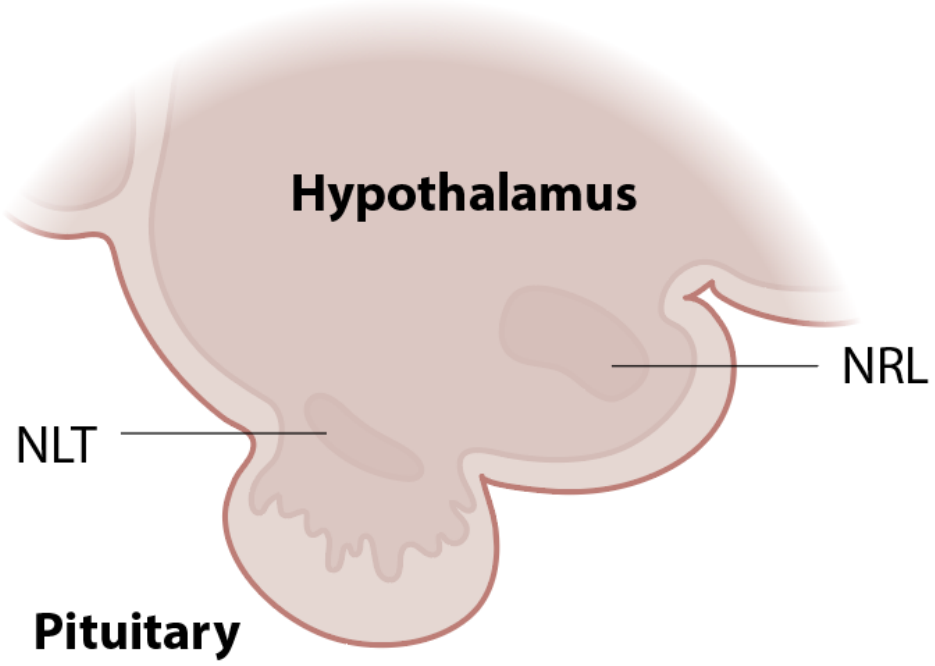

Figure 2. A schematic diagram outlining the teleost hypophyseal arrangement. nLT: Lateral tuberal nucleus; nRL: Nucleus of the lateral recess.

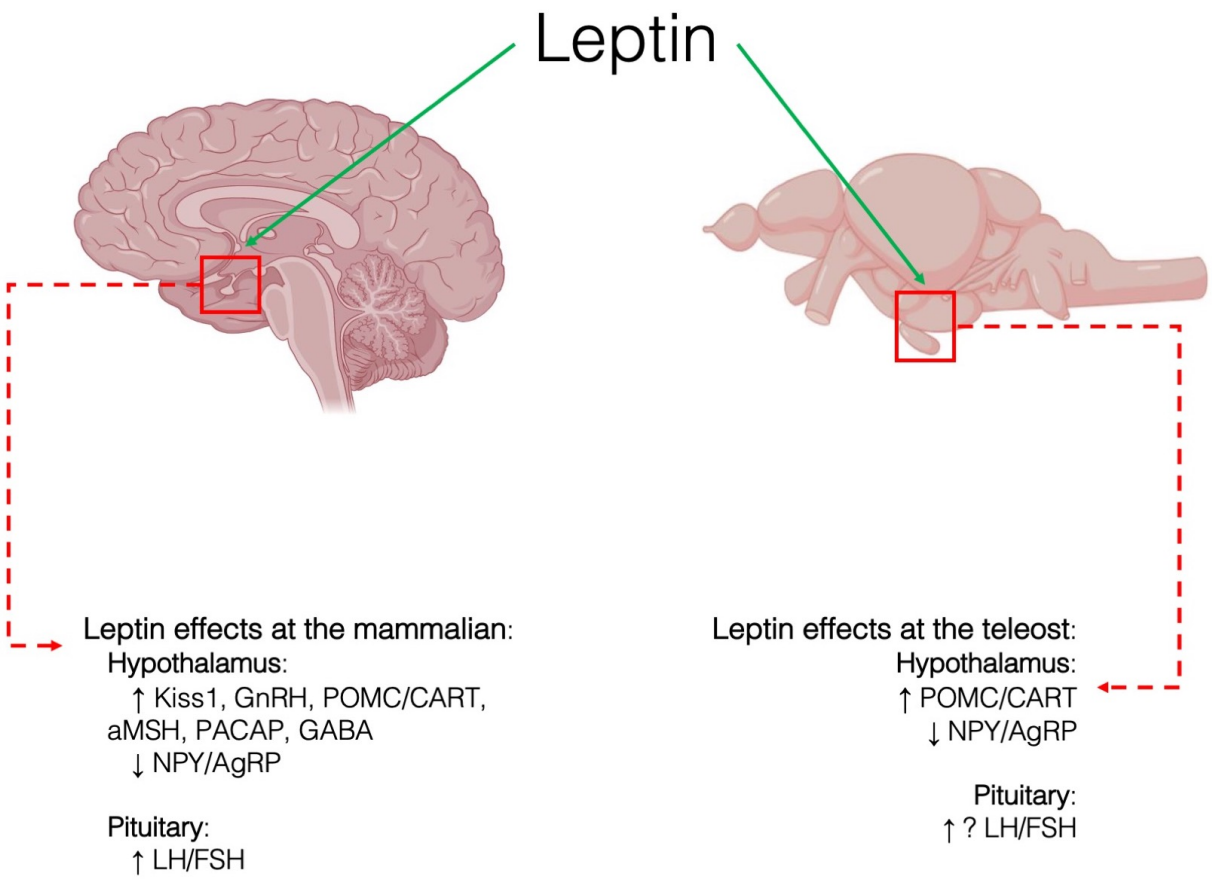

Figure 3. A schematic diagram outlining currently-understood reproductive actions of leptin at the mammalian and teleost hypothalamus and pituitary.

Table 1. An overview of the source, appetite-regulatory actions, and common and divergent reproductive properties of leptin among vertebrate groups. The summarized details in this table reflect an overview of the current state of knowledge regarding the dominant source of circulating leptin, the hormone's described reproductive targets, and currently known reproductive actions. Question marks indicate areas that have not yet been studied or remain unclear. Adapted from Davies *et al*. (2014), Friedman *et al*. (2014), Deck *et al*. (2017), and Friedman & Seroussi (2019).

					
Major Source	Fat	Liver and fat	Liver and fat bodies	?	Liver
Appetite Regulation	Anorexigenic	Anorexigenic	Anorexigenic	Anorexigenic	Anorexigenic
Reproductive Targets	Hypothalamus, pituitary, gonads	LepR expressed in hypothalamus, pituitary, gonads	?	Pre-optic area, hypothalamus, pituitary	Hypothalamus, pituitary, liver, gonads
Reproductive Actions	Permissive of puberty; can be inhibitory when in excess	Increases clutch size Generally unclear	Increases clutch size; Decreases with testicular regression Generally unclear	Increases with puberty and metamorphosis Generally unclear	+ Pituitary GTHs - Hepatic Vtgs Increases with puberty Generally unclear

CHAPTER 2

Leptin and cortisol regulation of food intake and appetite-regulatory neuropeptides in the Mozambique tilapia (*Oreochromis mossambicus*)

Abstract

Leptin and glucocorticoids are established regulators of appetite and food intake in mammals. Leptin reduces food intake by promoting anorexigenic neuropeptides, while glucocorticoids stimulate food intake by promoting orexigenic neuropeptides. While these appetite-regulatory properties are well-understood in mammals, their effects on food intake and regulation of central appetite-regulating targets requires elucidation in teleost fishes. In the current study, we investigated the behavioral actions of leptin and cortisol on food consumption, as well as their regulation of brain orexigens (*npya* and *agrp2*) and anorexigens (*pomc*, *cart*, *crf*) in the Mozambique tilapia (*Oreochromis mossambicus*). We also created a modified protocol for producing higher yields of biologically active recombinant tilapia leptin A (rtLepA), the dominant leptin paralog in teleosts. Acute treatment with 0.5 and 5.0 μg/g body weight (BW) rtLepA significantly decreased food intake at 2- and 6-hours post-injection. Acute cortisol treatment significantly reduced food intake initially at 2-hours, but promoted food intake at 6-hours post-injection. In a separate experiment, rtLepA at dosages of 0.5 and 5 μg/g BW increased brain *pomc* and *cart*, and reduced *agrp2* after 6-hours. The lower dosage of rtLepA stimulated, while the higher dosage suppressed *npya.* Acute cortisol injection significantly increased brain *npya* and *agrp2* after 2, 6 and 24-hours, but had no effect on *pomc* or *cart.* At the low dosage treatment, the steroid marginally increased *crf* over the first 6 hours. Our findings suggest that leptin may decrease appetite by stimulation of *pomc* and *cart* and inhibition of *agrp2*, while cortisol may promote appetite by stimulation of *npya* and *agrp2*. Overall, the current study indicates that leptin and glucocorticoids exert opposing appetite-regulatory actions in tilapia through mechanisms that may involve varied regulation of major orexigenic and anorexigenic neuropeptides in the brain.

Introduction

Regulation of food intake is key to maintaining normal energy homeostasis. Appetite and feeding are controlled by central circuits that sense energy sufficiency through metabolic and endocrine signaling (reviewed in Morley 1987). Among peripherally-secreted endocrine signals that exert control over appetite are leptin and cortisol. In mammals, leptin is an adipostatic hormone expressed and secreted in proportion to white adipose tissue (Zhang *et al*., 1994). Leptin's prototypic actions include enhanced energy mobilization and expenditure and post-prandial appetite suppression (Zhang *et al*., 1994; Masuzaki *et al*., 1995; reviewed in Margetic *et al*., 2002). Cortisol is an adrenal glucocorticoid and major effector hormone of the endocrine stress axis that mobilizes energy stores needed to meet increased metabolic demand. The stress response is known to induce changes in appetite across vertebrates (reviewed in Ans *et al*., 2018), attributed to endocrine signaling involving central circuits and glucocorticoids.

Food intake and the sequential changes associated with the post-prandial state are linked with leptin signaling at appetite-regulatory centers at the brain. Leptin acts on its first order targets at the hypothalamus including agouti-related peptide (AgRP), neuropeptide Y (NPY), and proopiomelanocortin (POMC) to inhibit appetite by decreasing the activity of orexigenic AgRP/NPY (Baskin *et al*., 1999; Korner *et al*., 2001; Morisson *et al*., 2005; Baver *et al*., 2014) neurons and stimulating the anorexigenic POMC derivative alpha-melanocyte-stimulating hormone (aMSH) (Baskin *et al*, 1999; Elias *et al*., 1999; Cowley *et al*., 2001; Balthasar *et al*, 2004). The anorexigenic model for leptin action in mammals has been well-established and emergent evidence continues to indicate an anorectic role for leptin on appetite in teleost fishes.

Leptin administration is reported to decrease food intake in various teleosts, reflective of its prototypic actions reported in other vertebrates (i.e., mice, humans – Zhang *et* al., 1994; Elias

et al., 1999; Ahima *et al*., 2001). Leptin administration in goldfish (*Carassius auratus* – Volkoff *et al*., 2003), rainbow trout (*Onchorhynchus mykiss* – Murashita *et al*., 2008), grass carp (*Ctenopharyngodon idella* – Li *et al*., 2010), Atlantic salmon (*Salmo salar* – Murashita *et al*., 2011), striped bass (*Morone saxatilis* – Won *et al*., 2012), and Nile tilapia (*Oreochromis niloticus* – Liu *et al*., 2018) has been shown to reduce food intake. This anorectic role for leptin in teleosts is further corroborated by evidence of its interactions with central appetite-regulatory neuropeptides. Notably, studies indicate that the hormone promotes hypothalamic POMC mRNA levels (e.g., *S. salar* – Murashita *et al*., 2011), while others show that it decreases hypothalamic NPY (e.g., *O. mykiss* – Murashita *et al*, 2008; *C. idellus* – Lu *et al*., 2015; *C. auratus* – Volkoff *et al*, 2006). The disparate effects observed across teleosts to date necessitates further study of its appetite-regulatory actions.

Leptin is highly responsive to metabolic state. In mammals, the hormone rises postprandially. In teleosts, postprandial increase to leptin expression has been reported in some species (e.g., *C. auratus* – Tinoco *et al*, 2012; *Epinephelus coioides* – Zhang *et al*, 2013) while others have indicated an absence of a postprandial leptin response (e.g., *Schizothorax prenanti* – Yuan *et al*., 2014). Greater variability has been reported with regards to leptin response to metabolic challenges presented through fasting or food restriction. In mammals, the metabolic challenge of fasting/starvation coincides with decreased leptin activity (Ahima *et al*., 1996), while fasting increases leptin expression in the preponderance of teleosts studied to date (reviewed in Deck *et al*., 2017 – e.g., *E. coioides* – Zhang *et* al, 2012; O. *mossambicus* – Douros *et al*., 2017). In other teleosts including the striped bass (*M. saxatilis* – Won *et al*., 2012) and red-bellied piranha (*Pygocentrus nattereri* – Volkoff 2015), leptin behaves in line with the current vertebrate paradigm by decreasing in response to fasting. Taken together, the variable

leptin responses to feeding and to metabolic challenge suggest there may be a vast array of species-specific functions for the hormone in the endocrine regulation of appetite and food intake.

In contrast to food abundance and satiation, a paucity of food acts as a major stressor and stimulates glucocorticoid actions for the mobilization of energy resources. Acute stress reduces appetite primarily through the potent anorexigenic effect of hypothalamic corticotropin-releasing factor (CRF) (Heinrichs & Richard, 1999; Richard *et al*., 2002; Heinrichs 2005). The chronic stress and metabolic challenge associated with starvation is linked to elevated levels of circulating glucocorticoids that can alter the appetite-regulatory component of the stress response by reducing hypothalamic CRF sensitivity to systemic glucocorticoid levels and stimulating hypothalamic factors including AgRP and NPY (Williams *et al*., 2001; Johnstone *et al*., 2005).

Interactions between leptin and glucocorticoids have also been reported in mammals and other vertebrates including fishes, in which leptin exerts potent inhibitory actions on multiple levels of the endocrine stress axis. Studies indicate leptin downregulation of hypothalamic CRF and cortisol secretion in mice (Bornstein *et al*., 1997; Gardner *et al*., 1998; Arvaniti *et al*., 2001; reviewed in Roubos *et al*., 2012). Similar actions have been reported in teleosts, with leptin inhibition of ACTH-induced cortisol secretion *in vivo* and suppression of CRF-induced ACTH *in vitro* in the common carp (*C. carpio* – Gorissen *et al*., 2012; Bernier *et al*., 2012). Hence, it is possible that this inhibitory effect of leptin may be conserved amongst vertebrate classes.

Fishes are the most abundant group within the vertebrate taxonomy, within which the bony fishes (Teleostei) represent more than half of all known vertebrate species. The immense diversity of teleosts is also reflected in the various adaptations to feeding and gastrointestinal function (Hart 1983), as well as drastically different metabolic needs of a largely ectothermic

taxa compared to homeothermic mammals (Angilletta *et al*., 2002). Further, anatomical differences reflected in direct innervation of the pituitary by the hypothalamus (Peter *et al*., 1990) may indicate a divergence in response to endocrine signaling typically reported in the established mammalian paradigm for the endocrinology of appetite regulation. Overall, fishes may demonstrate vastly different species-specific appetite-regulatory systems.

Many studies into leptin actions in teleost fishes have involved the use of mammalian protein (e.g., Baker *et al*., 2000; Londraville & Duvall, 2002; de Pedro *et al*., 2006). While they have produced valuable insights into leptin biology, the efficacy of using heterologous hormone is questionable due to the substantial differences that are observed between mammalian and teleostean proteins including poor conservation of gene and amino acid sequences, as well as variations in structure and folding of the mature protein. These variations may result in discrepancies in 'true' function when attempting to study hormone action using heterologous proteins. Investigations of leptin actions in teleosts using mammalian leptin sometimes indicate an absence of food intake response or a response only when hyperphysiological to near-pharmacological doses are applied (Baker *et al*., 2000; Londraville & Duvall, 2002). While the importance/necessity of using homologous hormones cannot be overstated, the practicality doing so is fairly limited when using non-typical study organisms (i.e., tilapia) such as fishes that are not classical biomedical models (i.e., zebrafish). For the most part, recombinant proteins for most teleost species are not commercially available. Our group has previously developed a technique for producing and purifying biologically-active tilapia leptin (Douros *et al*., 2014; Baltzegar *et al.,* 2014). While recombinant protein production using conventional techniques like affinity purification is highly feasible and practical, slight modifications to buffer systems can be implemented to improve yield.

Albeit abundant and diverse, the endocrine regulation of feeding in fishes has only been investigated in a few teleost species. Leptin and cortisol effects on appetite have been reported in some teleosts (reviewed in Volkoff 2019). However, the mechanisms of action and behavioral effects of these two hormones remain generally unresolved for teleost fishes, including in the Mozambique tilapia. In the current study, we modified a previously-developed recombinant leptin purification method to increase yield, investigated the effect of acute leptin and cortisol treatment on behavioral food intake and their effects on hypothalamic transcript levels of orexigenic (*npya*, *agrp2*) and anorexigenic (*pomc*, *cart, crf*) neuropeptides.

Materials and methods

Experimental animals

Adult male tilapia (*Oreochromis mossambicus*, ~88–103 g) were housed in freshwater (FW) recirculating tank systems (salinity 0–0.5 ppt, hardness 74–84 mg/L, alkalinity 126–178 mg/L, pH 8.0) at 24–26°C with a photoperiod of 12:12 h of light and dark and fed daily (1–2% body weight/day). Fish were anesthetized in buffered tricaine methanesulfonate (MS-222) prior to intraperitoneal injection and decapitated before brain removal. All animal protocols were approved by the NC State University Institutional Animal Care and Use Committee.

Recombinant tilapia leptin A (rtLepA) production and purification

Recombinant tilapia leptin A, the dominant paralog in tilapia (Douros *et al.,* 2014; Shpilman *et al*., 2014) was produced according to previously published methods (Baltzegar *et al.* 2014; Douros *et al*., 2014) and modified to increase yield of biologically active hormone (Figure 1). The Mozambique tilapia *lepa* complete coding sequence (462bp, GenBank ID: KC354702.1)

was amplified from total liver cDNA and inserted into a pET151b D-TOPO directional topo-isomerase vector. The sequence was confirmed via Sanger sequencing using the T7 promoter site on the pET151b backbone. The pET151b-*lepa* vector was transformed into TOP10 high copy *Escherichia coli* cells for plasmid propagation and purified via plasmid DNA mini-prep.

BL21 DE3 protein expression *E. coli* cells were transformed with pET151b-*lepa*. These cells were grown in 500 ml LB broth at 37°C until OD600, after which protein expression was induced using 100 nM IPTG for 10 hours at 26°C. Cells were pelleted at 15,000 x g, resuspended in a denaturing guanidine hydrochloride solution (6 M guanidine hydrochloride in 20 mM PBS pH 8.0), and sonicated. Cell debris was removed by centrifugation at 20,000 x g and the resulting supernatant containing total protein including rtLepA was applied to a Ni NTA column. Guanidine hydrochloride was removed by application of stepwise-decreased concentrations of urea buffers (8 M, 5 M, 1 M urea in 20 mM PBS pH 8.0), followed by protein refolding by application of native sodium phosphate buffer (20 mM PBS pH 8.0) with 2.5 mM L-cysteine. rtLepA was eluted using 500 mM imidazole suspended in a pH 8.0 PBS. The resulting eluate was dialyzed six times over 2 days at 4°C against 1 L of 0.5 nM PBS at pH 8.0, snap frozen on liquid nitrogen, and lyophilized. Following elution and dialysis, protein concentration was measured using bicinchoninic acid assay. SDS-PAGE was performed in a 4-20% gradient tris-glycine polyacrylamide gel (Novex Wedgewell SDS Gel, Invitrogen, Carlsbad, California, USA) to confirm expected size of rtLepA. The gel was stained with SyPro Ruby, imaged under UV light, and purity was quantified using ImageJ. The biological activity of purified rtLepA was verified by assessment of glucose response *in vivo* and *in vitro igf1* induction in primary tilapia hepatocytes (Baltzegar et al., 2014; Won *et al*., 2016; Douros *et al*., 2017). Fish were injected intraperitoneally with rtLepA (0.5 μg/g BW) and blood glucose was measured 4 hours later using

a blood glucometer (Bayer AG, Leverkusen, Germany). For *in vitro* analysis, liver was removed from fish and diced in Hank's buffered salt solution (HBSS) containing 0.3 mg/ml type IV collagenase (Sigma-Aldrich, St. Louis, Missouri, USA). The tissue homogenate was incubated in the HBSS-collagenase solution for 30 minutes at 24°C with gentle agitation. The collagenase-digested tissue was sequentially filtered through a 260 μm and 60 μm mesh, therein yielding a filtered hepatocyte suspension. The hepatocytes were washed in Dulbecco's PBS containing 3 mM $CaCl_2$ and 1x MEM solution (Gibco, Carlsbad, California, USA) and then allowed to recover for 1 hour. Tilapia hepatocytes were then cultured in 24-well plates at a density of 1x10^6 cells/ml at 3.5x10^5 cells/cm per well in with RPMI 1640 media containing L-glutamine and 1% streptomycin/penicillin. After a 4-hour acclimation incubation at 26°C, media was removed and replaced with fresh experimental media containing either rtLepA or no hormone. Cells were then incubated for 18 hours at 26°C. At termination, cells were taken up and lysed in Tri-Reagent (Molecular Research Center, Cincinnati, OH) for subsequent extraction and determination of *igf1* mRNA.

Regulation of food intake by rtLepA and cortisol

Two separate studies evaluated the effect of rtLepA and cortisol on food intake in tilapia. For each experiment, similarly-sized fish were housed in groups of 6 in 12 identical 135-liter rectangular tanks within a recirculating system, with each tank containing fish that varied by a maximum within-tank weight of +/- 1g. For 8 weeks preceding the experiment, fish were fed daily at 1100 h to entrain them on a regular feeding schedule. Food was withheld from the fish 24 hours prior to the start of the experiment. The recirculating tanks were divided into one control and two treatment groups (N = 4 tanks/group, 6 fish/tank) for each experiment. On the

day of the experiments, fish were anaesthesized in bicarbonate-buffered MS-222, weighed, and injected intraperitoneally with either rtLepA (0, 0.5, 5 μg/g BW in phosphate-buffered saline PBS) or cortisol (0, 5, 20 μg/g BW in sesame oil) using a 25-gauge needle at 0900 h. Fish were returned to their tanks and allowed to recover for two hours before the first feeding. Feeding was measured at 2- and 6-hours post-injection. Feed (Zeigler Finfish Bronze - 35% protein, 5% fat) was applied to satiation. Uneaten pellets were counted, multiplied by mean dry pellet weight, and subtracted from the weight of feed applied. Group feeding was quantified as a percentage of mean body weight.

Leptin effects on hypothalamic neuropeptide gene expression

Fish were anaesthesized, weighed, and injected intraperitoneally with 0.5 or 5.0 μg/g BW rtLepA, 5 μg/g recombinant human leptin (rhLep) or PBS vehicle alone (n = 8 fish/treatment). Six hours post-injection, fish were anaesthesized, decapitated, and fore- and mid-brain were collected and stored in RNAlater prior to RNA extraction and measurement of transcript levels by real time quantitative PCR (RT-qPCR). The fore- and mid-brain were dissected and collected for analysis as they contain the central regions encompassing the neuronal populations of NPYa, AgRP2, POMC, CART, and CRF (Delgado *et al*., 2017).

Cortisol effects on hypothalamic neuropeptide gene expression

Fish were anaesthesized, weighed, and treated with 5 or 20 μg/g BW cortisol in sesame oil or sesame oil vehicle (n = 8 fish/treatment). 2-, 6-, and 24-hours post-injection, the fish were anaesthesized, decapitated, and fore- and mid-brain were collected and stored in RNAlater prior to RNA extraction and RT-qPCR.

RNA isolation and quantification of gene expression

Brain tissues and hepatocytes were homogenized and total RNA was extracted using Tri-reagent, isopropanol precipitation, and DNase treatment. RNA purity and concentration were measured via an absorbance spectrophotometer (260/280 nm > 1.8; NanoDrop 1000, Thermo Fisher Scientific). Total RNA (1 μg) was reverse transcribed to cDNA using random hexamers (High Capacity cDNA Synthesis Kit, Life Technologies). Non-reverse transcribed samples were used as a control for genomic contamination. The mRNA levels of brain *npya, agrp2, pomc, cart, crf,* and hepatocyte *igf1* were determined by quantitative real-time PCR (qPCR) using gene-specific primers (Appendix Table 1). All cDNA samples, standards, and negative control reactions were run in triplicate on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems), with Brilliant II SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA). The qPCR cycling conditions were applied as follows: 10 minutes at 95°C, 40 cycles of 95°C for 30 seconds, and annealing for 1 minute at 60°C for 1 minute. A terminal dissociation melt curve step was used to verify a single PCR product. The absence of genomic DNA contamination was confirmed using water (No Template Control; NTC) and DNase treated RNA with no reverse transcriptase enzyme (No-Amplification Control; NAC) as negative controls. Cycle threshold (Ct) values were transformed against a standard curve of serially-diluted cDNA versus Ct values, normalized to *b-actin*, and reported as fold change relative to control treatments.

Statistical Analyses

In experiments with single time-point treatment comparisons, data was analyzed using Student's t-test for comparisons of two groups and one-way analysis of variance (ANOVA)

followed by Dunnett's Multiple Comparisons for comparisons of more than two groups. For comparisons including treatment and time, two-way ANOVA was employed followed by Fisher's Least Significant Difference test for within-treatment comparisons and Dunnett's Multiple Comparisons for comparisons of treatments over time. Statistical significance for all analyses was denoted by an alpha of $P < 0.05$ and data values are presented as means ± SEM. All analyses were performed using SPSS Statistics 25 (IBM, Armonk, New York, USA).

Results

Recombinant leptin purification and assessment of biological activity

Purification via a hybrid chaotrope-to-refolding buffer system generated soluble highly pure 6xHis-rtLepA, with little to no loss during initial lysis, binding, and wash phases. Final monomeric yield was 101.3 mg/L *E. coli* culture with a purity of at least 96%. Cleavage of the His-tag using TEV protease yielded a ~16 kDa band consistent with what has been previously reported for rtLepA (Douros *et al*., 2017).

We found that rtLepA stimulated *igf1* mRNA levels in primary hepatocytes (Figure 2; $P < 0.001$). The low dose of 1 nM elevated *igf1* by up to 11-fold, while 10 and 100 nM promoted *igf1* by up to approximately 5-fold relative to controls. Treatment of tilapia *in vivo* elevated blood glucose levels by around 50% compared to saline-injected control (Figure 3; $P < 0.01$).

Regulation of food intake by recombinant tilapia leptin and cortisol

During the rtLepA 2- and 6-hour post-injection feeding period, feed intake significantly decreased in comparison to saline control (2 h: 10.11±0.735; 6 h: 2.166±0.401 % BW) with both 0.5 μg/g (2 h: 1.603±0.306; 6 h: 1.517±0.275 % BW) and 5 μg/g BW (2 h: 1.231±0.703; 6 h:

1.033±0.030 % BW) rtLepA treatment (Figure 4; $P < 0.01$). Cortisol treatment at 5 μg/g and 20 μg/g BW reduced feed intake at 2-hours post-injection (Figure 5; $P < 0.05$), but significantly increased feed consumption at 6-hours compared with vehicle control ($P < 0.01$; control = 3.204±0.341; 5 μg/g BW cortisol = 10.06±0.439; and 20 μg/g BW cortisol = 8.64±0.361 % BW).

Leptin effects on hypothalamic neuropeptide gene expression

Brain mRNA levels of *npya* were increased by approximately 1.5-fold with 0.5 μg/g BW rtLepA, but decreased by nearly 50% with 5 μg/g BW rtLepA and rhLep treatment (Figure 6; $P < 0.05$). *agrp2* was significantly decreased by both doses ($P < 0.01$), with a notable effectiveness at 0.5 μg/g BW inducing an approximately 75% decline. Transcription of *pomc* ($P < 0.01$) was significantly increased by rtLepA and rhLep by approximately 3-fold at all treatments. Likewise, *cart* ($P < 0.01$) was significantly increased by rtLepA and rhLep by approximately 4-fold at all treatments ($P < 0.01$).

Cortisol effects on hypothalamic neuropeptide gene expression

Brain mRNA levels of *npya* and *agrp2* increased in response to cortisol treatment, demonstrating a significant effect of time and treatment (Figure 7; $P < 0.01$). The *npya* response was more apparent at 6-hours post-injection, with 5 μg/g BW eliciting a 13-fold increase and 20 μg/g BW a 15-fold increase. *npya* levels returned to near-control levels after 24-hours. There was no significant effect of cortisol treatment on *pomc* or *cart* transcript levels. 5 μg/g BW cortisol increased *crf* transcript levels by up to 1.5-fold at 2- and 6-hours post-injection (Figure 8; $P < 0.05$), but not at 24-hours.

Discussion

In the current study, we employed a hybrid buffer method for producing high yields of bioactive recombinant tilapia leptin with potential translatability to production of other cysteine-rich cytokines. Further, we show leptin reduction of behavioral feed intake, complemented by brain transcript evidence for leptin inhibition of orexigenic neuropeptides and promotion of anorexigenic neuropeptides. By contradistinction, cortisol increases feed intake and orexigenic neuropeptide transcript levels.

Immobilized metal affinity chromatography (IMAC) is a technically uncomplicated and cost-efficient method for the production of recombinant proteins. While the use of IMAC is ideal when producing peptides or small proteins (Sulkowski 1985; Bornhorst & Falke, 2000), the production and purification of complex full length proteins at a scale appropriate for use in *in vivo* studies often generates technical challenges in the form of non-specific binding to native proteins from the expression system in use (i.e., *E. coli*, *Pichia pastoris*) and multimerization (Block *et al*., 2009).

Early efforts into the production of recombinant leptin from various vertebrate systems encountered issues with aggregation (both to total bacterial proteins and leptin-to-leptin multimerization) and insolubility (Fawsi *et al.*, 1996; Jeong & Lee, 1999; personal communication, Daniel Hardy, Texas Tech University). Unsurprisingly, these issues are observed across efforts to purify recombinant cytokines (Kipriyanov *et al*., 1995; Shen *et al*., 2011; Di Cesare *et al*., 2013) due to a notable universality in the presence of cysteine residues responsible for the formation of disulfide bridges.

Here, we have modified a method previously established in our laboratory to purify recombinant tilapia leptin through the use of a hybrid buffer system on a conventional nickel

IMAC medium. The application of denaturing chaotropes allows for complete lysis and release of total bacterial protein while obstructing molecular dynamics that would promote aggregation (Wells *et al*., 1994; Haneskog 2006). First, the use of guanidine hydrochloride during lysis allows for the complete solubilization of aggregates and typically-reported inclusion bodies when overexpressing cysteine-rich cytokines in bacterial expression systems. Second, the use of urea-based wash buffers allows for comprehensive removal of concomitantly-expressed bacterial proteins that often bind to metal-bound proteins of interest during purification of cysteine-rich proteins. Finally, on-column native sodium phosphate buffers – coupled with dialysis into imidazole-free sodium phosphate buffers – allows for a direct one-step method to refold the protein into its native conformation in the absence of contaminating bacterial proteins and degraded fragments of the protein of interest. Taken together, the current procedure allows for consistent production of pure monomeric leptin without sacrificing yield while also circumventing the traditional aggregation and solubility problems that are associated with recombinant cytokine protein purification using IMAC systems. The method yielded bioactive rtLepA, as the hormone was effective in inducing hyperglycemia *in vivo* and increasing hepatic *igf1 in vitro*, responses identical to those previously reported in tilapia by our group (Baltzegar *et al.*, 2014; Douros *et al*., 2017). Its suppression of appetite as shown herein further substantiates bioactivity.

In the current study, we found that recombinant tilapia leptin A was effective in suppressing food consumption by as much as 80% over the first 2 hours, a response that diminished at 6 hours. In amphibians (*Xenopus laevis*: Cui *et al*., 2014; Brenes-Soto *et al*., 2018; Bender *et al*., 2018), reptiles (Paolucci *et al*., 2001), avians (Friedman-Einat & Seroussi, 2019), mammals (mice, humans – Zhang *et* al., 1994; Elias *et al*., 1999; Ahima *et al*., 2001), and

various teleosts (*C. auratus* – Volkoff *et al*., 2003; *O. mykiss* – Murashita *et al*., 2008; *C. idella* – Li *et al*., 2010; *S. salar* – Murashita *et al*., 2011; *M. saxatilis* – Won *et al*., 2012), leptin treatment suppresses appetite and decreases feed intake. Notably, the appetite-suppressing role for leptin in teleosts may occur with administration of either homologous or heterologous hormone. Overall, the anorexigenic effect of leptin reported in the current study provides further evidence for conservation of the appetite-regulatory properties of the hormone across vertebrates (see Chapter 1 Table 1; reviewed in Londraville *et al*., 2014 and Volkoff 2019).

The anorexigenic properties of leptin are further reflected in its effect on appetite-regulatory neuropeptide transcript levels. Studies indicate that the hormone promotes hypothalamic POMC (e.g., *S. salar* – Murashita *et al*., 2011) and CART mRNA levels (Volkoff *et al*., 2003), while others show that it decreases hypothalamic NPY (e.g., *O. mykiss* – Murashita *et al*, 2008; *C. idellus* – Lu *et al*., 2015; *C. auratus* – Volkoff *et al*, 2006). Here we found the hormone reduces *npya* and *agrp2* while stimulating *pomc* and *cart* in the tilapia, suggesting that the appetite-reducing actions of the hormone may be mediated by stimulation of these anorexigenic factors while suppressing orexigenic neuropeptides. A recent study by Liu *et* al. (2018) investigated leptin effects on appetite and hypothalamic NPY in the closely-related *O. niloticus*. This study showed leptin suppression of feed intake and suggested that the anorectic effect was mediated through the NPY pathway, as leptin treatment decreased hypothalamic NPY expression. Here, we report an overall decrease in NPYa mRNA levels, suggesting that NPYa may be the dominant leptin-responsive NPY paralog in tilapia. Taken together, the current data indicates that leptin exerts a potent anorexigenic effect likely mediated through stimulatory actions on POMC and CART and inhibitory effects on NPYa and AgRP2. Collectively, the

current study provides evidence to suggest that the centrally-mediated anorexigenic actions of leptin have been conserved among vertebrates.

In the current study, we treated fish with a single bolus injection of cortisol at either 5 µg/g or 20 µg/g BW and measured effects on feed intake and appetite-regulatory neuropeptides. While cortisol caused an initial decline in feed intake at 2-hours, it stimulated substantially greater food consumption by 6-hours. This observed increase provides evidence for a biphasic appetite-regulatory effect of cortisol in a teleost fish. In teleosts studied to date, cortisol stimulates appetite in goldfish (*C. auratus* – Bernier *et al*., 2004), while inhibiting appetite in rainbow trout (*O. mykiss* – Madison *et al*., 2015) and Mozambique tilapia (Janzen *et al*., 2012).

In the context of cortisol-induced appetite stimulation, the central mechanism of action remains generally unresolved. Current evidence in mammals and goldfish (Bernier *et al*., 2004) suggests that cortisol promotes hypothalamic orexigenic NPY while inhibiting CRF (Woods *et al*., 1998; Bernier *et al*., 2004; Bazahn & Zelena, 2013), a potent anorexigenic signal and apex signal of the hypothalamic-pituitary-adrenal/interrenal (mammals - HPA; fishes - HPI) axis (De Pedro *et al*., 1993; Volkoff *et al*., 2009; Matsuda *et al*., 2013; Ortega *et al*, 2013; reviewed in Lovejoy 2013, Ronnestad *et al*., 2017). Here, we provide evidence for cortisol stimulation of *npya* and *agrp2*, suggesting that the orexigenic actions of cortisol in tilapia are mediated by the NPY/AgRP pathway. These data support the notion that cortisol acts as an orexigenic signal in tilapia through actions on NPYa and AgRP2.

In contrast to a previous study evidencing cortisol reduction of feed intake concurrent with downregulation of *npy* in *O. mossambicus* (Janzen *et al*., 2012), our data show an overall increase in feed intake and stimulation of *npya* by peripheral cortisol administration. In the study by Janzen *et al*. (2012), a dosage of 2 µg/g BW cortisol was administered and cortisol effects on

feed intake and neuropeptide responses were measured 24-hours post-injection. In the current study, we administered 5 and 20 μg/g BW cortisol and measured effects on neuropeptide transcripts at 2-, 6-, and 24-hours and effects on feed intake at 2- and 6-hours post-injection. For both the current study and Janzen *et al*. (2012), dosages were determined based off effective ranges ascertained from a previous study on cortisol actions in *O. mossambicus* by Kajimura *et al*. (2003), as well as work from our group (Baltzegar *et al*., 2014). Collectively, the mechanisms mediating glucocorticoid action on food intake and appetite-regulatory central circuits are highly complex and dependent on factors including dosage, exposure-period, nutritional status, and holding conditions (Bernier & Peter, 2001; Lovejoy 2013). Accordingly, further study will be necessary to determine the appetite-regulatory effects of glucocorticoids in teleosts including the determination of systemic glucocorticoid levels following hormone treatment, duration of sustained glucocorticoid levels, and how the tandem factors of time and circulating levels following hormone administration may interact with appetite and appetite-regulatory neuropeptides.

The other appetite-regulatory neuropeptides measured in the current study – *pomc* and *cart* – were not significantly altered by cortisol treatment. However, *crf* was promoted at 5 μg/g BW at 2-hours and 6-hours. Behaviorally, feed intake was decreased at 2-hours, while *crf* was elevated at 2-hours post-injection. These data suggest that the initial anorexigenic effect of cortisol may be partly mediated by *crf*. While elevated *crf* is sustained at 6-hours, feed intake was substantially increased at this time point and the appetite-stimulatory actions of cortisol here were likely transduced through actions on *npya*, which was considerably upregulated at 6-hours by up-to 15-fold relative to control. Interestingly, the *crf* response to cortisol treatment presents a puzzling contraposition to the established paradigm of glucocorticoid negative feedback within

the HPA/HPI axis. Typically, elevations in peripheral glucocorticoids evoke a negative feedback response at central circuits therein resulting in a decline in CRF activity. Here, it is possible that the regulatory feedback mechanism is mediated through cortisol action at other targets (i.e., pituitary), but will necessitate further elucidation and future study.

Previous work demonstrates leptin elevation during fasting in tilapia and other fishes (e.g., *E. coioides* – Zhang *et* al, 2012; *O. mossambicus* – Douros *et al*., 2017; reviewed by Deck et al. 2017) and suggests that the hormone may be involved in the activation of catabolic pathways required for mobilization of energy substrates like glycogen stores (and in some fishes, lipid stores – Johansson *et al*., 2016; Gong *et al*., 2016). From an ecological standpoint, food availability for most fishes fluctuates regularly and hence, access to food is irregular (Ronnestad *et al*., 2013). Hence, in instances of starvation, it may be energetically advantageous to avoid the pursuit of food while instead mobilizing already-available internal energy stores through leptin-involved catabolic processes, particularly when coupled with the lack of need to support body temperature in order to maintain physiological processes. This postulation is further supported by evidence from the current study indicating leptin inhibition of brain *npya* and *agrp2* (albeit necessitating corroboration from a fasting study – a possible avenue of future work). In mammals, the largely co-expressed NPY and AgRP promote appetite and anabolic processes like fat deposition. In fishes, there is emergent – but limited – evidence that the orexigenic and anabolic actions of NPYa and AgRP2 have been conserved (with neofunctionalization and nonfunctionalization observed in paralogs like NPYb, PYYb – Sundstrom *et al*., 2008; Chen *et al*., 2014; Yan *et al*., 2017). Hence, leptin inhibition of these neuropeptides, while concurrently promoting the anorexigenic and catabolic POMC and CART, may be involved in mobilization of present energetic stores until food becomes available again.

In summary, the results from the current study provide a modified method for purifying pure monomeric high yield bioactive recombinant leptin using practical and affordable techniques. Further, the current study shows leptin reduction in feed intake in tilapia concurrent with stimulation of anorexigenic POMC and CART and inhibition of orexigenic NPYa and AgRP2. Moreover, the current study evidences a temporally biphasic cortisol effect on feed intake with an initial decline at 2-hours – possibly mediated by elevated CRF – followed by a substantial increase at 6-hours in overall food consumption concomitant with significant elevation NPYa and AgRP2 mRNA levels. Overall, the present study suggests a conservation of the appetite-regulatory actions of leptin and a possible orexigenic role for cortisol in the tilapia *O. mossambicus*.

REFERENCES

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., & Flier, J. S. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature*, *382*(6588), 250-252.

Alario, P., Gamallo, A., Beato, M. J., & Trancho, G. (1987). Body weight gain, food intake and adrenal development in chronic noise stressed rats. *Physiology & behavior*, *40*(1), 29-32.

Allen, A. P., Brown, J. H., & Gillooly, J. F. (2002). Global biodiversity, biochemical kinetics, and the energetic-equivalence rule. *Science*, *297*(5586), 1545-1548.

Angilletta Jr, M. J., Niewiarowski, P. H., & Navas, C. A. (2002). The evolution of thermal physiology in ectotherms. *Journal of thermal Biology*, *27*(4), 249-268.

Ans, A. H., Anjum, I., Satija, V., Inayat, A., Asghar, Z., Akram, I., & Shrestha, B. (2018). Neurohormonal regulation of appetite and its relationship with stress: a mini literature review. *Cureus*, *10*(7).

Arvaniti, K., Huang, Q., & Richard, D. (2001). Effects of leptin and corticosterone on the expression of corticotropin-releasing hormone, agouti-related protein, and proopiomelanocortin in the brain of ob/ob mouse. *Neuroendocrinology*, *73*(4), 227-236.

Baker, D. M., Larsen, D. A., Swanson, P., and Dickhoff, W. W. (2000). Long-term peripheral treatment of coho salmon (Oncorhynchus kisutch) with human leptin has no clear physiologic effect. Gen. Comp. Endocrinol. 118, 134–138.

Balthasar, N., Coppari, R., McMinn, J., Liu, S. M., Lee, C. E., Tang, V., & Lowell, B. B. (2004). Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron*, *42*(6), 983-991.

Baltzegar, D. A., Reading, B. J., Douros, J. D., & Borski, R. J. (2014). Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. *J Endocrinol*, *220*(1), 61-72.

Baskin, D. G., Hahn, T. M., & Schwartz, M. W. (1999). Leptin sensitive neurons in the hypothalamus. *Hormone and Metabolic Research*, *31*(05), 345-350.

Baver, S. B., Hope, K., Guyot, S., Bjørbaek, C., Kaczorowski, C., & O'Connell, K. M. (2014). Leptin modulates the intrinsic excitability of AgRP/NPY neurons in the arcuate nucleus of the hypothalamus. *Journal of Neuroscience*, *34*(16), 5486-5496.

Bazhan, N., & Zelena, D. (2013). Food-intake regulation during stress by the hypothalamo-pituitary-adrenal axis. *Brain research bulletin*, *95*, 46-53.

Bender, M. C., Hu, C., Pelletier, C., & Denver, R. J. (2018). To eat or not to eat: ontogeny of hypothalamic feeding controls and a role for leptin in modulating life-history transition in

amphibian tadpoles. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1875), 20172784.

Bernier, N. J. (2006). The corticotropin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. *General and comparative endocrinology*, *146*(1), 45-55.

Bernier, N. J., & Peter, R. E. (2001). The hypothalamic–pituitary–interrenal axis and the control of food intake in teleost fish. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *129*(2-3), 639-644.

Bernier, N. J., Gorissen, M., & Flik, G. (2012). Differential effects of chronic hypoxia and feed restriction on the expression of leptin and its receptor, food intake regulation and the endocrine stress response in common carp. *Journal of Experimental Biology*, *215*(13), 2273-2282.

Block, H., Maertens, B., Spriestersbach, A., Brinker, N., Kubicek, J., Fabis, R., & Schäfer, F. (2009). Immobilized-metal affinity chromatography (IMAC): a review. In *Methods in enzymology* (Vol. 463, pp. 439-473). Academic Press.

Bornhorst, J. A., & Falke, J. J. (2000). [16] Purification of proteins using polyhistidine affinity tags. In *Methods in enzymology* (Vol. 326, pp. 245-254). Academic Press.

Bornstein, S. R., Uhlmann, K., Haidan, A., Ehrhart-Bornstein, M., & Scherbaum, W. A. (1997). Evidence for a novel peripheral action of leptin as a metabolic signal to the adrenal gland: leptin inhibits cortisol release directly. *Diabetes*, *46*(7), 1235-1238.

Brenes-Soto, A., Dierenfeld, E. S., & Janssens, G. P. (2018). The interplay between voluntary food intake, dietary carbohydrate-lipid ratio and nutrient metabolism in an amphibian,(Xenopus laevis). *PloS one*, *13*(12).

Cao, Y. B., Xue, J. L., Wu, L. Y., Jiang, W., Hu, P. N., & Zhu, J. (2011). The detection of 3 leptin receptor isoforms in crucian carp gill and the influence of fasting and hypoxia on their expression. *Domestic animal endocrinology*, *41*(2), 74-80.

Cartana, J., Huget, J., Arola, L., & Alemany, M. (1989). Effects of a high lipidic diet on murine energetic reserves in food deprivation. *Hormone and metabolic research*, *21*(11), 606-611.

Chen, Y., Pandit, N. P., Fu, J., Li, D., & Li, J. (2014). Identification, characterization and feeding response of peptide YYb (PYYb) gene in grass carp (Ctenopharyngodon idellus). *Fish physiology and biochemistry*, *40*(1), 45-55.

Chu, D. L. H., Li, V. W. T., & Yu, R. M. K. (2010). Leptin: clue to poor appetite in oxygen-starved fish. *Molecular and cellular endocrinology*, *319*(1-2), 143-146.

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdán, M. G., Diano, S., Horvath, T. L., & Low, M. J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the

arcuate nucleus. *Nature*, *411*(6836), 480-484.

Cui, M. Y., Hu, C. K., Pelletier, C., Dziuba, A., Slupski, R. H., Li, C., & Denver, R. J. (2014). Ancient origins and evolutionary conservation of intracellular and neural signaling pathways engaged by the leptin receptor. *Endocrinology*, *155*(11), 4202-4214.

Dalmolin, C., Almeida, D. V., Figueiredo, M. A., & Marins, L. F. (2015). Food intake and appetite control in a GH-transgenic zebrafish. *Fish physiology and biochemistry*, *41*(5), 1131-1141.

De Pedro, N., Alonso-Gómez, A. L., Gancedo, B., Delgado, M. J., & Alonso-Bedate, M. (1993). Role of corticotropin-releasing factor (CRF) as a food intake regulator in goldfish. *Physiology & behavior*, *53*(3), 517-520.

de Pedro, N., Martinez-Alvarez, R., and Delgado, M. J. (2006). Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). *J. Endocrinol.* 188, 513–520.

Di Cesare, S., Binder, U., Maier, T., & Skerra, A. (2013). High-yield production of PASylated human growth hormone using secretory E. coli technology. *Bioprocess Int*, *11*(4), 30-38.

Dickson, K. A., & Graham, J. B. (2004). Evolution and consequences of endothermy in fishes. *Physiological and Biochemical Zoology*, *77*(6), 998-1018.

Douros, J. D., Baltzegar, D. A., Mankiewicz, J., Taylor, J., Yamaguchi, Y., Lerner, D. T., & Borski, R. J. (2017). Control of leptin by metabolic state and its regulatory interactions with pituitary growth hormone and hepatic growth hormone receptors and insulin like growth factors in the tilapia (Oreochromis mossambicus). *General and comparative endocrinology*, *240*, 227-237.

El-Haschimi, K., Pierroz, D. D., Hileman, S. M., Bjørbæk, C., & Flier, J. S. (2000). Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *The Journal of clinical investigation*, *105*(12), 1827-1832.

Elias, C. F., Aschkenasi, C., Lee, C., Kelly, J., Ahima, R. S., Bjorbæk, C., & Elmquist, J. K. (1999). Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron*, *23*(4), 775-786.

Ellis, B. J., Jackson, J. J., & Boyce, W. T. (2006). The stress response systems: Universality and adaptive individual differences. *Developmental Review*, *26*(2), 175-212.

Fawzi, A. B., Zhang, H., Van Heek, M., & Graziano, M. P. (1996). Purification of milligram quantities of human leptin from recombinant E. coli. *Hormone and metabolic research*, *28*(12), 694-697.

Gardner, J., Rothwell, N., & Luheshi, G. (1998). Leptin affects food intake via CRF-receptor-mediated pathways. *Nature neuroscience*, *1*(2), 103-103.

Geiser, F., Stawski, C., Wacker, C. B., & Nowack, J. (2017). Phoenix from the ashes: fire, torpor, and the evolution of mammalian endothermy. *Frontiers in physiology*, *8*, 842.

Gorissen, M., Bernier, N. J., Manuel, R., de Gelder, S., Metz, J. R., Huising, M. O., & Flik, G. (2012). Recombinant human leptin attenuates stress axis activity in common carp (Cyprinus carpio L.). *General and comparative endocrinology*, *178*(1), 75-81.

Haneskog, L. (2006). Purification of Histidine-Tagged Proteins under Denaturing Conditions Using IMAC. *Cold Spring Harbor Protocols*, *2006*(1), pdb-prot4221.

Hart, P. J. (1993). Teleost foraging: facts and theories. *Behaviour of teleost fishes*, *2*, 253-284.

Heinrichs, S. C. (2005). Behavioral consequences of altered corticotropin-releasing factor activation in brain: a functionalist view of affective neuroscience. In *Techniques in the Behavioral and Neural Sciences* (Vol. 15, pp. 155-177). Elsevier.

Heinrichs, S. C., & Richard, D. (1999). The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. *Neuropeptides*, *33*(5), 350-359.

Honeycutt, J. L. (2018). Environmental and Endocrine Regulators of Stress Effects in Teleost Fishes.

Janzen, W. J., Duncan, C. A., & Riley, L. G. (2012). Cortisol treatment reduces ghrelin signaling and food intake in tilapia, Oreochromis mossambicus. *Domestic animal endocrinology*, *43*(3), 251-259.

Jeong, K. J., & Lee, S. Y. (1999). High-level production of human leptin by fed-batch cultivation of recombinant Escherichia coli and its purification. *Appl. Environ. Microbiol.*, *65*(7), 3027-3032.

Johansson, M., Morgenroth, D., Einarsdottir, I. E., Gong, N., & Björnsson, B. T. (2016). Energy stores, lipid mobilization and leptin endocrinology of rainbow trout. *Journal of Comparative Physiology B*, *186*(6), 759-773.

Johnstone, L. E., Srisawat, R., Kumarnsit, E., & Leng, G. (2005). Hypothalamic expression of NPY mRNA, vasopressin mRNA and CRF mRNA in response to food restriction and central administration of the orexigenic peptide GHRP-6. *Stress*, *8*(1), 59-67.

Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K., & Grau, E. G. (2003). Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, Oreochromis mossambicus. *Journal of endocrinology*, *178*(1), 91-100.

Kipriyanov, S. M., Dübel, S., Breitling, F., Kontermann, R. E., Heymann, S., & Little, M. (1995). Bacterial expression and refolding of single-chain Fv fragments with C-terminal cysteines. *Cell biophysics*, *26*(3), 187-204.

Kobayashi, K., Forte, T. M., Taniguchi, S., Ishida, B. Y., Oka, K., & Chan, L. (2000). The db/db mouse, a model for diabetic dyslipidemia: molecular characterization and effects of Western diet feeding. *Metabolism*, *49*(1), 22-31.

Korner, J., Savontaus, E., Chua Jr, S. C., Leibel, R. L., & Wardlaw, S. L. (2001). Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus. *Journal of neuroendocrinology*, *13*(11), 959-966.

Leal-Cerro, A., Soto, A., Martínez, M. A., Dieguez, C., & Casanueva, F. F. (2001). Influence of cortisol status on leptin secretion. *Pituitary*, *4*(1-2), 111-116.

Levin, B. E., & Dunn-Meynell, A. A. (1997). Dysregulation of arcuate nucleus preproneuropeptide Y mRNA in diet-induced obese rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *272*(5), R1365-R1370.

Li, G. G., Liang, X. F., Xie, Q., Li, G., Yu, Y., & Lai, K. (2010). Gene structure, recombinant expression and functional characterization of grass carp leptin. *General and comparative endocrinology*, *166*(1), 117-127.

Lin, S., Storlien, L. H., & Huang, X. F. (2000). Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain. *Brain research*, *875*(1-2), 89-95.

Liu, C. Z., He, A. Y., Ning, L. J., Luo, Y., Li, D. L., Zhang, M. L., & Du, Z. Y. (2018). Leptin selectively regulates nutrients metabolism in nile tilapia fed on high carbohydrate or high fat diet. *Frontiers in endocrinology*, *9*, 574.

Londraville, R. L., and Duvall, C. S. (2002). Murine leptin injections increase intracellular fatty acid-binding protein in green sunfish (*Lepomis cyanellus*). *Gen. Comp. Endocrinol.* 129, 56–62.

Lovejoy, D. A. (2013). CRH Family. In *Handbook of Biologically Active Peptides* (pp. 752-759). Academic Press.

Lu, R. H., Zhou, Y., Yuan, X. C., Liang, X. F., Fang, L., Bai, X. L., & Zhao, Y. H. (2015). Effects of glucose, insulin and triiodothyroxine on leptin and leptin receptor expression and the effects of leptin on activities of enzymes related to glucose metabolism in grass carp (Ctenopharyngodon idella) hepatocytes. *Fish physiology and biochemistry*, *41*(4), 981-989.

Margetic, S., Gazzola, C., Pegg, G. G., & Hill, R. A. (2002). Leptin: a review of its peripheral actions and interactions. *International journal of obesity*, *26*(11), 1407-1433.

Masuzaki, H., Ogawa, Y., Isse, N., Satoh, N., Okazaki, T., Shigemoto, M., & Jingami, H. (1995). Human obese gene expression: adipocyte-specific expression and regional differences in the adipose tissue. *Diabetes*, *44*(7), 855-858.

Matsuda, K. (2013). Regulation of feeding behavior and psychomotor activity by corticotropin-releasing hormone (CRH) in fish. *Frontiers in neuroscience*, *7*, 91.

McCue, M. D. (2010). Starvation physiology: reviewing the different strategies animals use to survive a common challenge. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *156*(1), 1-18.

Morley, J. E. (1987). Neuropeptide regulation of appetite and weight. *Endocrine reviews*, *8*(3), 256-287.

Morrison, C. D., Morton, G. J., Niswender, K. D., Gelling, R. W., & Schwartz, M. W. (2005). Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling. *American Journal of Physiology-Endocrinology and Metabolism*, *289*(6), E1051-E1057.

Murashita, K., Jordal, A. E. O., Nilsen, T. O., Stefansson, S. O., Kurokawa, T., Björnsson, B. T., & Rønnestad, I. (2011). Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *158*(1), 79-86.

Murashita, K., Uji, S., Yamamoto, T., Rønnestad, I., & Kurokawa, T. (2008). Production of recombinant leptin and its effects on food intake in rainbow trout (Oncorhynchus mykiss). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *150*(4), 377-384.

Newcomer, J. W., Selke, G., Melson, A. K., Gross, J., Vogler, G. P., & Dagogo-Jack, S. (1998). Dose-dependent cortisol-induced increases in plasma leptin concentration in healthy humans. *Archives of general psychiatry*, *55*(11), 995-1000.

Ortega, V. A., Lovejoy, D. A., & Bernier, N. J. (2013). Appetite-suppressing effects and interactions of centrally administered corticotropin-releasing factor, urotensin I and serotonin in rainbow trout (Oncorhynchus mykiss). *Frontiers in neuroscience*, *7*, 196.

Peter, R. E., Yu, K. L., Marchant, T. A., & Rosenblum, P. M. (1990). Direct neural regulation of the teleost adenohypophysis. *Journal of Experimental Zoology*, *256*(S4), 84-89.

Piato, Â. L., Capiotti, K. M., Tamborski, A. R., Oses, J. P., Barcellos, L. J., Bogo, M. R., & Bonan, C. D. (2011). Unpredictable chronic stress model in zebrafish (Danio rerio): behavioral and physiological responses. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *35*(2), 561-567.

Poggioli, R., Vergoni, A. V., & Bertolini, A. (1986). ACTH-(1–24) and α-MSH antagonize feeding behavior stimulated by kappa opiate agonists. *Peptides*, *7*(5), 843-848.

Richard, D., Lin, Q., & Timofeeva, E. (2002). The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. *European journal of pharmacology*, *440*(2-3), 189-197.

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology & Evolution*, *19*(5), 249-255.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., & Boglione, C. (2013). Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. *Reviews in Aquaculture*, *5*, S59-S98.

Rønnestad, I., Gomes, A. S., Murashita, K., Angotzi, R., Jönsson, E., & Volkoff, H. (2017). Appetite-controlling endocrine systems in teleosts. *Frontiers in endocrinology*, *8*, 73.

Roubos, E. W., Dahmen, M., Kozicz, T., & Xu, L. (2012). Leptin and the hypothalamo-pituitary–adrenal stress axis. *General and comparative endocrinology*, *177*(1), 28-36.

Satoh, S., Takeuchi, T., & Watanabe, T. (1984). Effects of starvation and environmental temperature on proximate and fatty acid compositions of Tilapia nilotica. *Bulletin of the Japanese Society of Scientific Fisheries (Japan)*.

Shen, M., Siu, S., Byrd, S., Edelmann, K. H., Patel, N., Ketchem, R. R., & Hasegawa, H. (2011). Diverse functions of reactive cysteines facilitate unique biosynthetic processes of aggregate-prone interleukin-31. *Experimental cell research*, *317*(7), 976-993.

Shpilman, M., Hollander-Cohen, L., Ventura, T., Gertler, A., & Levavi-Sivan, B. (2014). Production, gene structure and characterization of two orthologs of leptin and a leptin receptor in tilapia. *General and comparative endocrinology*, *207*, 74-85.

Stevanović, D., Milošević, V., Starčević, V. P., & Severs, W. B. (2007). The effect of centrally administered ghrelin on pituitary ACTH cells and circulating ACTH and corticosterone in rats. *Life sciences*, *80*(9), 867-872.

Sulkowski, E. (1985). Purification of proteins by IMAC. *Trends in Biotechnology*, *3*(1), 1-7.

Sundström, G., Larsson, T. A., Brenner, S., Venkatesh, B., & Larhammar, D. (2008). Evolution of the neuropeptide Y family: new genes by chromosome duplications in early vertebrates and in teleost fishes. *General and comparative endocrinology*, *155*(3), 705-716.

Swart, I., Jahng, J. W., Overton, J. M., & Houpt, T. A. (2002). Hypothalamic NPY, AGRP, and POMC mRNA responses to leptin and refeeding in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *283*(5), R1020-R1026.

Tinoco, A. B., Nisembaum, L. G., Isorna, E., Delgado, M. J., & de Pedro, N. (2012). Leptins and leptin receptor expression in the goldfish (Carassius auratus). Regulation by food intake and fasting/overfeeding conditions. *Peptides*, *34*(2), 329-335.

Tsigos, C., & Chrousos, G. P. (2002). Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. *Journal of psychosomatic research*, *53*(4), 865-871.

Volkoff, H. (2015). Cloning, tissue distribution and effects of fasting on mRNA expression levels of leptin and ghrelin in red-bellied piranha (Pygocentrus nattereri). *General and comparative endocrinology*, *217*, 20-27.

Volkoff, H. (2019). Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. *Molecular and cellular endocrinology*.

Volkoff, H., Eykelbosh, A. J., & Peter, R. E. (2003). Role of leptin in the control of feeding of goldfish Carassius auratus: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. *Brain research*, *972*(1-2), 90-109.

Volkoff, H., Xu, M., MacDonald, E., & Hoskins, L. (2009). Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: notes on actions and responses to nutritional, environmental and reproductive changes. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *153*(1), 8-12.

Volpato, G. L., & Fernandes, M. O. (1994). Social control of growth in fish. *Brazilian Journal of Medical and Biological Research*, *27*, 797-797.

Wabitsch, M., Jensen, P. B., Blum, W. F., Christoffersen, C. T., Englaro, P., Heinze, E., & Hauner, H. (1996). Insulin and cortisol promote leptin production in cultured human fat cells. *Diabetes*, *45*(10), 1435-1438.

Wells, P. A., Garlick, R. L., Lyle, S. B., Tuls, J. L., Poorman, R. A., Brideau, R. J., & Wathen, M. W. (1994). Purification of a recombinant human respiratory syncytial virus chimeric glycoprotein using reversed-phase chromatography and protein refolding in guanidine hydrochloride. *Protein Expression and Purification*, *5*(4), 391-401.

Williams, G., Bing, C., Cai, X. J., Harrold, J. A., King, P. J., & Liu, X. H. (2001). The hypothalamus and the control of energy homeostasis: different circuits, different purposes. *Physiology & behavior*, *74*(4-5), 683-701.

Won, E. T., Baltzegar, D. A., Picha, M. E., & Borski, R. J. (2012). Cloning and characterization of leptin in a Perciform fish, the striped bass (Morone saxatilis): control of feeding and regulation by nutritional state. *General and comparative endocrinology*, *178*(1), 98-107.

Won, E. T., Douros, J. D., Hurt, D. A., & Borski, R. J. (2016). Leptin stimulates hepatic growth hormone receptor and insulin-like growth factor gene expression in a teleost fish, the hybrid striped bass. *General and comparative endocrinology*, *229*, 84-91.

Yan, P., Jia, J., Yang, G., Wang, D., Sun, C., & Li, W. (2017). Duplication of neuropeptide Y and peptide YY in Nile tilapia Oreochromis niloticus and their roles in food intake regulation. *Peptides*, *88*, 97-105.

Yuan, D., Wang, T., Zhou, C., Lin, F., Chen, H., Wu, H., & Li, Z. (2014). Leptin and cholecystokinin in Schizothorax prenanti: molecular cloning, tissue expression, and mRNA expression responses to periprandial changes and fasting. *General and comparative endocrinology*, *204*, 13-24.

Zhang, H., Chen, H., Zhang, Y., Li, S., Lu, D., Zhang, H., & Lin, H. (2013). Molecular cloning, characterization and expression profiles of multiple leptin genes and a leptin receptor gene in orange-spotted grouper (Epinephelus coioides). *General and comparative endocrinology*, *181*, 295-305.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, *372*(6505), 425-432.

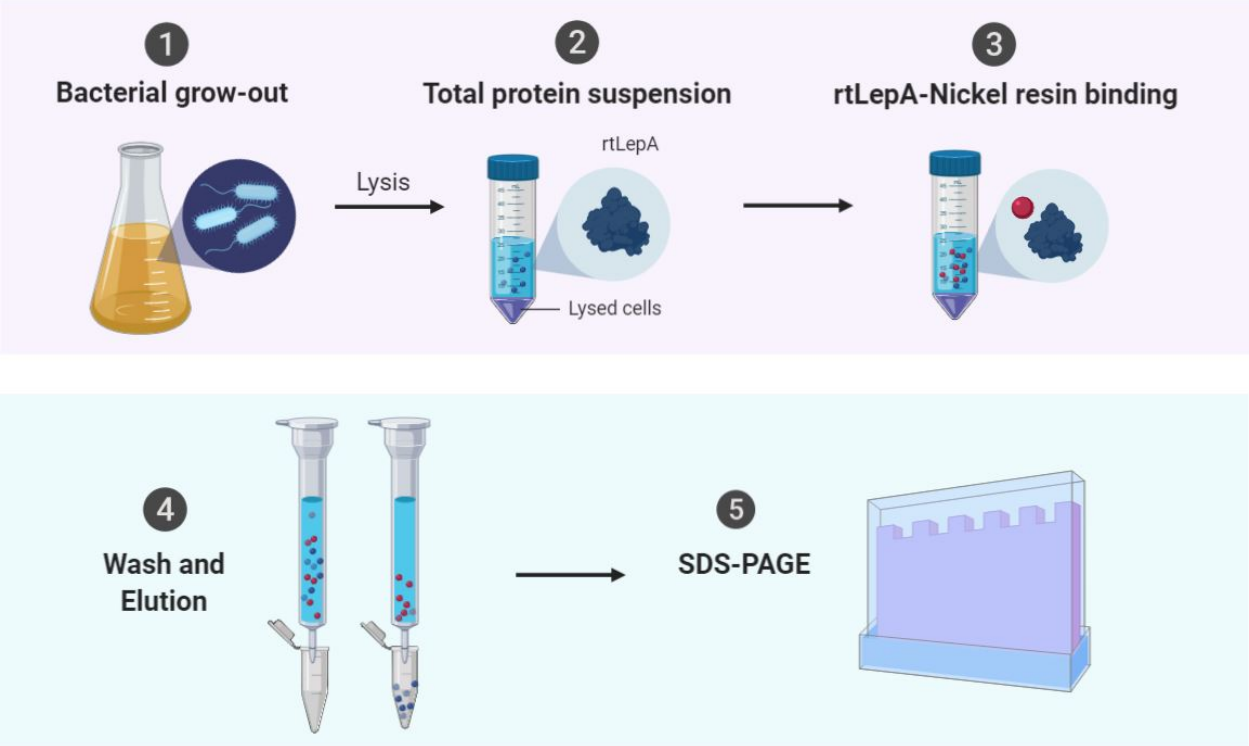

Figure 1. Conceptual diagram outlining the rtLepA purification protocol.

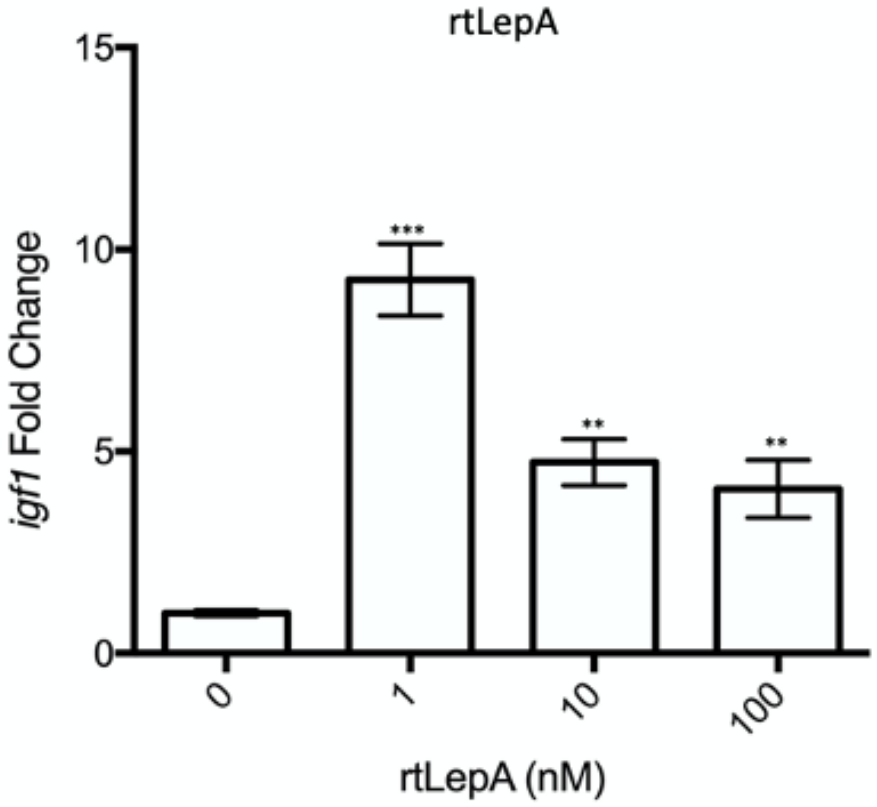

Figure 2. Effect of rtLepA on *igf1* mRNA levels in primary hepatocytes after 18-hour incubation (shown as fold change from 0 nM treatment). Values reported as means ± SEM. * denotes significant difference from 0 nM treatment. (n = 6/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

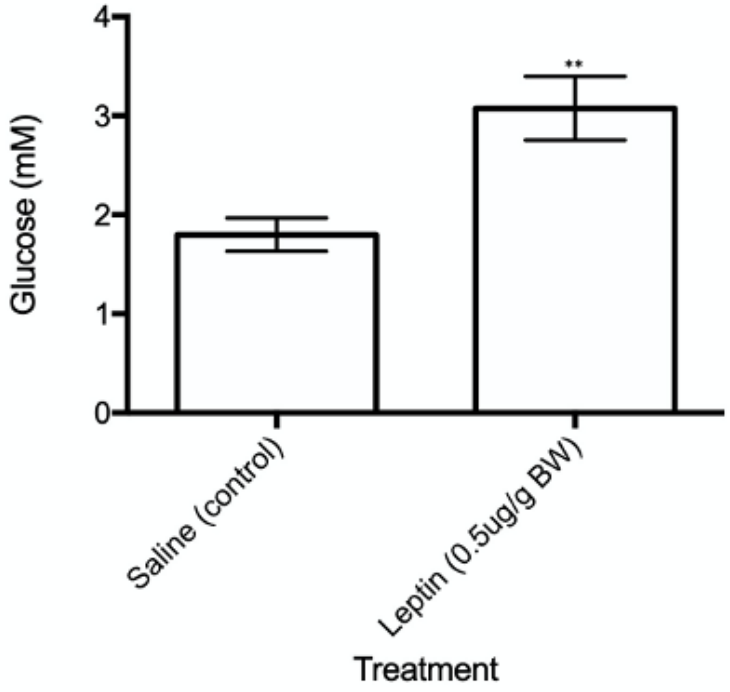

Figure 3. Effect of rtLepA on blood glucose levels at 4-hours following bolus intraperitoneal injection. Values reported as means ± SEM. * denotes significant difference from saline control (n = 8/treatment; ** $P < 0.01$).

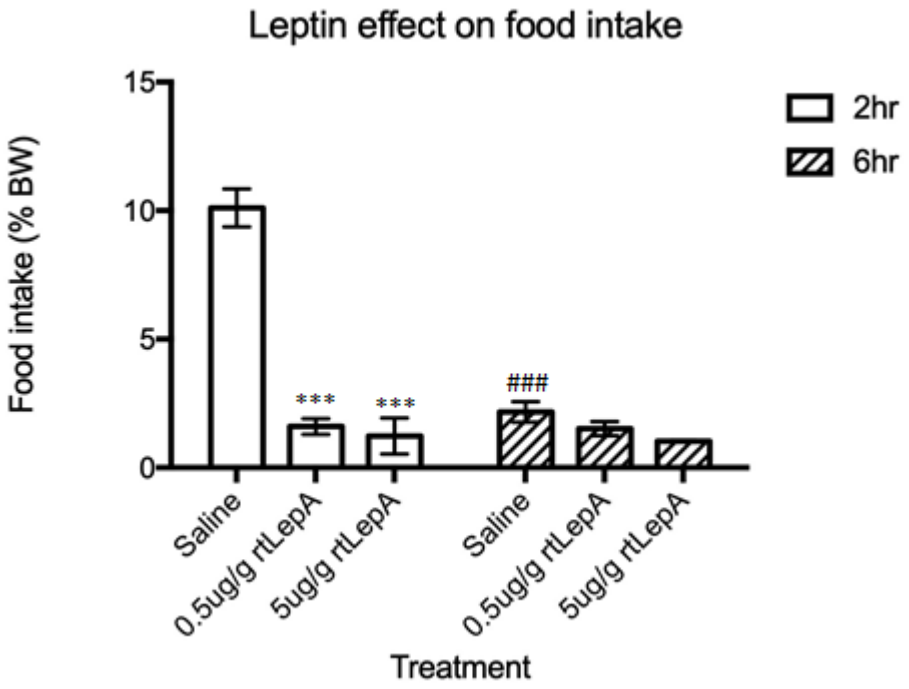

Figure 4. Effect of rtLepA on food intake at 2- and 6-hours following bolus intraperitoneal injection. Values reported as percentage body weight means ± SEM. * denotes significant differences of treatments compared to saline control at each time point, while # denotes significant differences within treatments over time (n = 8/treatment; *** ### $P < 0.001$).

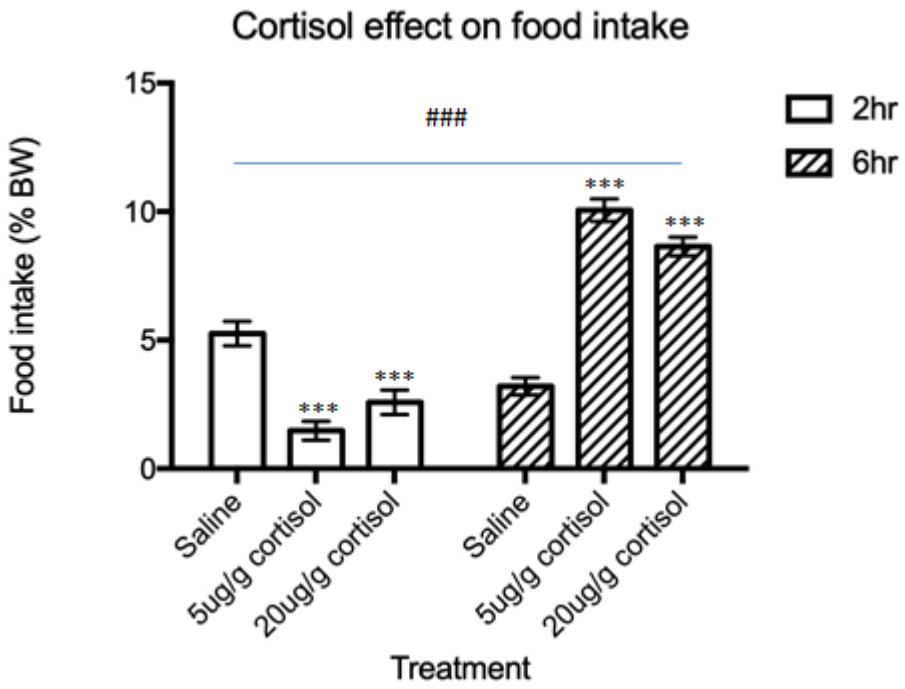

Figure 5. Effect of cortisol on food intake at 2- and 6-hours following intraperitoneal bolus injection. Values reported as percentage body weight means ± SEM. * denotes significant differences of treatments compared to saline control at each time point, while # denotes significant differences within treatments over time (n = 8/treatment; *** ### $P < 0.001$). All treatments were significantly different over time ($P < 0.001$) and are represented with a single ### above data bars for clarity.

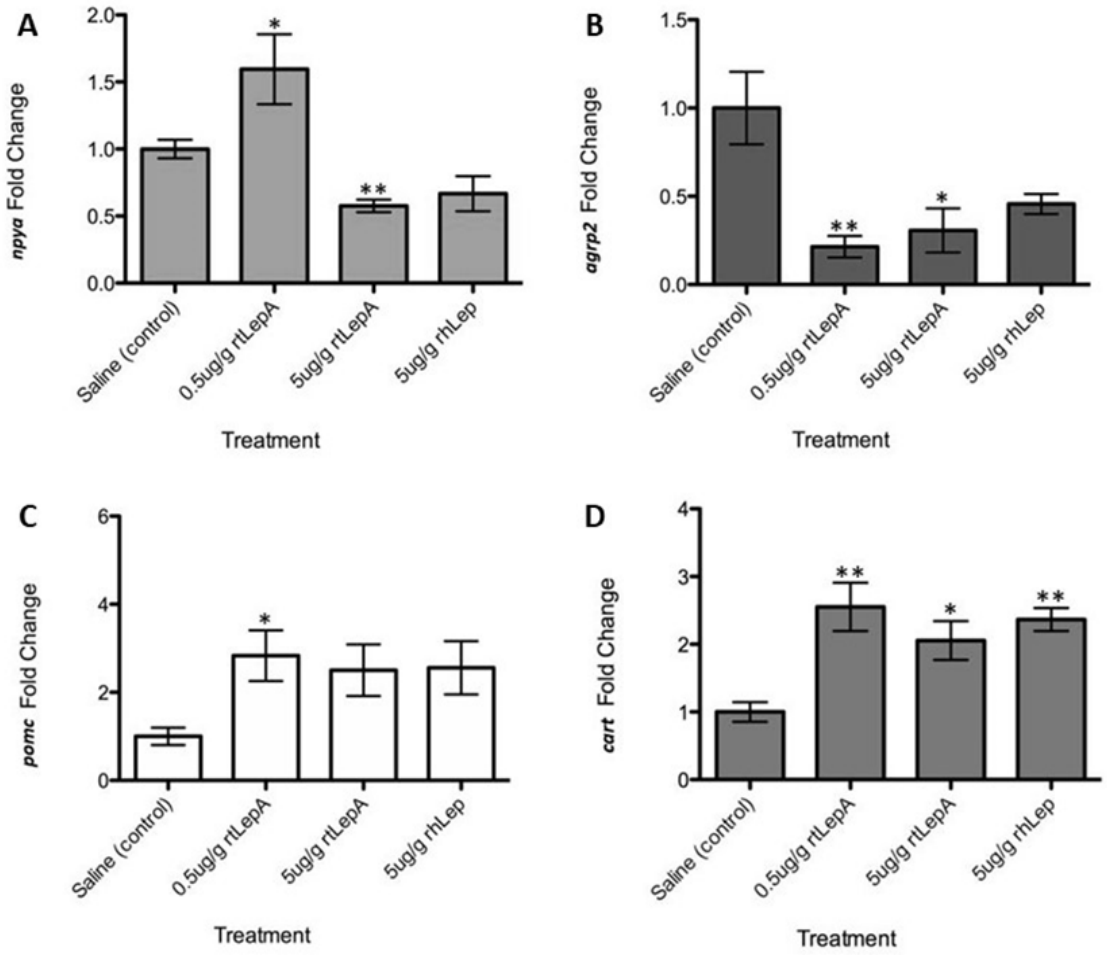

Figure 6. Effect of rtLepA on fore/mid-brain (A) *npya,* (B) *agrp2,* (C) *pomc,* and (D) *cart* levels at 6-hours following intraperitoneal injection. Values reported as means ± SEM. * denotes significant difference from saline control (n = 8/treatment; * $P < 0.05$, ** $P < 0.01$).

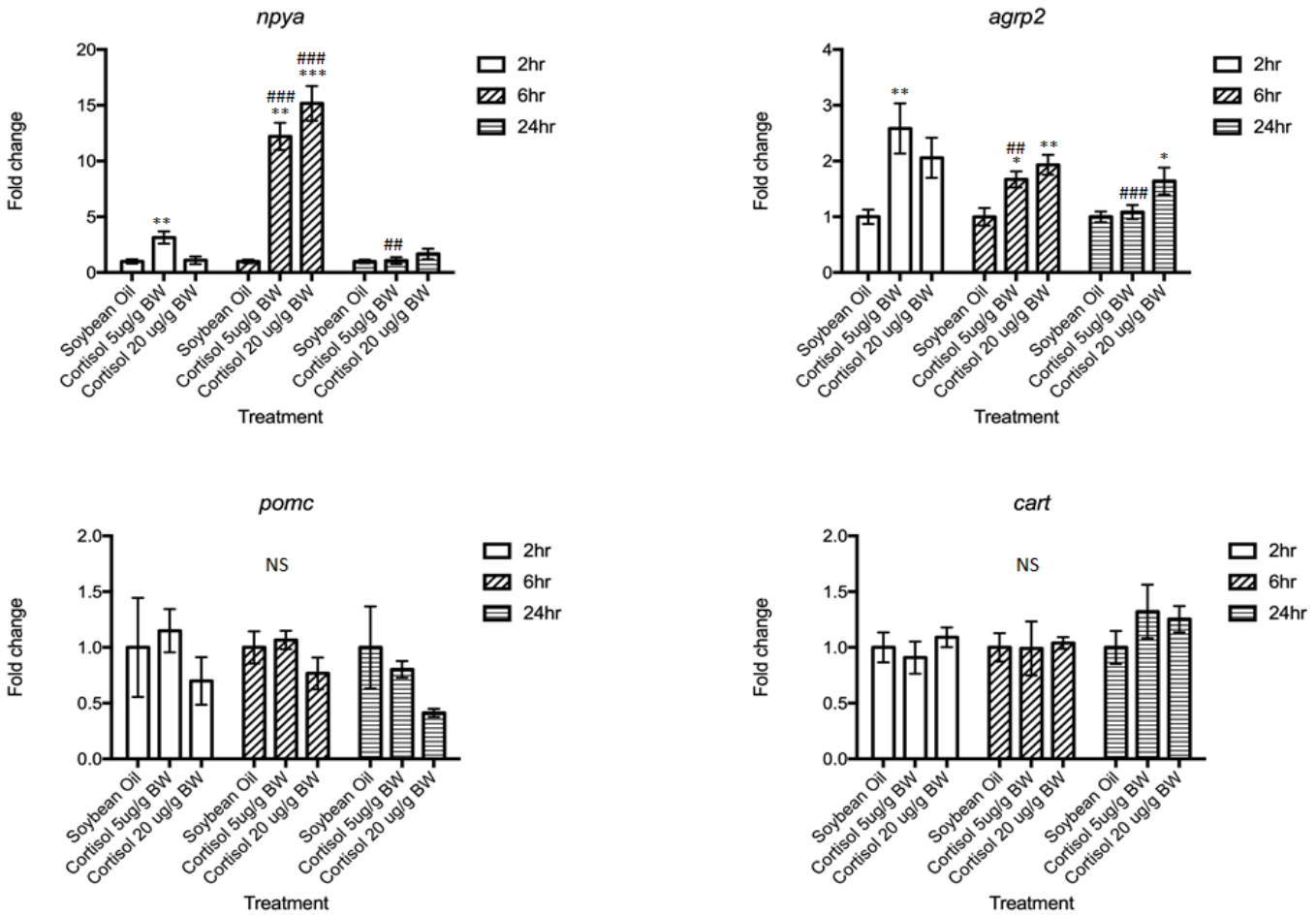

Figure 7. Effect of cortisol on fore/mid-brain mRNA levels of *npya, agrp2, pomc,* and *cart* at 2-, 6-, and 24-hours following intraperitoneal injection. Values reflect fold change from soybean oil control and are reported as means ± SEM. * denotes significant differences between treatments relative to Soybean Oil control, while # denotes significant differences within treatments over time (n = 8/treatment; * # $P < 0.05$, ** ## $P < 0.01$, *** ### $P < 0.001$).

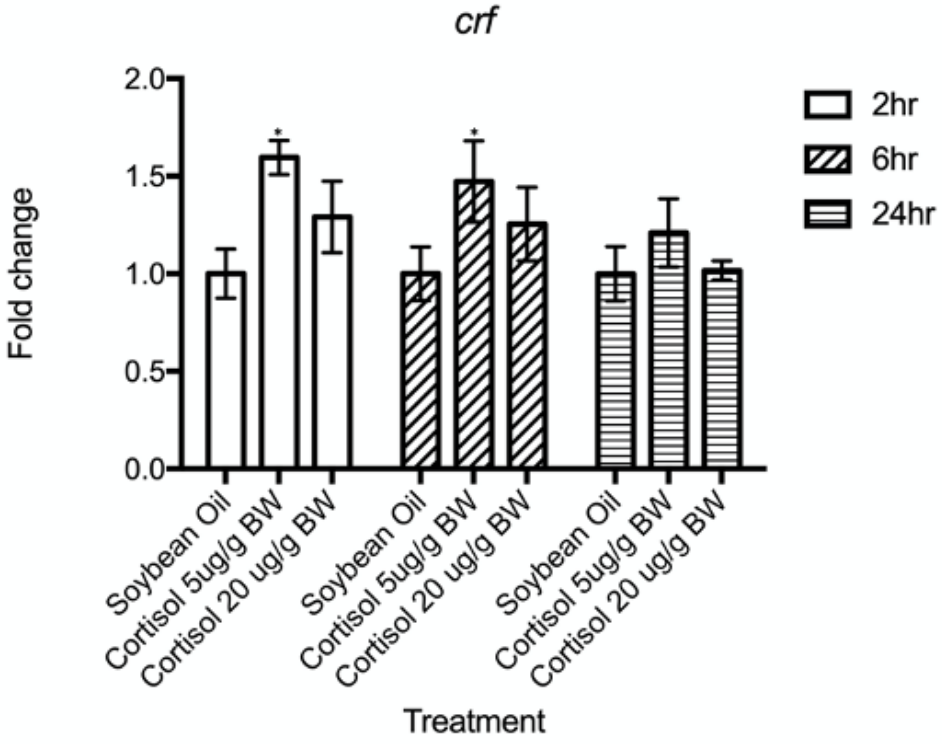

Figure 8. Effect of cortisol treatment on fore/mid-brain mRNA levels of *crf* at 2-, 6-, and 24-hours following bolus intraperitoneal injection. Values reflect fold change from soybean oil control and are reported as means ± SEM. * denotes significant differences between treatments relative to soybean oil control at each time point (n = 8/treatment; * $P < 0.05$).

CHAPTER 3

A role for leptin in sexual maturation and regulation of kisspeptin, gonadotropin-releasing hormone, and gonadal sex steroids in the Mozambique tilapia *Oreochromis mossambicus*

Abstract

Leptin is a key regulator of energy homeostasis and a permissive signal for puberty and reproductive function in mammals. The role for leptin in regulation of the reproductive axis in ectotherms and teleost fishes, specifically, remains poorly understood. We evaluated developmental profiles of brain kisspeptin (Kiss2), gonadotropin-releasing hormone (GnRH1), and hepatic leptin (LepA) expression over the course of sexual maturation, as well as the hormone's role in regulating these key hypothalamic reproductive signals (Kiss2, GnRH1, gonadotropin-inhibiting hormone - GnIH) and sex steroids in the Mozambique tilapia (*Oreochromis mossambicus*). Transcript levels of *lepa*, the dominant paralog of leptin in teleosts, in the liver, the primary source of circulating hormone, positively correlated with expression of *kiss2* and *gnrh1*, gonadosomatic index, and sexual maturation of male and female tilapia beginning at 13-14 weeks post-hatch. Intraperitoneal injection and 6-hour treatment with recombinant tilapia leptin A in adult male fish increased brain *kiss2* and *gnrh1* levels, while reducing circulating 17β-estradiol, testosterone, and 11-ketotestosterone levels relative to saline control. In testicular explant incubations, rtLepA suppressed testosterone and 11-ketotestosterone similarly to results observed *in vivo*. Estradiol response could not be ascertained due to low production levels in testes. Our findings suggest that leptin expression rises with sexual maturation and that the hormone correlates strongly with apex hypothalamic gonadotropic factors during pubertal onset. Strikingly, we show for the first time that leptin reduces sex steroid levels with evidence for direct action as shown in our testicular explant culture. We speculate that this latter effect may be associated with limiting energy partitioning to reproduction in instances of negative energy balance when leptin has been shown to increase.

Introduction

Reproduction is a critical component of an organism's lifespan and key to the perpetuation of species. The energetic costliness of reproduction necessitates its dispensability in instances of disruption to energy homeostasis. Accordingly, reproductive function is sensitive to an array of endogenous signals that provide information on metabolic and nutritional sufficiency. Within this premise, the onset of puberty in mammals is known to be permitted by the acquisition of an optimal threshold of adiposity to maintain reproductive capacity, while conditions of metabolic challenges and energy insufficiency can delay pubertal onset and gonadal function and development (Kennedy & Mitra, 1963; Cameron 1996). Although metabolic gating of reproductive function, as evidenced by studies demonstrating delayed puberty and perturbed fertility (Kennedy & Mitra, 1963; reviewed in Roa *et al*., 2010), has long been understood to be a function of energy sufficiency, elucidation and understanding of the putative endocrine regulators of this relationship have emerged more recently.

Puberty is a complex physiological event during which reproductive competence is achieved through the growth and eventual maturation of sexual and somatic tissues within an individual (Marshall 1978; Sisk & Foster, 2004). The energetic costliness of puberty and reproductive function necessitates signaling of energy sufficiency to the hypothalamic-pituitary-gonadal axis (HPG axis) from the periphery (Schwartz 2000; Tena-Sempere 2003). Leptin is a pleiotropic cytokine hormone that – in mammals – circulates in direct proportion to an individual's adiposity and is understood for its prototypic actions in regulating appetite and serving as a key signal of energy sufficiency (Zhang *et al*., 1994). In the context of mammalian reproduction, leptin informs the HPG axis of energy sufficiency and serves a permissive role in the metabolic gating of puberty onset particularly at the level of the hypothalamus (Chehab *et al*.,

1996; Ahima *et al*., 1997; Cheung *et al*., 1997). Kisspeptins (Kiss) are a family of neuropeptides positioned as apex effectors of the HPG axis that serve a key role in the control of pubertal onset and reproductive function via interactions with gonadotropin-releasing hormone (GnRH) neurons (Messager *et al*., 2005; Navarro & Tena-Sempere, 2012). Instances of negative energy balance (and accordingly, reduced leptin levels) have been reported to coincide with suppression of hypothalamic kisspeptins, thereby inhibiting the overall function of the HPG axis (Castellano *et al*., 2005; Luque *et al*., 2007). Studies have suggested that leptin may directly regulate hypothalamic Kiss1 neurons, as evidenced by expression of the leptin receptor (LepR) in these neurons (Smith *et al*., 2006; Backholer *et al*., 2010). In spite of the complementary and experimental evidence to support this proposed pathway, recent work has indicated that the elimination of LepR from Kiss1 neurons does not impede pubertal onset nor fertility, suggesting that the reproductive actions of leptin do not occur entirely through direct targeting of Kiss1 neurons, but involve interactions with other upstream neuronal populations (Donato *et al*., 2011; Zuure *et al*., 2013). Nonetheless, bona fide evidence from these various studies implicate leptin in the metabolic control of puberty and reproduction in mammals whether through direct or indirect interfacing with apex components of the HPG axis. While the importance of central leptin signaling in the control of puberty and reproductive function is well-established, the hormone's role in the regulation of reproduction in non-mammalian vertebrates remains unresolved and largely uninvestigated.

Reproduction in teleost fishes – as in all vertebrates studied to date – is regulated by the highly conserved HPG axis. In teleosts, there are several isoforms of key reproductive neuropeptides. Ongoing studies indicate that genome duplication events have led to the production of paralogs (Taylor *et al.*, 2003), some of which may have undergone non- and/or

neo-functionalization (Weber *et al.*, 1997; Steven *et al.*, 2003; reviewed in Somoza *et al.*, 2002, Lethimonier *et al.*, 2004, and Zohar *et al.*, 2010). In teleosts, the apex neuronal regulators of the HPG axis at the brain – Kiss and GnRH – demonstrate several distinct forms. Two forms of kisspeptin have been identified in fishes including Kiss1 and Kiss2. Overall, there appear to be wide species-specific differences in terms of the capacity for Kiss1 and Kiss2 to bind to their cognate receptor (GPR54), as well as variable potency in stimulating hypothalamic GnRH and pituitary gonadotropins (reviewed in Tena-Sempere *et al*., 2012). GnRH also retains three distinct forms in teleosts: GnRH1, GnRH2, and GnRH3 (reviewed in Shahjahan *et al*., 2014). In teleosts studied to date, GnRH1 appears to be the dominant isoform due to its pituitary gonadotropin-stimulatory actions and direct innervation of the pituitary (Okubo *et al*., 2000; Andersson *et al*., 2001). By comparison, GnRH2 is generally lowly-expressed and does not appear to have hypophysiotropic properties (Shahjahan *et al*., 2014), while GnRH3 may be involved in neuromodulatory actions associated with aggressive and mating behaviors like nest-building (e.g., *Colisa lalia*: Yamamoto *et al*., 1997; *Oreochromis niloticus*: Ogawa *et al*., 2006). Studies using *in situ* hybridization and immunocytochemistry have identified Kiss and GnRH localizations in the teleost brain. Kiss and GnRH neuronal populations are generally located at the forebrain in the pre-optic area and habenula, as well as the midbrain with major populations at the lateral tuberal nucleus (nLT) of the hypothalamus – the putative teleost homolog of the mammalian arcuate nucleus (Tena-Sempere *et al*., 2012; Shahjahan *et al*., 2014).

Albeit fishes demonstrate some distinctive differences in the organization, structure, and genomic duplicity of the HPG axis, the overall neuroendocrinology of reproduction in fishes has been the subject of exhaustive on-going study and the core components are fairly well-understood. By contrast, the integration of information on metabolic status and energy

sufficiency for the activation and function of the HPG axis has largely been ignored. While there is overwhelming evidence for Kiss and GnRH sensitivity to metabolic signals in mammals (Kennedy & Mitra, 1963; reviewed in Roa *et al*., 2010), virtually nothing is known on how the reproductive axis in teleost fishes senses metabolic cues critical to its activation and function. Although the fundamental mechanisms and pathways of the HPG axis reported in mammals are highly conserved and shared between vertebrate classes including teleost fishes, the nature and mechanism of action for metabolic regulation through endocrine signals and particularly a role for leptin in teleost reproduction remain unclear. In the current study, we measured changes to brain *kiss2* – the singular form of Kiss in tilapia (Parhar *et al*., 2004), *gnrh1* – the dominant reproductive paralog of GnRH in tilapia (Uchida *et al*., 2005), and liver *lepa* expression over the course of sexual maturation. Additionally, we tested for a functional role for leptin in the stimulation of hypothalamic elements of the reproductive axis via *in vivo* injection and the regulation of sex steroids both *in vivo* and in *ex vivo* testes culture with recombinant tilapia leptin treatment.

Materials and methods

Experimental animals

Adult male tilapia (~75–85 g) were housed in freshwater (FW) recirculating tank systems (salinity 0–0.5 ppt, hardness 74–84 mg/L, alkalinity 126–178 mg/L, pH 8.0) at 24–26°C with a photoperiod of 12:12 h of light and dark and fed daily (1–2% body weight/day). A separate group of mixed sex juvenile tilapia (5-weeks post-hatch) were obtained from North Carolina State Pamlico Field Lab (Aurora, NC) and housed in the separate recirculating tank systems under the same conditions as described for the adult males. These juveniles were allowed two

weeks to acclimate to tank conditions at our animal facility before sampling. Fish were anesthetized in buffered tricaine methanesulfonate (MS-222) prior to intraperitoneal injection and decapitated before tissue removal. All animal protocols were approved by the NC State University Institutional Animal Care and Use Committee.

Developmental expression profile and anatomical indices

To determine the developmental expression profile of *kiss2*, *gnrh1*, and *lepa*, tilapia were sacrificed (n = 16/time point, 8 males and 8 females) and sampled from 7-weeks post-hatch onward for brain, liver, gonads, and plasma at biweekly incremental time points over the known period of sexual maturation (7, 9, 11, 13 weeks post-hatch) with weekly sampling during the critical maturation period (14, 15, 16, 17 weeks post-hatch) and again at 50 weeks post-hatch for known mature timepoint. Additionally, body mass, total length, gonad mass, and gonadosomatic index (GSI = [gonad mass/body mass]*100) were collected for each fish.

In vivo leptin treatment

Fish were anaesthesized, weighed, and injected intraperitoneally with 0.5 or 5 μg/g BW recombinant tilapia leptin A (rtLepA; physiologically-relevant dosages previously validated in Baltzegar *et al*., 2014; Douros *et al*, 2017), 5 μg/g recombinant human leptin (rhLep; National Hormone and Peptide Program, Harbor– UCLA Medical Center, Torrance, CA, USA), or vehicle control saline (n = 8 fish/treatment). Recombinant human leptin was used in the current study to assess the efficacy of using heterologous hormone versus homologous species-specific hormone. 6-hours post-injection, the fish were anaesthetized, decapitated, and fore- and mid-brain – areas within which *kiss2* and *gnrh1* are localized to – were collected and stored in RNAlater prior to

RNA extraction and RT-qPCR. Plasma was collected and frozen at -20°C until sex steroid analysis.

Ex vivo testes culture

Using a technique adapted from Miura *et al*. (1991) in Japanese eel, testes were cultured *ex vivo* to test the direct effects of leptin treatment on sex steroid secretion. Testes were removed from adult male tilapia, washed in ice cold Dulbecco's PBS, and cut into small pieces at ~3 mm length. Approximately 100 mg of testicular fragments were placed into separate wells of a Falcon 24-well plate in 1 ml of L-15 medium supplemented with L-glutamine (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After a 4-hour acclimation pre-incubation at 26°C , the culture medium was replaced with fresh medium containing 15 U/ml human chorionic gonadotropin (hCG, Chorulon, Merck & Co., Inc, Kenilworth, New Jersey, USA), 1 or 10 nM rtLepA, or a no treatment control (n = 6/treatment). After 6-hours of treatment incubation, media was removed and frozen at -20°C prior to steroid analyses.

Steroid measurements

Plasma steroid hormones were extracted using ether-based extraction. 5 mL of diethyl ether (Sigma Aldrich, St. Louis, Missouri, USA) was added to 100 µL of plasma or culture media and vortexed for 10 minutes. The solvent layer was allowed to separate for 5 minutes, after which samples were snap-frozen on liquid nitrogen. The unfrozen liquid phase was removed, stored in a clean glass tube, and dried overnight in a fume hood. Dried samples were reconstituted in ELISA buffer (100 mM PBS, 400 mM NaCl, 1mM EDTA, 0.01% NaN_3). Samples were measured for testosterone, 11-ketotestosterone (11-KT), and 17β-estradiol (E2) in

duplicate by ELISA using commercially available kits (Cayman Chemical, Ann Arbor, Michigan, USA). Estradiol was not measured from *ex vivo* testes incubations due to lower endogenous estradiol activity in these tissues (Hess & Carnes, 2004; Thomas *et al*., 2006; Nishimura & Tanaka, 2014).

RNA isolation and quantification of gene expression

Brain and liver were homogenized and total RNA was extracted using Trizol reagent, isopropanol precipitation, and DNase treatment. RNA purity and concentration were measured via an absorbance spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific). ~1 µg of total RNA was reverse transcribed to cDNA using random hexamers (High Capacity cDNA Synthesis Kit, Life Technologies). Non-reverse transcribed samples were used as a control for genomic contamination. Gene expression was quantified using gene specific primers (Appendix Table 1) via SYBR Green qPCR in triplicate for all cDNA samples, standards, and negative controls. All qPCR reactions were performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The qPCR cycling conditions were applied accordingly: 10 minutes at 95°C, 40 cycles of 95°C for 30 seconds, and annealing for 1 minute at 60°C for 1 minute. A terminal dissociation melt curve step was used to verify a single PCR product. Cycle threshold (Ct) values were transformed against a standard curve of serially-diluted cDNA versus Ct values, normalized to *b-actin*, and reported as fold change relative to control treatments. For developmental expression profiles, expression was reported as fold change relative to sexually mature adults.

Statistical Analyses

Data collected from the developmental expression profile study were analyzed via Spearman's rank correlation coefficient to ascertain correlational relationships over time. In experiments with single time-point treatment comparisons, data was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparisons. For comparisons including treatment and time, two-way ANOVA was employed followed by *a priori* Fisher's Least Significant Difference test for within-treatment comparisons and Dunnett's Multiple Comparisons for comparisons of treatments over time. Statistical significance for all analyses was denoted by an alpha of $P < 0.05$ and data values are presented as means ± SEM. All analyses were performed using SPSS Statistics 25 (IBM, Armonk, New York, USA).

Results

Expression of hepatic *lepa* and brain *kiss2* and *gnrh1* during sexual maturation in relation to anatomical indices

In males and females, hepatic *lepa* mRNA levels strongly correlated with gonadosomatic index (GSI) (Figure 1; male: $r^2 = 0.75$; female: $r^2 = 0.65$) with observed increases beginning at 13 weeks, peaking at 14-16 weeks post-hatch, and remaining elevated thereafter. This period is the typical timeframe for sexual maturation in tilapia (Weber & Grau, 1999; Coward & Bromage, 2000). *lepa* mRNA levels in females rose by up to almost 6-fold during sexual maturation compared to males, in which *lepa* rose approximately 2-fold. Brain *kiss2* (male: $r^2 = 0.62$, female: $r^2 = 0.667$) and *gnrh1* (male: $r^2 = 0.92$, female: $r^2 = 0.8$) also highly correlated with *lepa* with observed increases at week 13 post-hatch onward. *kiss2* (male: $r^2 = 0.52$, female: $r^2 = 0.633$) and *gnrh1* (male: $r^2 = 0.55$, female: $r^2 = 0.8$) also correlated strongly with GSI. For both

males and females, increases to *lepa*, *kiss2*, and *gnrh1* occurred approximately 1 to 2 weeks prior to observable changes in GSI.

In vivo leptin effects on brain neuropeptides and circulating sex steroid levels

Brain mRNA levels of *kiss2* and *gnrh1* were significantly increased by 0.5 μg/g BW rtLepA and rhLep (Figure 2; $P < 0.05$). Albeit not statistically significant, this effect was also observed with the 5 μg/g BW rtLepA dose. There was no significant effect of rtLepA on *gnih*. By contrast, rhLep increased *gnih* by up to 4-fold.

Recombinant tilapia LepA at a dosage of 0.5 μg/g reduced plasma estradiol levels to 0.3 ng/ml relative to control levels of 1.3 ng/ml (Figure 3; $P < 0.01$). The administration of 5 μg/g rhLep also reduced estradiol levels to 0.4 ng/ml. Plasma testosterone levels were significantly reduced by the 0.5 μg/g rtLepA treatment ($P < 0.05$). The higher dose 5 μg/g rtLepA and rhLep treatment also caused a sustained decrease but not significantly different from control. Leptin treatment significantly reduced 11-ketotestosterone to approximately 3 ng/ml compared to 23 ng/ml in the untreated control group ($P < 0.001$).

Leptin effects on sex steroid levels in testicular explant culture

Incubation of testicular fragments with rtLepA reduced media testosterone by approximately 80% (0.037 pg/mg tissue ± 0.011) at 1 nM and approximately 67% (0.05 pg/mg tissue ± 0.01) at 10 nM concentration (Figure 4; $P < 0.012$). rtLepA at 1 and 10 nM concentration similarly decreased 11-ketotestosterone levels by approximately 80% (0.544 pg/mg tissue ±0 .226) and 65% (0.935 pg/mg tissue±0.3749), respectively ($P < 0.01$). For both androgens, media levels showed the largest decrease with 1 nM rtLepA treatment. Treatment

with the positive control hCG resulted in a significant increase of up-to 105% in media testosterone levels but had no effect on 11-ketotestosterone.

Discussion

The current study provides the first evidence of a role for leptin in the regulation of central factors and inhibition of gonadal steroids of the reproductive axis in a teleost fish. We show that hepatic leptin expression positively correlates with increases in GSI and brain *kiss2* and *gnrh1* in both males and females and demonstrate the hormone stimulates transcript levels of these key neuroendocrine factors. A striking finding of the current study is that leptin markedly reduces circulating sex steroid levels *in vivo* and directly suppresses androgen release from cultured testicular fragments.

In vertebrates studied to date, nutritional and energy sufficiency play a fundamental role in the regulation of the onset of puberty and reproductive function. The energetic costliness of reproduction necessitates sufficient energy stores for the metabolic expenditure associated with the various physiological and behavioral processes that coincide with reproduction (i.e., gamete production, lactation, parental care – Calow 1979; Vezina & Williams, 2005; Harshman & Zera, 2007). In mammals, a role for leptin in the metabolic control of reproduction became clear soon after the hormone's initial discovery as evidenced through correlative studies on circulating leptin levels, adiposity, and pubertal onset, as well as reproductive abnormalities observed in murine models with disrupted leptin signaling (i.e., *ob/ob* and *db/db* mice – Zhang *et al*., 1994; Rosenbaum & Leibel, 1998; Farooqi *et al*., 2007). Continuing work over the past few decades has indicated that while the targets and sites of action for leptin are notably diverse, the majority

of the hormone's reproductive actions are centrally regulated at key hypothalamic nuclei (Manfredi-Lozano *et al*., 2016; reviewed in Sanchez-Garrido & Tena-Sempere, 2013).

Soon after the reproductive properties of leptin in mammals began to emerge, early studies in teleosts - even before leptin was cloned and characterized in any teleosts - attempted to evaluate similar HPG-permissive/stimulatory properties of the hormone via application of high doses of recombinant human leptin to primary cultured pituitary cells. Evidence from this early work suggested that leptin might promote GTH secretion (*Dicentrarchus labrax* – Peyon *et al*., 2001, 2003; *Onchorynchus mykiss* – Weil *et al*., 2003). Sequential studies in the past decade have suggested correlations between leptin expression and gonadotropin activity and sexual maturation (*Salmo salar* – Trombley & Schmitz, 2013, 2014). A recent study evidenced *in vivo* leptin stimulation of pituitary gonadotropins in chub mackerel (*Scomber japonicus* – Ohga *et al*., 2020). That said, no studies to date have considered the functional properties of homologous leptin on teleost reproduction at key effector levels including the brain and gonads.

Previous work in tilapia has shown that Kiss2 – the singular Kiss isoform in tilapia – stimulates GnRH1 – the dominant GnRH isoform in tilapia regulating gonadotropin secretion (Tena-Sempere *et al*., 2012; Shahjahan *et al*., 2014; Park *et al*., 2016). In the current study, we report leptin stimulation of *kiss2* and *gnrh1* mRNA levels in the brain, indicating that the cytokine may promote these neuropeptides in regulating downstream gonadotropin secretion. The current state of knowledge of GnIH is largely variable with studies suggesting that the neuropeptide can both stimulate and inhibit gonadotropic cascades. Here, absence of a *gnih* response to homologous leptin treatment suggests that leptin may not exert any particular action on the neuropeptide within the narrow physiological parameters of a mature adult male fish (Son

et al., 2019). Nonetheless, the central effect of leptin on Kiss2 and GnRH1 is consistent with the established paradigm for its action on apex components of the HPG axis in mammals.

In teleosts studied to date, as in mammals, Kiss and GnRH are known to be sensitive to metabolic state. For example, teleosts experiencing a metabolic challenge (i.e., starvation) are likely to have a suppressed Kiss/GnRH response (e.g., *Dicentrarchus labrax*: Gonzalez-Martinez *et al*., 2002; *Danio rerio*: Busby *et al*., 2010; reviewed in Van Der Kraak 2009, Shahjahan *et al*., 2014). While initial reports in mammals indicated a putative direct action of leptin on these neuronal populations, recent work has shown that metabolic sensing by Kiss and GnRH through leptin occurs via indirect signaling (i.e., through POMC-related GABAergic pathways – Vong *et al*., 2011; Zuure *et al*., 2013). Notably, the leptin receptor is scarcely expressed on approximately 13% of hypothalamic Kiss neurons (Hakansson *et al*., 1998; Cravo *et al*., 2011; Sanches-Garrido & Tena-Sempere, 2013). Work in teleosts continues to corroborate the notion that the majority of the HPG axis is highly conserved across vertebrate classes with recent studies showing similar metabolic sensitivity by Kiss and GnRH, albeit differential responses between paralogs and between species (Adams *et al*., 2002; Vickers *et al*., 2004; Pasquier *et al*., 2013; Shahjahan *et al*., 2014). However, localization of the leptin receptor and the hormone's sites of action (through pSTAT3 staining for instance) has not been performed. Hence, whether the classically identified first-order targets of the hormone in mammals are conserved in teleosts remains unclear.

While stimulation of *kiss2* and *gnrh1* by leptin treatment observed in the current study suggests a possible stimulation of the hormone on central targets, leptin's reduction of circulating sex steroid levels in the same experimental animals indicates inhibitory actions at peripheral tissues. We found that treatment of fish with leptin caused a marked decline in circulating

testosterone, 11-KT and 17β-estradiol. Similar responses were observed in *ex vivo* testicular culture suggesting leptin likely acts directly on the testes to suppress sex steroid secretion.

In the current study, we evidence leptin suppression of sex steroids. In teleost fishes, studies from our group and others indicate that stressors may enhance leptin activity (Baltezegar *et al*., 2014; Deck *et al*, 2017). Recent studies on the teleost leptin response and leptin action during fasting (e.g., *E. coioides* – Zhang *et* al, 2012; *O. mossambicus* – Douros *et al*., 2017), hyperosmotic stress (Baltezegar *et al*., 2014), and hypoxia (Chu *et al*., 2010; Cao *et al*., 2011) indicate that transcription and circulating levels of the hormone may increase in order to mobilize energy stores to respond to the demand of the incurring stressor (Copeland *et al*., 2011; Deck *et al*., 2017). These stressors – among others – typically coincide with disruptions to gonadotropic signaling and reproductive systems.

The evidence from the current study positions leptin as a possible signal mediating disruption to the HPG axis during stress. Similar dynamics can be observed in instances of metabolic abnormality in mammals. Obesity in mammals retains similar energy-demanding physiological properties attributed to chronic stress including elevated glucocorticoid levels, chronic inflammation, increased activity of inflammatory cytokines (IL-1, IL-6, TNFa), as well as disrupted HPG function (Das 2001; Wisse 2004; Makki *et al*., 2013). In both humans and rodent models, hypogonadotropic hypogonadism (HH) resulting from morbid obesity is typically characterized by low circulating sex steroid levels (Jarow *et al*., 1993; Tchernof & Despres, 2000; Bianco & Kaiser, 2009; Giton *et al*., 2015). Mechanistically, this can occur from leptin dysfunction at central circuits and/or leptin action at the gonads (Bianco & Kaiser, 2009). In both males and females, hyperleptinemia in HH as a consequence of obesity can result in a downregulation of the leptin receptor at neuronal targets that regulate the HPG axis, therein

disrupting all levels of gonadotropic signaling (Pasquali 2006; Olivares *et al*., 2010; Lainez & Coss, 2019). Interruptions in normal gonadotropic signaling therein causes reduction in the overall sensitivity of apex components to typical agonists (i.e., leptin to kisspeptin-relevant circuits), as well as circulating sex steroid levels. At the gonads, inhibitory action of leptin has been reported in mammalian models through both the observed expression of functional leptin receptor at both the testes and ovaries, notably localized to steroidogenic Leydig and Sertoli cells (Isidori *et al*., 1999; Giovambattista *et al*., 2003). In the case of obesity-related hyperleptinemia and HH, chronically elevated leptin levels can reduce gonadal steroidogenesis as evidenced by reduced mRNA levels of steroidogenic factor-1, StAR, and other P450 side-chain cleavage enzymes (Tena-Sempere *et al*., 2001). In normal non-obese mammals, leptin exerts a stimulatory effect on gonadotropic signaling and the inhibitory effects observed in individuals with hyperleptinemia attributed to metabolic abnormalities typically do not occur (Ahima *et al*., 1997; Yu *et al*., 1997). Hence, possibility of a role for leptin as an inhibitor of key facets of the HPG axis via inhibition of steroidogenic pathways and circulating sex steroid levels during stress in teleosts may be supported by the currently understood disrupted reproductive phenotype associated with obese hyperleptinemic HH individuals.

When interpreting the inhibitory effect that leptin had on sex steroids in the *in vivo* experiment from the current study, it is tempting to assert that reduction in sex steroid levels may be due to acute exposure to a high enough concentration of leptin resulting in disruptions to gonadal steroidogenesis, as reported in HH mammals. However, the evidence generated from our *ex vivo* testicular culture reduced media androgen levels with the application of physiological levels of recombinant tilapia leptin (~1-10 nM) that would not necessarily be reflective of chronically-elevated stress or hyper-leptinemic levels (Douros *et al*., 2014, 2017). A similar

study in rats reports comparable results, with leptin inhibiting androgen secretion in cultured testes independent and regardless of the prevailing nutritional state and dose (Tena-Sempere *et al*., 1999). Given the current mammalian model for leptin action at the gonads in both males and females (reviewed in Chou & Mantzoros, 2014), as well as the data reported in the current study for leptin reduction of sex steroid levels in normally-fed adult male fish both *in vivo* and in cultured testicular fragments, it is conceivable that leptin exerts a basal inhibitory effect specifically at the gonads independent of its actions on central components of the HPG axis.

The evidence from the current study points to complex and multiple modes of action for leptin at the various levels and targets of the HPG axis. The concurrent stimulation of Kiss and GnRH and reduction of circulating sex steroid levels indicates that the hormone is exerting different actions at central and peripheral targets. The disparity between stimulation of the apex components of the HPG axis while inhibiting major steroidal effectors suggests multimodal and target-specific actions by the hormone: possibly through disruption of hypothalamic signaling circuits, targeting of pituitary gonadotropes, or inhibition of steroidogenic pathways. Alternatively, the sharp decline in circulating sex steroid levels may be prompting a stimulatory response at the level of the brain.

Within the vertebrate paradigm for reproduction, the onset of puberty and sustained reproductive function are metabolically gated due to the immense energetic cost affiliated with investment in all aspects of reproduction (i.e., gonadal growth, parental care – Calow 1979; Vezina & Williams, 2005; Harshman & Zera, 2007). Given the established permissive role for leptin in the onset of puberty and reproduction in mammals (Ahima *et al*., 1997; Cheung *et al*., 1997), our postulation here for a putative role for leptin in the regulation of reproduction in teleost fishes is not inconceivable. In the present study, we report concurrent increases to and

strong positive correlations between hepatic *lepa*, brain *kiss2* and *gnrh1*, and gonadal mass, suggesting a possible role for leptin in the onset of puberty in tilapia. In mammals, metabolic gating of puberty is surmounted by sufficient leptin activity, which circulates in proportion to adiposity thereby signaling overall energy availability. However, this adipostatic property of the hormone likely evolved in mammals as there is little evidence to support a similar role in teleosts (see Gorissen & Flik, 2014; Michel *et al.,* 2016; Deck *et al*., 2017 for review), this despite some conserved glucoregulatory or liporegulatory functions among these vertebrates (Li *et al*., 2010; Song *et al*., 2015; Zhang *et al*., 2015; Michel *et al*. 2016; Deck *et al.,* 2017). Indeed, depletion of energy reserves through fasting enhances leptin synthesis and secretion in tilapia and other teleosts (Chapter 2, Douros *et al*., 2017) suggesting the hormone is not likely relaying energy sufficiency to central circuits as seen with mammals. Nonetheless, while the correlational increase with *kiss2* and *gnrh1* suggests a role for leptin in the onset of puberty, possible mechanisms underlying its action on sexual maturation in teleosts remains to be explored.

The present study shows leptin elevation in conjunction with sexual maturation and suggests that the hormone may act at central targets to stimulate hypothalamic factors like Kiss and GnRH during pubertal onset. Remarkably, we found for the first time that leptin reduces sex steroid levels and that this inhibitory action may be direct as shown by our testicular explant culture. There is ongoing evidence to suggest that instances of stress stimulate leptin activity in teleost fishes (reviewed in Deck *et al*, 2017). While leptin may act in a stimulatory manner on central targets during normal homeostatic circumstances, we speculate that the inhibitory effect of the hormone on gonadal sex steroids may be to limit energy partitioning to expensive reproductive systems during instances of negative energy balance associated with the stress response during which leptin has been shown to increase.

REFERENCES

Adams, B. A., Vickers, E. D., Warby, C., Park, M., Fischer, W. H., Grey Craig, A., & Sherwood, N. M. (2002). Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, Coregonus clupeaformis. *Biology of Reproduction*, *67*(1), 232-239.

Ahima, R. S., Dushay, J., Flier, S. N., Prabakaran, D., & Flier, J. S. (1997). Leptin accelerates the onset of puberty in normal female mice. *The Journal of clinical investigation*, *99*(3), 391-395.

Backholer, K., Smith, J. T., Rao, A., Pereira, A., Iqbal, J., Ogawa, S., & Clarke, I. J. (2010). Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. *Endocrinology*, *151*(5), 2233-2243.

Baltzegar, D. A., Reading, B. J., Douros, J. D., & Borski, R. J. (2014). Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. *J Endocrinol*, *220*(1), 61-72.

Bianco, S. D., & Kaiser, U. B. (2009). The genetic and molecular basis of idiopathic hypogonadotropic hypogonadism. *Nature Reviews Endocrinology*, *5*(10), 569.

Calow, P. (1979). The cost of reproduction–a physiological approach. *Biological Reviews*, *54*(1), 23-40.

Cameron, J. L. (1996). Nutritional determinants of puberty. *Nutrition reviews*, *54*(2), S17.

Casabiell, X., Piñeiro, V., Vega, F., Luis, F., Diéguez, C., & Casanueva, F. F. (2001). Leptin, reproduction and sex steroids. *Pituitary*, *4*(1-2), 93-99.

Castellano, J. M., Navarro, V. M., Fernandez-Fernandez, R., Nogueiras, R., Tovar, S., Roa, J., & Pinilla, L. (2005). Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology*, *146*(9), 3917-3925.

Chehab, F. F., Lim, M. E., & Lu, R. (1996). Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature genetics*, *12*(3), 318-320.

Cheung, C. C., Thornton, J. E., Kuijper, J. L., Weigle, D. S., Clifton, D. K., & Steiner, R. A. (1997). Leptin is a metabolic gate for the onset of puberty in the female rat. *Endocrinology*, *138*(2), 855-858.

Chou, S. H., & Mantzoros, C. (2014). Role of leptin in human reproductive disorders. *J. Endocrinol*, *223*(1), T49-T62.

Cravo, R. M., Margatho, L. O., Osborne-Lawrence, S., Donato Jr, J., Atkin, S., Bookout, A. L., & Zigman, J. M. (2011). Characterization of Kiss1 neurons using transgenic mouse models. *Neuroscience*, *173*, 37-56.

Das, U. N. (2001). Is obesity an inflammatory condition?. *Nutrition*, *17*(11-12), 953-966.

Deck, C. A., Honeycutt, J. L., Cheung, E., Reynolds, H. M., & Borski, R. J. (2017). Assessing the functional role of leptin in energy homeostasis and the stress response in vertebrates. *Frontiers in endocrinology*, *8*, 63.

Donato, J., Cravo, R. M., Frazão, R., Gautron, L., Scott, M. M., Lachey, J., & Richardson, J. A. (2011). Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. *The Journal of clinical investigation*, *121*(1), 355-368.

Douros, J. D., Baltzegar, D. A., Breves, J. P., Lerner, D. T., Seale, A. P., Grau, E. G., & Borski, R. J. (2014). Prolactin is a major inhibitor of hepatic leptin A synthesis and secretion: studies utilizing a homologous leptin A ELISA in the tilapia. *General and comparative endocrinology*, *207*, 86-93.

Farooqi, I. S., Wangensteen, T., Collins, S., Kimber, W., Matarese, G., Keogh, J. M., & Dattani, M. T. (2007). Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. *New England Journal of Medicine*, *356*(3), 237-247.

Giovambattista, A., Suescun, M. O., Nessralla, C. C., França, L. R., Spinedi, E., & Calandra, R. S. (2003). Modulatory effects of leptin on leydig cell function of normal and hyperleptinemic rats. *Neuroendocrinology*, *78*(5), 270-279.

Giton, F., Trabado, S., Maione, L., Sarfati, J., Le Bouc, Y., Brailly-Tabard, S., & Young, J. (2015). Sex steroids, precursors, and metabolite deficiencies in men with isolated hypogonadotropic hypogonadism and panhypopituitarism: a GCMS-based comparative study. *The Journal of Clinical Endocrinology & Metabolism*, *100*(2), E292-E296.

Gorissen, M., & Flik, G. (2014). Leptin in teleostean fish, towards the origins of leptin physiology. *Journal of chemical neuroanatomy*, *61*, 200-206.

Håkansson, M. L., Brown, H., Ghilardi, N., Skoda, R. C., & Meister, B. (1998). Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *Journal of Neuroscience*, *18*(1), 559-572.

Harshman, L. G., & Zera, A. J. (2007). The cost of reproduction: the devil in the details. *Trends in ecology & evolution*, *22*(2), 80-86.

Hess, R. A., & Carnes, K. (2004). The role of estrogen in testis and the male reproductive tract: a review and species comparison. *Animal Reproduction (AR)*, *1*(1), 5-30.

Isidori, A. M., Caprio, M., Strollo, F., Moretti, C., Frajese, G., Isidori, A., & Fabbri, A. (1999). Leptin and androgens in male obesity: evidence for leptin contribution to reduced androgen levels. *The Journal of Clinical Endocrinology & Metabolism*, *84*(10), 3673-3680.

Jarow, J. P., Kirkland, J., Koritnik, D. R., & Cefalu, W. T. (1993). Effect of obesity and fertility status on sex steroid levels in men. *Urology*, *42*(2), 171-174.

Kennedy, G. C., & Mitra, J. (1963). Body weight and food intake as initiating factors for puberty in the rat. *The Journal of physiology*, *166*(2), 408-418.

Lainez, N. M., & Coss, D. (2019). Obesity, Neuroinflammation, and Reproductive Function. *Endocrinology*, *160*(11), 2719-2736.

Li, G. G., Liang, X. F., Xie, Q., Li, G., Yu, Y., & Lai, K. (2010). Gene structure, recombinant expression and functional characterization of grass carp leptin. *General and comparative endocrinology*, *166*(1), 117-127.

Luque, R. M., Kineman, R. D., & Tena-Sempere, M. (2007). Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. *Endocrinology*, *148*(10), 4601-4611.

Makki, K., Froguel, P., & Wolowczuk, I. (2013). Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN inflammation*, *2013*.

Manfredi-Lozano, M., Roa, J., Ruiz-Pino, F., Piet, R., Garcia-Galiano, D., Pineda, R., & Dieguez, C. (2016). Defining a novel leptin–melanocortin–kisspeptin pathway involved in the metabolic control of puberty. *Molecular metabolism*, *5*(10), 844-857.

Marshall, W. A. (1978). Puberty. In *Human growth* (pp. 141-181). Springer, Boston, MA.

Messager, S., Chatzidaki, E. E., Ma, D., Hendrick, A. G., Zahn, D., Dixon, J., & Colledge, W. H. (2005). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proceedings of the National Academy of Sciences*, *102*(5), 1761-1766.

Navarro, V. M., & Tena-Sempere, M. (2012). Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. *Nature Reviews Endocrinology*, *8*(1), 40.

Nishimura, T., & Tanaka, M. (2014). Gonadal development in fish. *Sexual Development*, *8*(5), 252-261.

Ohga, H., Ito, K., Matsumori, K., Kimura, R., Ohta, K., & Matsuyama, M. (2020). Leptin stimulates gonadotropin release and ovarian development in marine teleost chub mackerel. *General and Comparative Endocrinology*, *292*, 113442.

Olivares, A., Méndez, J. P., Zambrano, E., Cárdenas, M., Tovar, A., Perera-Marín, G., & Ulloa-Aguirre, A. (2010). Reproductive axis function and gonadotropin microheterogeneity in a male rat model of diet-induced obesity. *General and comparative endocrinology*, *166*(2), 356-364.

Pasquali, R. (2006). Obesity, fat distribution and infertility. *Maturitas*, *54*(4), 363-371.

Pasquier, J., Lafont, A. G., Tostivint, H., Vaudry, H., Rousseau, K., & Dufour, S. (2012). Comparative evolutionary histories of kisspeptins and kisspeptin receptors in vertebrates reveal both parallel and divergent features. *Frontiers in endocrinology*, *3*, 173.

Peyon, P., de Celis, S. V. R., Gómez-Requeni, P., Zanuy, S., Pérez-Sánchez, J., & Carrillo, M. (2003). In vitro effect of leptin on somatolactin release in the European sea bass (Dicentrarchus labrax): dependence on the reproductive status and interaction with NPY and GnRH. *General and comparative endocrinology*, *132*(2), 284-292.

Roa, J., García-Galiano, D., Castellano, J. M., Gaytan, F., Pinilla, L., & Tena-Sempere, M. (2010). Metabolic control of puberty onset: new players, new mechanisms. *Molecular and cellular endocrinology*, *324*(1-2), 87-94.

Rosenbaum, M., & Leibel, R. L. (1998). Leptin: a molecule integrating somatic energy stores, energy expenditure and fertility. *Trends in Endocrinology & Metabolism*, *9*(3), 117-124.

Sanchez-Garrido, M. A., & Tena-Sempere, M. (2013). Metabolic control of puberty: roles of leptin and kisspeptins. *Hormones and behavior*, *64*(2), 187-194.

Schwartz, N. B. (2000). Neuroendocrine regulation of reproductive cyclicity. In *Neuroendocrinology in physiology and medicine* (pp. 135-145). Humana Press, Totowa, NJ.

Shahjahan, M., Kitahashi, T., & Parhar, I. S. (2014). Central pathways integrating metabolism and reproduction in teleosts. *Frontiers in endocrinology*, *5*, 36.

Sisk, C. L., & Foster, D. L. (2004). The neural basis of puberty and adolescence. *Nature neuroscience*, *7*(10), 1040-1047.

Smith, J. T., Acohido, B. V., Clifton, D. K., & Steiner, R. A. (2006). KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *Journal of neuroendocrinology*, *18*(4), 298-303.

Song, Y. F., Wu, K., Tan, X. Y., Zhang, L. H., Zhuo, M. Q., Pan, Y. X., & Chen, Q. L. (2015). Effects of recombinant human leptin administration on hepatic lipid metabolism in yellow catfish Pelteobagrus fulvidraco: in vivo and in vitro studies. *General and comparative endocrinology*, *212*, 92-99.

Tchernof, A., & Després, J. P. (2000). Sex steroid hormones, sex hormone-binding globulin, and obesity in men and women. *Hormone and Metabolic Research*, *32*(11/12), 526-536.

Tena-Sempere, M. (2003). Gonadotropins and gonadotropin receptors. *Reproductive Medicine-Molecular, Cellular and Genetic Fundamentals*, 225-244.

Tena-Sempere, M., Manna, P. R., Zhang, F. P., Pinilla, L., Gonzalez, L. C., Dieguez, C., & Aguilar, E. (2001). Molecular mechanisms of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *Journal of Endocrinology*, *170*(2), 413-424.

Tena-Sempere, M., Pinilla, L., Gonzalez, L. C., Dieguez, C., Casanueva, F. F., & Aguilar, E. (1999). Leptin inhibits testosterone secretion from adult rat testis in vitro. *Journal of Endocrinology*, *161*, 211-218.

Thomas, P., Dressing, G., Pang, Y., Berg, H., Tubbs, C., Benninghoff, A., & Doughty, K. (2006). Progestin, estrogen and androgen G-protein coupled receptors in fish gonads. *Steroids*, *71*(4), 310-316.

Trombley, S., & Schmitz, M. (2013). Leptin in fish: possible role in sexual maturation in male Atlantic salmon. *Fish physiology and biochemistry*, *39*(1), 103-106.

Trombley, S., Mustafa, A., & Schmitz, M. (2014). Regulation of the seasonal leptin and leptin receptor expression profile during early sexual maturation and feed restriction in male Atlantic salmon, Salmo salar L., parr. *General and Comparative Endocrinology*, *204*, 60-70.

Tulipano, G., Vergoni, A. V., Soldi, D., Muller, E. E., & Cocchi, D. (2004). Characterization of the resistance to the anorectic and endocrine effects of leptin in obesity-prone and obesity-resistant rats fed a high-fat diet. *Journal of endocrinology*, *183*(2), 289-298.

Vézina, F., & Williams, T. D. (2005). The metabolic cost of egg production is repeatable. *Journal of Experimental Biology*, *208*(13), 2533-2538.

Vickers, E. D., Laberge, F., Adams, B. A., Hara, T. J., & Sherwood, N. M. (2004). Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, Coregonus clupeaformis. *Biology of reproduction*, *70*(4), 1136-1146.

Vong, L., Ye, C., Yang, Z., Choi, B., Chua Jr, S., & Lowell, B. B. (2011). Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. *Neuron*, *71*(1), 142-154.

Weil, C., Le Bail, P. Y., Sabin, N., & Le Gac, F. (2003). In vitro action of leptin on FSH and LH production in rainbow trout (Onchorynchus mykiss) at different stages of the sexual cycle. *General and comparative endocrinology*, *130*(1), 2-12.

Wisse, B. E. (2004). The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity. *Journal of the American society of nephrology*, *15*(11), 2792-2800.

Yu, W. H., Kimura, M., Walczewska, A., Karanth, S., & McCann, S. M. (1997). Role of leptin in hypothalamic–pituitary function. *Proceedings of the National Academy of Sciences*, *94*(3), 1023-1028.

Zhang, L. H., Tan, X. Y., Wu, K., Zhuo, M. Q., Song, Y. F., & Chen, Q. L. (2015). Regulation and mechanism of leptin on lipid metabolism in ovarian follicle cells from yellow catfish Pelteobagrus fulvidraco. *General and comparative endocrinology*, *222*, 116-123.

Zohar, Y., Muñoz-Cueto, J. A., Elizur, A., & Kah, O. (2010). Neuroendocrinology of reproduction in teleost fish. *General and comparative endocrinology*, *165*(3), 438-455.

Zuure, W. A., Roberts, A. L., Quennell, J. H., & Anderson, G. M. (2013). Leptin signaling in GABA neurons, but not glutamate neurons, is required for reproductive function. *Journal of Neuroscience*, *33*(45), 17874-17883.

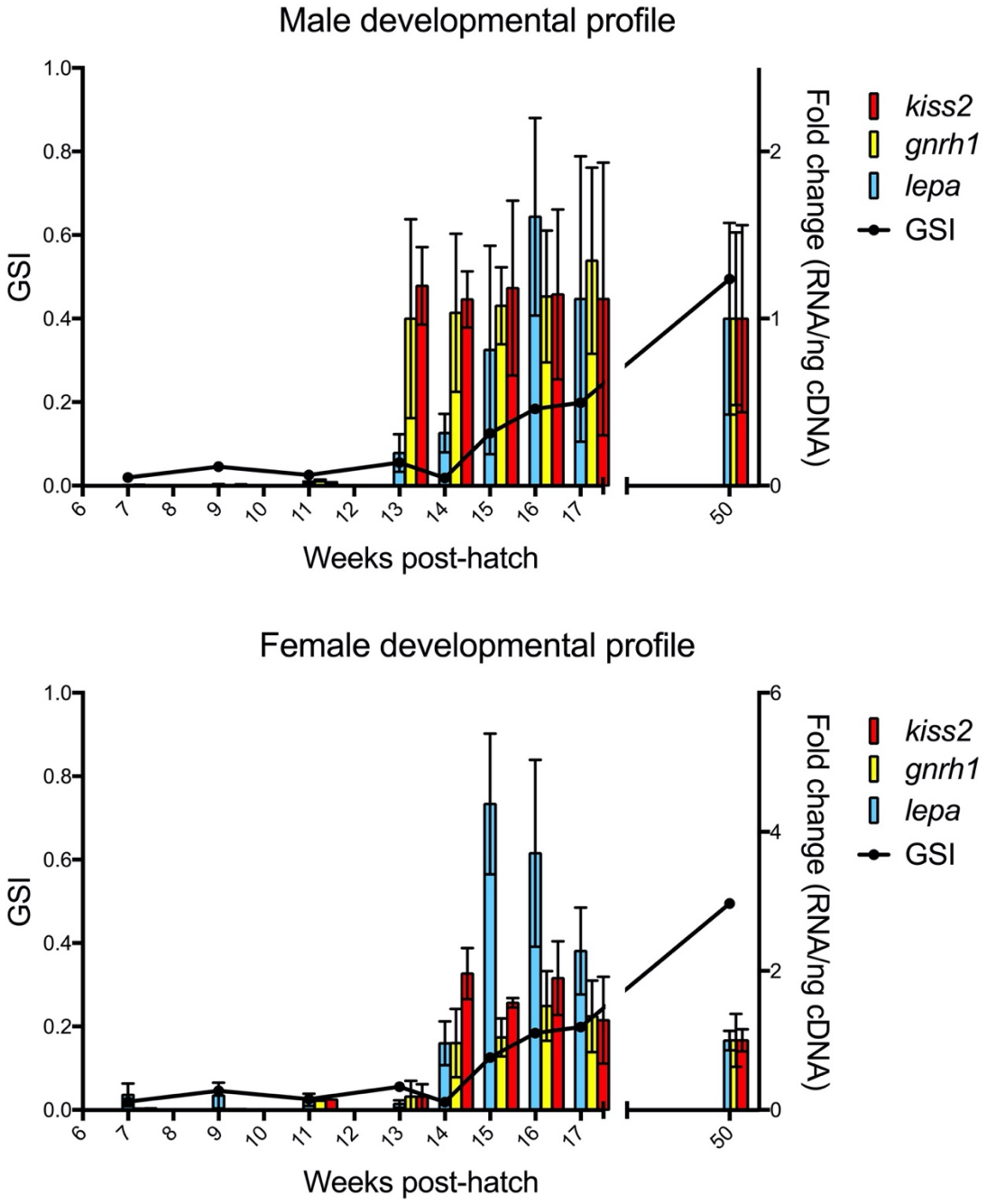

Figure 1. Expression profiles of brain *kiss2*, *gnrh1*, and liver *lepa* (as fold change from 50-weeks post-hatch mature adult n = 8/time point) of male and female tilapia plotted over GSI from 7-weeks post-hatch to 50-weeks post-hatch. Values reported as means ± SEM.

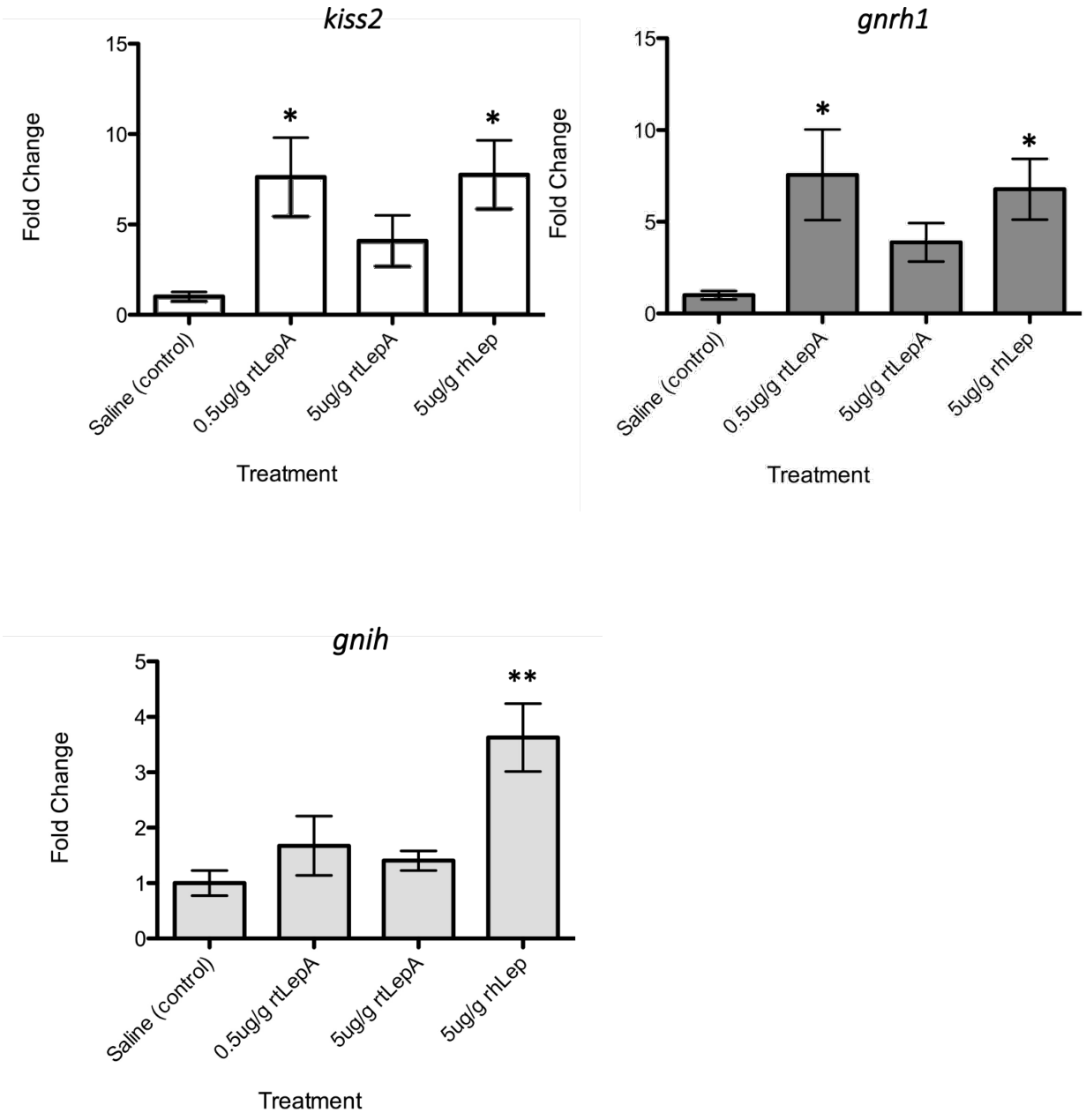

Figure 2. Effect of acute rtLepA injection on fore/mid-brain *kiss2, gnrh1,* and *gnih* mRNA levels (as fold change from Saline control) at 6-hours following bolus injection. Values reported as means ± SEM. * denotes significant difference from saline control (n = 8/treatment; * $P < 0.05$, ** $P < 0.01$).

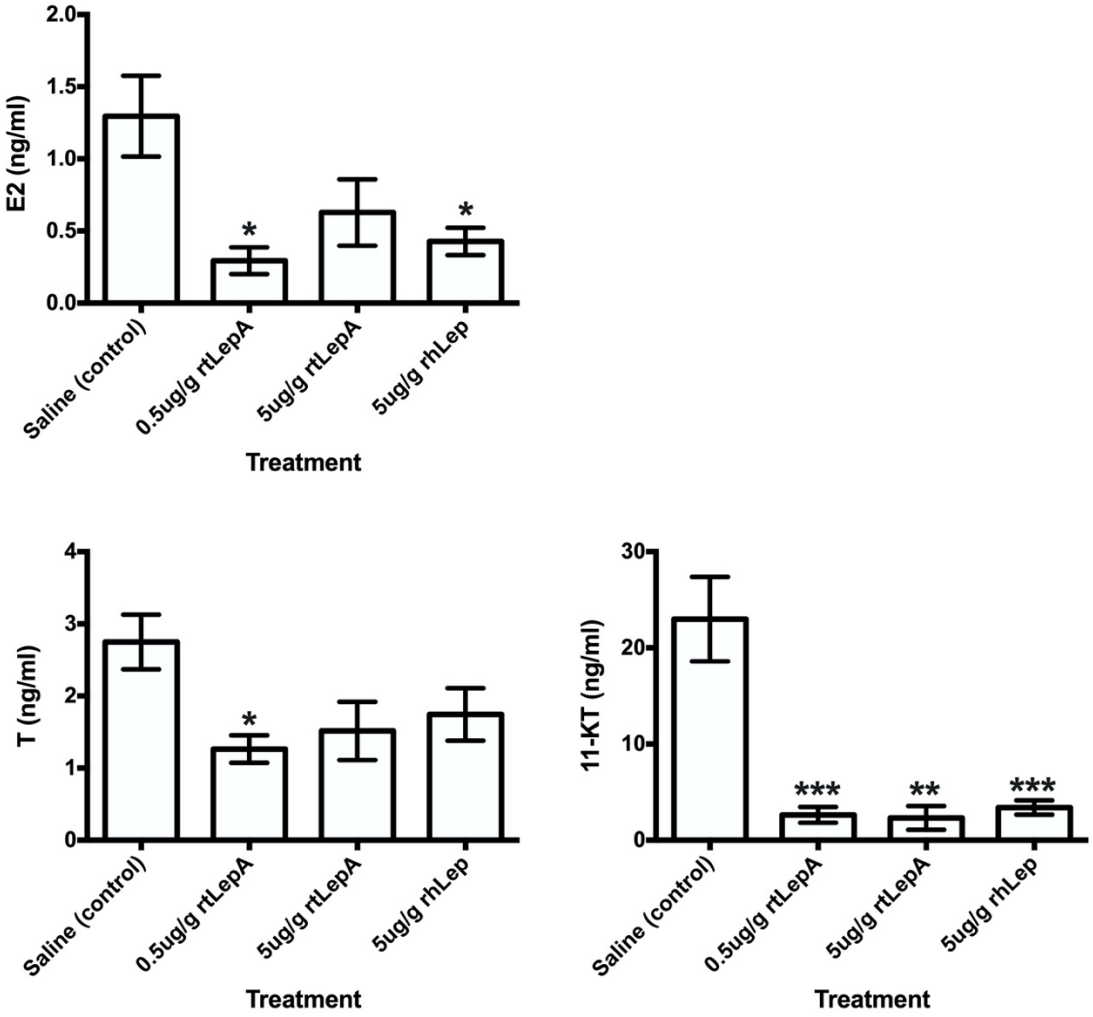

Figure 3. Effect of acute rtLepA injection on circulating estradiol (E2), testosterone (T), and 11-ketotestosterone (11KT) levels at 6-hours following bolus injection. Values reported as means ± SEM. * denotes significant difference from saline control (n = 8/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

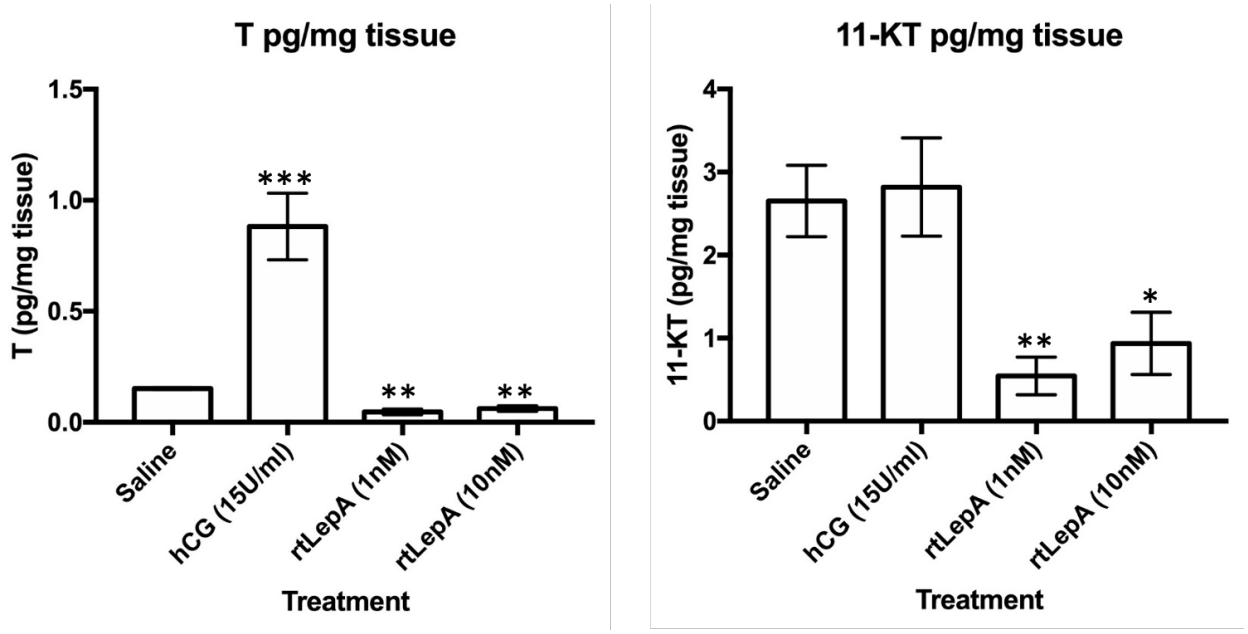

Figure 4. Effect of hCG and rtLepA treatment in *ex vivo* testicular fragment culture on media testosterone (T) and 11-ketotestosterone (11KT) levels at 6-hours following treatment application. Values reported as means ± SEM. * denotes significant difference from saline control (n = 8/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

CHAPTER 4

Vitellogenins, sex steroids, and maternal mouthbrooding: Interdependent dynamics with leptin in the Mozambique tilapia *Oreochromis mossambicus*

Abstract

Structural growth and reproduction are physiologically demanding processes that require substantial energetic input, the lack of which can result in the suppression of one or both of these systems. The pleiotropic cytokine hormone leptin has been implicated in the integration of central and peripheral signals that mediate the function of and energy allocation between the brain-pituitary-gonadotropic and brain-pituitary-somatotropic axes. Its role in mediating resource allocation between growth and reproduction in fishes or other ectotherms and control by sex steroids is poorly understood. The current study assesses (i) *in vivo* estradiol and *in vitro* sex steroid regulation of leptin (*lepa*) and insulin-like growth factor (*igf1*) at the liver, the primary source of circulating leptin and IGF1 (IGFs are central regulators of somatic growth) (ii) leptin effects on hepatic vitellogenins (*vtgaa*, *vtgab*, *vtgc*) and *igf1*, and (iii) the expression profiles of *lepa, igf1*, and vitellogenins in fed-gravid, fasted, and mouthbrooding females. Hepatic transcript levels of *lepa*, the dominant paralog of leptin in teleosts, and *igf1* were decreased in response to estradiol both *in vivo* and *in vitro*. In primary hepatocyte culture, leptin suppressed *vtgab* and *vtgc* transcript levels, while it stimulated *igf1*. When comparing the hepatic expression profiles of gravid, mouthbrooding, and fasted females, gravid and mouthbrooding females shared highly similar *lepa*, *igf1*, and *vtg* levels. By contrast, fasted females demonstrated significantly elevated *lepa*, decreased *igf1* and *vtg levels*. These studies evidence that leptin suppresses vitellogenins in teleost fishes, opposite to the well-established stimulatory effect of estradiol, while it enhances elements of the growth regulatory axis. We postulate that estradiol may enhance vitellogenesis and promote reproductive growth, in part, by antagonizing leptin synthesis. Estradiol and testosterone exert disparate effects on elements of the growth-regulatory axis, with estradiol suppressing and testosterone stimulating *igf1*, consistent with the sexually dimorphic growth

observed in tilapia where females grow more slowly than males. Strikingly, our results also demonstrate differences in the leptin response to fasting and mouthbrooding. Our previous work demonstrates leptin increases with stressors and hence it may suppress reproductive growth (vitellogenesis) during catabolic periods while preserving structural growth via enhancement of growth factors. We postulate that the metabolic and reproductive challenges associated with mouthbrooding may be regulated by estradiol rather than leptin, though this estrogenic effect necessitates further study.

Introduction

Growth and reproduction are highly interconnected biological processes that are regulated by endocrine networks that control the allocation of resources between these two fundamental systems. In vertebrates studied to date, available energetic resources are generally allocated into either somatic or gonadal growth (Parent *et al*., 2003; reviewed in Sanches-Garrido & Tena-Sempere, 2013). While somatotropic signaling (i.e., growth hormone, GH and IGFs) participates in some facets of gonadal development and growth (reviewed in Wootton & Smith, 2014), there is substantial experimental and clinical evidence demonstrating its disruption of reproductive growth and function. For example, GH treatment and overexpression in both mammalian and teleost models can result in reduced fertility and hindered gonadal development (Bartke *et* al., 1994; Hull & Harvey, 2000; Chen *et al*., 2018).

Competitive investment between somatic and gonadal growth is markedly more evident in females (e.g., Kozlowski & Wiegert, 1986; Lardner & Loman, 2003; Kuzawa 2007). In a variety of vertebrates studied to date, sexually dimorphic growth following the onset of puberty and eventual acquisition of sexual maturity can be attributed to the actions of gonadal sex steroids (Rljnsdorp & Ibelings, 1989; Wehrenberg & Giustina, 1992; Imsland *et al*., 1997; Fontana *et al*., 2016; Ma *et al*., 2016). In many species, androgens promote anabolic processes that increase somatic growth in males, while estrogen signaling in mature females often coincides with the suppression of somatotropic signaling in favor of investment into reproductive tissues. While the endocrine regulation between the interdependent growth and reproductive axes is readily evidenced by models and studies of sexually dimorphic growth, the regulatory mechanisms driving this interaction are not well understood.

Teleost fishes represent more than half of all known vertebrate species. The immense diversity of teleosts is also reflected in the various reproductive strategies and adaptations that they possess. The Mozambique tilapia (*Oreochromis mossambicus*) is a well-established comparative model for the study of the endocrine regulation of growth and reproduction. Previous work by Davis *et al*. (2007, 2008) has shown distinct effects of estradiol on hepatic elements, in which male fish were studied to simulate the transition from somatic to gonadal growth without a prevailing estradiol-primed physiology. These studies indicated estradiol inhibition of hepatic insulin-like growth factor 1 (IGF1) while promoting egg yolk precursor proteins, vitellogenins (Vtg). Hence, these studies suggested that estradiol likely shifts energy investment away from somatic growth towards gonadal growth via induction of Vtgs. While the interaction between growth and reproductive processes at the hepatic level in tilapia has become increasingly apparent, the mechanisms regulating the shift between somatic and gonadal growth factors remain unclear.

Leptin is a cytokine hormone well-established for its prototypic actions in regulating food intake. In most teleost fishes, it is generally produced at the liver, rather than white adipose tissue in mammals. Under normal non-stress homeostatic circumstances, post-prandial elevation in leptin activity promotes energy expenditure and stimulates primary endocrine factors integral to growth control in vertebrates (Zhang *et al*., 1994; Weigle *et al*., 1997 Copeland *et al*., 2011; reviewed in Fernandez-Formoso *et al*., 2015; Gorissen & Flik, 2014; Deck *et al.*, 2017). In the tilapia model, leptin stimulates hepatic GH receptor and/or hepatic IGF1 both *in vivo* and in primary hepatocyte culture (Won *et al*., 2012; Baltzegar *et al*., 2014; Douros *et al*., 2017), suggesting the hormone may be important in the coordination of somatic growth with energy sufficiency and metabolic status. While somatotropic interactions for leptin at the liver have been

evidenced in some teleost fishes, only one study has considered a possible role for the hormone in regulating Vtgs (Paolucci *et al*., 2020), which are coexpressed at the teleost liver. Additionally, while leptin appears to have a major role in gonadotropic function, notably at central circuits, few studies have examined its regulation by gonadal sex steroids.

The endocrine stress response mobilizes energy resources in order to face elevated energetic demands associated with stressors. In most vertebrates studied to date, leptin appears to have an inhibitory effect on facets of the endocrine stress axis including corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and glucocorticoids (Heiman *et* al., 1997; Leal-Cerro *et al*., 2001). Some suggest that leptin attenuation of the endocrine stress response may be critical to reducing energy consumption during instances that energy allocation to critical biological processes may be necessary (Heiman *et al*., 1997). In teleosts, leptin increases in response to various stressors including salinity challenge (Baltzegar *et al*., 2014), hypoxia (Chu *et al*., 2010; Cao *et al*., 2011), and notably, starvation (e.g., *E. coioides* – Zhang *et* al, 2012; *O. mossambicus* – Douros *et al*., 2017; reviewed in Deck *et al*, 2017). Feed restriction results in a substantial elevation in hepatic leptin expression and circulating leptin levels in tilapia as well as other teleosts (Zhang *et* al, 2012; Douros *et al*., 2017).

Disruption to gonadotropic signaling and reproductive function is a hallmark condition of the stress response. These effects are markedly more evident in females. While increased leptin activity and disrupted reproductive function in response to stress have been reported in tilapia and other teleosts (Foo & Lam, 1993; Barcellos *et al*., 1999; Schreck *et al*., 2001), no studies to date have evaluated a possible role for interactions between leptin and gonadal sex steroids at the liver. In females, the role of the liver in metabolism, growth, and reproduction positions it as a key integrating site for a variety of endocrine signals from numerous physiological systems. As

mentioned earlier, gonadal estradiol induces hepatic vitellogenins while inhibiting growth factors (Davis *et al*., 2007, 2008). In the case of locally expressed and secreted leptin, the current vertebrate paradigm suggests, as mentioned above, that leptin increases energy expenditure towards somatic growth (Zhang *et al*., 1994; Weigle *et al*., 1997; Rosenbaum & Leibel, 1998; reviewed in Fernandez-Formoso *et al*., 2015). Leptin stimulation of hepatic IGF1 and its elevation in response to chronic stress suggests that it may play a role in the allocation of resources away from vitellogenesis and gonadal growth at the liver. Accordingly, leptin and estradiol may have major interdependent roles in the regulation of hepatic energy mobilization towards two separate competing physiological systems – growth and reproduction. However, this interaction remains to be explored.

As evidence continues to emerge on a role for leptin in the regulation of teleost appetite, reproduction, and stress, little is known on the hormone's interactions with gonadal sex steroids and its role in female reproductive physiology. Here, we investigated a possible reproductive role for leptin and its interactions with estradiol and estrogen-sensitive vitellogenins in the Mozambique tilapia *Oreochromis mossambicus*. In the current study, we evaluated changes to *in vivo* hepatic leptin and IGF1 in response to acute estradiol treatment, *in vitro* effects of gonadal sex steroids on leptin and IGF1, *in vitro* effects of leptin on hepatic vitellogenins, and measured changes to leptin expression and anatomical indices of metabolic state between gravid, mouthbrooding, and fasted female fish. This latter experiment was undertaken to ascertain a role for leptin in a reproductive behavior that coincides with changes to estradiol, vitellogenins, and self-imposed fasting (Wootton & Smith, 2014), a metabolic state during which leptin levels have been reported to increase in tilapia (Douros *et al*, 2017).

Materials and methods

Experimental animals

Adult male and female tilapia were separated by sex and housed in freshwater recirculating tank systems (salinity 0–0.5 ppt, hardness 74–84 mg/L, alkalinity 126–178 mg/L, pH 8.0) at 24–26°C with a photoperiod of 12:12 h of light and dark and fed daily (1–2% body weight/day). Unless otherwise noted. fish were fasted overnight and during the course of the experiment the following day. Fish were anesthetized in $NaHCO_3$ buffered tricaine methanesulfonate (MS-222) prior to intraperitoneal injection and decapitated before tissue removal. All animal protocols were approved by the NC State University Institutional Animal Care and Use Committee.

In vivo estradiol treatment

To test the *in vivo* effect of 17ß-estradiol (E2) on hepatic *lepa*, *igf1*, and *vtg* mRNA levels, male fish were utilized as they lack or have a lower prevailing estradiol-primed physiology relative to females. Fish were anaesthesized, weighed (75-80 g), and injected interperitoneally with either 5 µg/g BW estradiol (E_2, Sigma-Alrich, St. Louis, Missouri, USA) solubilized in sesame oil or sesame oil alone as the vehicle control (n = 8 fish/treatment). At 1- and 4-hours post-injection, fish were anaesthesized and blood was drawn by heparinized syringe. Animals were subsequently decapitated and a section of liver was collected and stored in RNAlater prior to RNA extraction and RT-qPCR.

In vitro effects of sex steroids and leptin during hepatocyte incubations

We evaluated the *in vitro* effects of sex steroids and recombinant tilapia leptin A (rtLepA) on primary tilapia hepatocytes according to previously described methods in Douros *et al*. (2014). Liver was removed from fish and diced in Hank's buffered salt solution (HBSS) containing 0.3 mg/ml type IV collagenase (Sigma-Aldrich, St. Louis, Missouri, USA). The tissue homogenate was incubated in the HBSS-collagenase solution for 30 minutes at 24°C with gentle agitation. The collagenase-digested tissue was sequentially filtered through a 260 μm and 60 μm mesh, therein yielding a filtered hepatocyte suspension. The hepatocytes were washed in Dulbecco's PBS containing 3 mM $CaCl_2$ and 1x MEM solution (Gibco, Carlsbad, California, USA) and then allowed to recover for 1-hour. Tilapia hepatocytes were then cultured in 24-well plates at a density of 1x10^6 cells/ml at 3.5x10^5 cells/cm per well in with RPMI 1640 media containing L-glutamine and 1% streptomycin/penicillin. After a 4-hour acclimation incubation at 26°C, media was removed and replaced with fresh experimental media containing either a hormone treatment or no hormone.

Primary hepatocytes cultured separately from adult male and female fish (95-100 g) were treated with 0, 1, 10, or 100 nM 17β-estradiol (E2), testosterone, or 11-ketotestosterone (11-KT), the latter reflecting the primary androgen in fishes. Cells were incubated for 4-hours at 26°C. At the termination of the culture, media was removed and cells were taken up and lysed in Tri-Reagent (Molecular Research Center, Cincinnati, OH) for subsequent extraction and determination of *lepa* and *igf1* mRNA levels.

Primary hepatocytes cultured from adult female fish were treated with 0, 1, 10, and 100 nM rtLepA. Cells were incubated for 18-hours at 26°C, and at termination, cells were taken up

and lysed in Tri-Reagent (Molecular Research Center, Cincinnati, OH) for subsequent extraction and determination of *vtgaa*, *vtgab*, *vtgc*, and *igf1* mRNA levels.

Hepatic expression profiles and anatomical indices of normally-fed gravid, fasted, and mouthbrooding females

Prior to the initiation of this experiment, establishment of a mouthbrooding female population was performed by housing ten groups of four females with one male in a recirculating tank system. Over the course of three months, a regular 30-day spawning cycle had been established for each female with approximately 16-18 days of mouthbrooding recorded immediately following spawning.

To compare hepatic expression profiles (*lepa, vtgaa, vtgab, vtgc*) and anatomical indices, normally-fed gravid, mouthbrooding, and fasted females of the same cohort were maintained in separate tanks (n = 10/group) for 16 days. All fish were fasted overnight and then sacrificed and sampled for liver, gonads, and plasma the next day. Body mass, total length, gonad mass, gonadosomatic index (GSI = [gonad mass/body mass]*100), hepatosomatic index (HSI = [liver mass/body mass]*100), and condition factor (K = [body mass/total length^3]*100) were collected for each fish. A section of liver was collected and stored in RNAlater prior to RNA extraction and RT-qPCR

RNA isolation and quantification of gene expression

Sections of liver or cell lysates were homogenized and total RNA was extracted using Trizol reagent, isopropanol precipitation, and DNase treatment. RNA purity and concentration were measured via an absorbance spectrophotometer (NanoDrop 1000, Thermo Fisher

Scientific). ~1 μg of total RNA was reverse transcribed to cDNA using random hexamers (High Capacity cDNA Synthesis Kit, Life Technologies). Non-reverse transcribed samples were used as a control for genomic contamination. Gene expression was quantified using gene specific primers (Appendix Table 1) via SYBR Green qPCR in triplicate for all cDNA samples, standards, and negative controls. All qPCR reactions were performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The qPCR cycling conditions were applied accordingly: 10 minutes at 95°C, 40 cycles of 95°C for 30 seconds, and annealing for 1 minute at 60°C for 1 minute. A terminal dissociation melt curve step was used to verify a single PCR product. Cycle threshold (Ct) values were transformed against a standard curve of serially-diluted cDNA versus Ct values, normalized to *b-actin*, and reported as fold change relative to control treatments.

Statistical Analyses

In experiments with single time-point treatment comparisons, data was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparisons. For comparisons including treatment and time, two-way ANOVA was employed followed by *a priori* Fisher's Least Significant Difference test for within-treatment comparisons and Dunnett's Multiple Comparisons for comparisons of treatments over time. Statistical significance for all analyses was denoted by an alpha of $P < 0.05$ and data values are presented as means ± SEM. All analyses were performed using SPSS Statistics 25 (IBM, Armonk, New York, USA).

Results

In vivo estradiol effect on hepatic *lepa*, *igf1*, and *vtg*

Liver *lepa* declined by almost 80% within 1 hour and remained substantially lower than controls after 4-hour exposure to estradiol (Figure 1, $P < 0.001$). Estradiol also suppressed *igf1* by almost 50%, albeit the effect was not statistically different from controls. By contrast, estradiol increased *vtgaa* and *vtgab* by 3- to 13-fold, a response that was further amplified with the longer exposure period (Figure 2, $P < 0.01$). There was no effect on *vtgc*.

Sex steroid effects on *lepa* and *igf1* in primary tilapia hepatocytes

Levels of *lepa* declined with estradiol treatment at a lowest effective dosage of 1 nM (Figure 3; $P < 0.01$). Testosterone similarly suppressed *lepa* in a dose-dependent fashion ($P < 0.01$). Treatment with 11-ketotestosterone on the other hand increased *lepa* at the highest dosage of 100 nM ($P < 0.05$). Similar to that observed for *lepa,* the same hepatocytes showed a decline in *igf1* with increasing concentrations of estradiol (Figure 3; $P < 0.01$). Testosterone and 11-ketotestosterone stimulated *igf1* at concentrations of 10 and 100 nM, respectively ($P < 0.05$).

Similar to that observed from male-derived cells, estradiol also reduced both *lepa* and *igf1* in hepatocytes derived from female fish (Figure 4; $P < 0.001$, $P < 0.01$). Testosterone also suppressed *lepa* and stimulated *igf1*, but with less efficacy than male-derived cells. Incubation with 11-ketotestosterone had no effect on *lepa*. However, it increased *igf1* at a lower effective concentration than seen with male derived hepatocytes.

Leptin effect on *vtg*s in primary female hepatocyte culture

Recombinant tilapia LepA reduced *vtgab* in a dose-dependent fashion in female hepatocytes over an 18-hour incubation (Figure 5, One-way ANOVA, $P < 0.001$). The hormone also suppressed *vtgc* ($P < 0.01$), but had little effect on *vtgaa*. By contrast, rtLepA stimulated *igf1* by 12-fold relative to controls (Figure 6, $P < 0.001$).

Anatomical indices and expression profiles of gravid, fasted, and mouthbrooding females

This study assessed differences in the anatomical and hepatic expression profiles between gravid, mouthbrooding, and fasted females. Fasted fish had a lower GSI and HSI and no significant change in condition factor compared to fed gravid females (Figure 7, $P < 0.01$). Mouthbrooding fish showed a significant decline in condition factor ($P < 0.01$) with little change in GSI or HSI compared with fed controls. Fasted females showed a 12-fold increase in hepatic *lepa* (Figure 8, $P < 0.01$) and concomitant declines in *igf1*, *vtgaa*, *vtgab*, and *vtgc* (Figures 8, 9; $P < 0.01$). Mouthbrooding females showed no significant difference in *lepa*, *igf1*, *vtgab*, or *vtgc*. However, mouthbrooders had significantly higher *vtgaa* levels compared to fed females (One-way ANOVA, $P < 0.01$).

Discussion

In the current study, we investigated the reproductive actions of leptin on estrogen-sensitive vitellogenins, the hormone's regulation by estradiol, and a possible role for the hormone in maternal mouthbrooding compared to gravid and fasted females. Here, we provide novel evidence for estradiol suppression of hepatic leptin and IGF1 expression and a contrasting effect of leptin suppression of vitellogenins. Results also suggest an apparent vacant role for

leptin during the forced starvation state that coincides with maternal mouthbrooding. Generally, we postulate that this interdependent dynamic between leptin and estradiol may be the mechanism driving energy status context-dependent competitive investment into either somatic or gonadal growth.

The present study provides the first *in vivo* evidence that estradiol suppresses hepatic leptin expression concurrent with its well-established function in inducing vitellogenins. As previously mentioned, male fish were used to simulate estradiol-induced transition from somatic growth to vitellogenic gonadal growth in a non-estradiol prevailing physiological system. Previous work by Davis *et al*. (2007, 2008) demonstrated estradiol inhibition of hepatic *igf1* while promoting vitellogenins, which is also presently reported. These studies evidenced estradiol alterations to GH sensitivity by downregulation of GH receptors. In the present study, concurrent inhibition of hepatic *lepa* and *igf1* expression suggests that leptin may act as an intermediary in regulating estradiol's suppression of GH sensitivity at the liver. We have previously demonstrated that leptin treatment promotes hepatic *igf1*, *igf2*, *ghr1*, and *ghr2* in tilapia as well as another teleost, the striped bass (Won *et al*., 2016*;* Douros *et al,* 2017). When considered together, the contrasting effects of estradiol and leptin on hepatic growth factors suggest that both hormones may be critical to the competitive interdependent dynamics between somatic and gonadal growth. Our evidence for estradiol suppression of leptin further indicates that leptin may be involved in mediating the shift from gonadal growth towards somatic growth.

The inhibitory effect of estradiol on *lepa* and *igf1* is reflected similarly in primary hepatocyte incubations. These data suggest that estradiol is likely acting directly at the liver to exert its inhibitory actions. That said, this does not preclude estradiol targeting of other upstream facets of the somatotropic axis particularly given the ubiquitous nature of estrogen receptors, as

well as the hormone's known actions at the hypothalamus and pituitary (Wehrenberg & Giustina, 1992; Borski *et al*., 1996; Roley *et al*., 2004; McCormick *et al*., 2006).

The tandem evidence provided by our *in vivo* and *in vitro* work and that of others suggests that estradiol inhibition of *igf1* and somatic growth (Borski *et* al, 1996; Riley *et al*., 2004; Davis *et al*, 2007, 2008; Norbeck & Sheridan, 2011) may be mediated, in part, through disruption of liver-localized leptin signaling. The current vertebrate paradigm for estradiol actions associated with somatic growth involve the disruption of cytokine signaling pathways. For example, estradiol is known to suppress phosphorylation of the JAK/STAT signaling pathway with some evidence for stimulation of suppressor of cytokine signaling (SOCS) family of cytokine inhibitors (Leung *et al*., 2003; Leong *et al*, 2004; Leung *et al*., 2004). In the case of leptin, estradiol disruption of the cytokine signal cascade would provide a mechanistic explanation as to why hepatic growth factors like *igf1* might also be downregulated. Overall, estradiol effects on cytokine signal transduction pathways will require further investigation.

The *in vitro* effects of testosterone and 11-ketotestosterone contrast to the presently reported inhibitory actions of estradiol on *lepa* and *igf1*. The stimulation of *igf1* in male- and female-derived hepatocytes reflect the prototypical and well-established anabolic growth-promoting effects of androgens. However, differential regulation of *lepa* might be explained by several prospective mechanisms necessitating further investigation. Here, testosterone and estradiol elicited a similar inhibitory effect on *lepa*. Generally, it is unlikely that testosterone induces the same competitive resource reallocation between somatic and gonadal growth as estradiol does in the liver with respect to vitellogenin production and subsequent oogenesis (Peter & Oommen, 1989; Hop *et al*., 1995; Habibi & Huggard, 1998). The observed *in vitro* testosterone suppression of *lepa* that occurs at a physiological relevant concentration (10 nM) in

O. mossambicus (Cornish 1998) may reflect aromatization to estradiol rather than through androgen receptor-mediated processes associated with *igf1* induction. This is supported indirectly by evidence that 11KT, a nonaromatizable and primary androgen of many fishes, had little effect on *lepa*. It is unclear whether aromatase activity is present in the liver of tilapia and additional evidence shows androgen-receptor mediated induction of vitellogenins in tilapia hepatocytes, albeit with substantially lower efficacy than estradiol (Kim *et al*., 2003; Piferrer & Blazquez, 2005). Future investigations will require evaluation of whether aromatase is locally expressed at the liver and, if so, how co-treatment of both testosterone and aromatase inhibitor might affect testosterone regulation of *lepa*.

A strong inhibitory effect of leptin on vitellogenins was found in the current study. Treatment of primary female hepatocytes with leptin resulted in a downregulation of *vtgab*, the dominant vitellogenin, and *vtgc* (Reading *et al*., 2011; Hara *et al*, 2016; Sullivan *et al*, 2018). This observed effect indicates that leptin may act directly at the liver to disrupt a fundamental component of female reproduction, vitellogenesis and oocyte growth (Reading *et al*., 2011). In humans and rodent models, elevated cytokine signaling (including leptin and interleukins) during abnormal metabolic states is associated with declines in oocyte growth and ovarian function (Ben-Rafael & Orvieto, 1992). When considered in the context of the teleost stress response, chronic stress typically coincides with major disruptions to gonadotropic signaling and gonadal growth. A substantial body of evidence demonstrates that fasting induces leptin synthesis and/or secretion in teleosts, including the tilapia (e.g., *E. coioides* – Zhang *et* al, 2012; *O. mossambicus* – Douros *et al*., 2017; reviewed by Deck *et al*., 2017). Hence, it is possible that under fasted conditions, vitellogenesis and ovarian growth may be suppressed, in part, through direct leptin action in the liver of females. While the direct effects of leptin on hepatic vitellogenins are clear

from the present study, we postulate that leptin may also disrupt estrogen signaling at the liver by downregulating estrogen receptors. Another possible mechanism is that this disruption of the female reproductive axis may occur through reduction of gonadal estradiol secretion via direct actions at the ovaries. We show that leptin markedly suppresses testicular steroid production in tilapia (Chapter 3) and evidence demonstrates leptin inhibition of gonadal sex steroids in mammals (Tena-Sempere *et al*., 1999). Overall, the present study provides support for an inhibitory effect of leptin on vitellogenesis. However, further investigation into the hormone's *in vivo* actions on female physiology is necessary.

In addition to evaluating leptin's regulation of hepatic vitellogenins, we sought to determine if the hormone might also be involved in maternal mouthbrooding, an energetically-costly reproductive behavior that involves a cessation in appetite and stimulation of vitellogenic processes (Hildemann 1959; Chong *et al*., 2005; Reading *et al*., 2011). Here, we provide the first description of the differential leptin responses to fed-gravid state, mouthbrooding, and fasted state. Some studies describing neuropeptide, hormonal, and anatomical profiles associated with mouthbrooding have used fasted animals in order to emulate the feed-restricted state associated with mouthbrooding (e.g., Mrowka & Schierwater, 1988; Smith & Wootton, 1994). However, there is substantial evidence that central properties including neuronal and neuropeptide profiles of mouthbrooding females across several teleost species studied to date are distinct compared to that of fasted females (Tacon *et al*., 2000; Grone *et al*, 2012; Das *et al*., 2019). Here, we provide evidence based on leptin – a major anorectic hormone – that mouthbrooding and the fasted state harbor different endocrine responses.

In fasted females, typical starvation effects were observed. Hepatic expression of vitellogenins and *igf1* were suppressed, while anatomical indices including GSI and HSI

declined as the fish continued to exhaust their energetic resources in the face of the metabolic challenge. Generally, these physiological responses were expected and have been well-documented in teleosts studied to date (reviewed in Tyler & Sumpter, 1996; Tocher 2003; Chatzifotis *et al*., 2011). When considering the leptin response, fasted females showed a sharp increase in hepatic *lepa*, corroborating our previous findings in feed-restricted male tilapia (Douros *et al*, 2017). Given the decline in GSI and depressed *vtg* levels, it is possible that in fasted animals leptin may be exerting its inhibitory effects on vitellogenins as demonstrated in hepatocyte incubations.

The hepatic expression profile of mouthbrooding females was near identical to that of fed-gravid females. Here, the absence of a leptin response alongside the concurrent stoppage in feed intake in mouthbrooding females presents an inherent confound as to the overall actions and role of the hormone in this particular reproductive context. Maternal mouthbrooding coincides with a sustained metabolic challenge (i.e., fasting) and appetite suppression (Mrowka 1984). Here, the lack of a leptin response suggests that other factors are likely exerting appetite-suppressing effects, as well as potential catabolic actions to meet the energetic demand associated with the fasted state.

In a variety of mouthbrooding fishes, it is understood that feed intake is extinguished for the duration of the mouthbrooding phase (Mrowka 1984; Verheyen *et al*., 2012). However, the fasted state incurred by this parental behavior does not seem to involve physiological responses typically attributed to starvation. While fasted females demonstrate elevated leptin activity, disrupted gonadotropic signaling, and gonadal atrophy, mouthbrooding females here show similar leptin profiles to fed-gravid fish, as well as sustained gonadal investment and

vitellogenesis. Hence, we postulate that these distinct physiological differences might be regulated by estradiol.

Sustained estradiol signaling is a well-established characteristic of maternal mouthbrooders. In the case of repeat spawning mouthbrooding species like cichlids, estradiol-induced vitellogenesis throughout mouthbrooding allows for investment into the sequential clutch (Smith & Haley, 1988). Vitellogenins are also important for the rearing of young. In tilapia, as in several other cichlid species with maternal care (e.g., discus *Symphysodon aequifasciata*: Hildemann 1959; Chong *et al*., 2005), estradiol-induced vitellogenins are secreted into the oral cavity and through the epidermis to feed brooding, free-swimming young (Kishida & Specker, 1994; Iq & Shu-Chien, 2011). In the current study, we report representative evidence of sustained estradiol signaling during mouthbrooding insofar as *vtg* levels remain elevated or increase. Notably, all three vitellogenin transcripts reflected those of fed-gravid females with a significant increase in *vtgaa*. The *vtgaa* response may imply a potential role for that particular paralog in mucosal feeding mechanism. Alternatively, it may simply be reflective of the oocyte vitellogenic growth phase that the animals were in at that point in time. Beyond its classically reported stimulation of vitellogenins, it is possible that estradiol may also be implicated in the regulation of appetite in mouthbrooding females.

In mammals, estradiol is known to exert anorexigenic actions at the arcuate nucleus (ARC) by targeting POMC neurons (reviewed in Lopez & Tena-Sempere, 2017). Generally, the appetite-regulatory effects of estradiol in fishes are not consistent (Malison *et al*, 1988; Woo *et al*., 1993; Wang *et al*., 2008). However, recent work on the cichlid, *Astatotilapia burtoni*, indicates that hypothalamic POMC neurons differ between fed-gravid and mouthbrooding females (Porter *et al*, 2017). Notably, anorectic POMC neurons are larger in mouthbrooding

females than fed-gravid females. However, other studies demonstrate that POMC transcript levels do not typically differ between fed-gravid and mouthbrooding females (Brone *et al*., 2012; reviewed in Volkoff 2016). Overall, it is possible that estradiol is regulating the anorexigenic melanocortin signaling system during mouthbrooding through modulation of POMC, its precursors, or melanocortin receptors. When considered with evidence from the current study, we postulate that sustained estradiol levels in mouthbrooding females, which has been previously documented in tilapia (Smith & Haley, 1988), may be acting at multiple levels to exert its reproductive and metabolic actions: at the hypothalamus, suppression of appetite via interfacing with POMC neurons and at the liver, stimulation of vitellogenins.

In summary, the present investigations demonstrate estradiol inhibition of *lepa* and *igf1*, leptin suppression of *vtg*s and promotion of *igf1*, and contrasting leptin dynamics between gravid, fasted, and mouthbrooding females and collectively suggest an interdependent oppositional relationship between leptin and estradiol. Overall, the evidence from the present study contributes to the current framework of understanding on the endocrine mechanisms that may drive stress-related suppression of gonadal growth, as well as possible mechanisms underlying sexually dimorphic growth in normal non-stress contexts. We postulate that estradiol may enhance vitellogenesis and promote reproductive growth through antagonization of leptin activity, while leptin may contribute to sustaining somatic growth through inhibition of estrogen-sensitive vitellogenins and stimulation of IGFs. Overall, the current study provides novel evidence for interactions between leptin and estradiol and suggests an inter-regulatory role for both hormones in the reproductive biology of teleost fishes.

Final remarks: Comparative perspectives on the reproductive actions of leptin

Within the paradigm for leptin biology, the current study contributes to the evolving understanding of leptin's role in the regulation of reproductive function in vertebrates. While it is tempting to assert that leptin is functionally divergent in teleosts due to its contrary action of increased expression and secretion in response to starvation rather than decreasing as is observed in mammals, leptin action in metabolically abnormal contexts is essentially identical across vertebrate classes studied to date. In mammals, leptin is expressed and secreted in proportion to white adipose tissue content in order to maintain adipostasis. In obese humans and rodents, persistently high leptin levels lead to leptin resistance and dysfunction of metabolic and reproductive systems including development of hypogonadotropic hypogonadism pathologies (Ahima *et al*., 1997; Yu *et al*., 1997; Chou & Mantzoros, 2014). Further, chronic low-grade inflammation, likely attributed to increased cytokine signaling and glucocorticoid activity are hallmarks of obesity (Shoelson *et al*., 2007; Estep *et al*., 2009). When the properties of obesity in mammals are compared to what is currently understood of the teleostean leptin response to metabolic abnormalities, many of these features are essentially identical. Metabolic challenge induced by feed restriction (rather than obesity and overfeeding) results in elevated leptin activity (Douros *et al*., 2017; reviewed in Deck *et al*., 2017) and disruptions to reproductive function. In the preceding and current studies, we provide evidence for leptin decrease of circulating sex steroid levels and potential disruption of ovarian growth through downregulation of hepatic vitellogenins. This disruption to the reproductive axis reflects similar properties to mammalian hypogonadotropic hypogonadism observed in obese individuals (Figure 10).

While inhibitory hyperleptinemic effects in both teleosts and mammals may appear to be fundamentally identical within the semantic lens of "metabolic abnormality", as well as the

context of chronic stress from which leptin is incontrovertibly linked regardless of taxonomic distinction, mammals and teleosts differ at one crucial juncture. In mammals, chronically elevated leptin levels occur during overnutrition and obesity while in teleosts, this happens during starvation (and other stressors). Discourse on the biological dynamics underlying this particular item sit far beyond the scope of this dissertation. However, we postulate that at some point in the course of vertebrate evolution, the divergence between poikilothermic ectotherms and endotherms may be attributed to some of the observed differences between mammalian and teleost leptin. With the emergence of endothermy and the need for constant nutritional intake to maintain homeostatic body temperature, mammalian leptin may have undergone neofunctionalization while retaining the hormone's core and 'original' functions that we continue to observe in teleosts now.

REFERENCES

Baltzegar, D. A., Reading, B. J., Douros, J. D., & Borski, R. J. (2014). Role for leptin in promoting glucose mobilization during acute hyperosmotic stress in teleost fishes. *J Endocrinol*, *220*(1), 61-72.

Barcellos, L. J. G., Nicolaiewsky, S. S. M. G., De Souza, S. M. G., & Lulhier, F. (1999). Plasmatic levels of cortisol in the response to acute stress in Nile tilapia, Oreochromis niloticus (L.), previously exposed to chronic stress. *Aquaculture Research*, *30*(6), 437-444.

Bartke, A., Cecim, M., Tang, K., Steger, R. W., Chandrashekar, V., & Turyn, D. (1994). Neuroendocrine and reproductive consequences of overexpression of growth hormone in transgenic mice. *Proceedings of the Society for Experimental Biology and Medicine*, *206*(4), 345-359.

Ben-Rafael, Z., & Orvieto, R. (1992). Cytokines—involvement in reproduction. *Fertility and sterility*, *58*(6), 1093-1099.

Borski, R. J., Tsai, W.., DeMott-Friberg, R., & Barkan, A. L. (1996). Regulation of somatic growth and the somatotropic axis by gonadal steroids: primary effect on insulin-like growth factor I gene expression and secretion. *Endocrinology*, *137*(8), 3253-3259.

Chatzifotis, S., Papadaki, M., Despoti, S., Roufidou, C., & Antonopoulou, E. (2011). Effect of starvation and re-feeding on reproductive indices, body weight, plasma metabolites and oxidative enzymes of sea bass (Dicentrarchus labrax). *Aquaculture*, *316*(1-4), 53-59.

Chen, J., Cao, M., Zhang, A., Shi, M., Tao, B., Li, Y., & Hu, W. (2018). growth hormone Overexpression Disrupts reproductive status Through actions on leptin. *Frontiers in endocrinology*, *9*, 131.

Chong, K., Ying, T. S., Foo, J., Jin, L. T., & Chong, A. (2005). Characterisation of proteins in epidermal mucus of discus fish (Symphysodon spp.) during parental phase. *Aquaculture*, *249*(1-4), 469-476.

Chou, S. H., & Mantzoros, C. (2014). Role of leptin in human reproductive disorders. *J. Endocrinol*, *223*(1), T49-T62.

Copeland, D. L., Duff, R. J., Liu, Q., Prokop, J., & Londraville, R. L. (2011). Leptin in teleost fishes: an argument for comparative study. *Frontiers in physiology*, *2*, 26.

Cornish, D. A. (1998). Seasonal steroid hormone profiles in plasma and gonads of the tilapia, Oreochromis mossambicus. *WATER SA-PRETORIA-*, *24*, 257-264.

Das, K., Ogawa, S., Kitahashi, T., & Parhar, I. S. (2019). Expression of neuropeptide Y and gonadotropin-releasing hormone gene types in the brain of female Nile tilapia (Oreochromis niloticus) during mouthbrooding and food restriction. *Peptides*, *112*, 67-77.

Davis, L. K., Hiramatsu, N., Hiramatsu, K., Reading, B. J., Matsubara, T., Hara, A., & Grau, E. G. (2007). Induction of three vitellogenins by 17beta-estradiol with concurrent inhibition of the growth hormone-insulin-like growth factor 1 axis in a euryhaline teleost, the tilapia (Oreochromis mossambicus). *Biology of Reproduction*, *77*(4), 614-625.

Davis, L. K., Pierce, A. L., Hiramatsu, N., Sullivan, C. V., Hirano, T., & Grau, E. G. (2008). Gender-specific expression of multiple estrogen receptors, growth hormone receptors, insulin-like growth factors and vitellogenins, and effects of 17β-estradiol in the male tilapia (Oreochromis mossambicus). *General and Comparative Endocrinology*, *156*(3), 544-551.

Deck, C. A., Honeycutt, J. L., Cheung, E., Reynolds, H. M., & Borski, R. J. (2017). Assessing the functional role of leptin in energy homeostasis and the stress response in vertebrates. *Frontiers in endocrinology*, *8*, 63.

Douros, J. D., Baltzegar, D. A., Mankiewicz, J., Taylor, J., Yamaguchi, Y., Lerner, D. T., & Borski, R. J. (2017). Control of leptin by metabolic state and its regulatory interactions with pituitary growth hormone and hepatic growth hormone receptors and insulin like growth factors in the tilapia (Oreochromis mossambicus). *General and comparative endocrinology*, *240*, 227-237.

Estep, J. M., Baranova, A., Hossain, N., Elariny, H., Ankrah, K., Afendy, A., & Younossi, Z. M. (2009). Expression of cytokine signaling genes in morbidly obese patients with non-alcoholic steatohepatitis and hepatic fibrosis. *Obesity surgery*, *19*(5), 617-624.

Fernández-Formoso, G., Pérez-Sieira, S., González-Touceda, D., Dieguez, C., & Tovar, S. (2015). Leptin, 20 years of searching for glucose homeostasis. *Life sciences*, *140*, 4-9.

Fontana, R., & Torre, S. D. (2016). The deep correlation between energy metabolism and reproduction: A view on the effects of nutrition for women fertility. *Nutrients*, *8*(2), 87.

Foo, J. T. W., & Lam, T. J. (1993). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, Oreochromis mossambicus. *Aquaculture*, *115*(1-2), 145-158.

Gorissen, M., & Flik, G. (2014). Leptin in teleostean fish, towards the origins of leptin physiology. *Journal of chemical neuroanatomy*, *61*, 200-206.

Grone, B. P., Carpenter, R. E., Lee, M., Maruska, K. P., & Fernald, R. D. (2012). Food deprivation explains effects of mouthbrooding on ovaries and steroid hormones, but not brain neuropeptide and receptor mRNAs, in an African cichlid fish. *Hormones and behavior*, *62*(1), 18-26.

Habibi, H. R., & Huggard, D. L. (1998). Testosterone regulation of gonadotropin production in goldfish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *119*(3), 339-344.

Heiman, M. L., Ahima, R. S., Craft, L. S., Schoner, B., Stephens, T. W., & Flier, J. S. (1997). Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology*, *138*(9), 3859-3863.

Hildemann, W. H. (1959). A cichlid fish, Symphysodon discus, with unique nurture habits. *The American Naturalist*, *93*(868), 27-34.

Hop, H., Trudeau, V. L., & Graham, M. (1995). Spawning energetics of Arctic cod (Boreogadus saida) in relation to seasonal development of the ovary and plasma sex steroid levels. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(3), 541-550.

Hull, K. L., & Harvey, S. (2000). Growth hormone: a reproductive endocrine-paracrine regulator?. *Reviews of Reproduction*, *5*(3), 175-182.

Imsland, A. K., Folkvord, A., Grung, G. L., Stefansson, S. O., & Taranger, G. L. (1997). Sexual dimorphism in growth and maturation of turbot, Scophthalmus maximus (Rafinesque, 1810). *Aquaculture Research*, *28*(2), 101-114.

Iq, K. C., & Shu-Chien, A. C. (2011). Proteomics of buccal cavity mucus in female tilapia fish (Oreochromis spp.): a comparison between parental and non-parental fish. *PLoS One*, *6*(4), e18555.

Izquierdo, A. G., Crujeiras, A. B., Casanueva, F. F., & Carreira, M. C. (2019). Leptin, Obesity, and Leptin Resistance: Where Are We 25 Years Later?. *Nutrients*, *11*(11), 2704.

Kim, B. H., Takemura, A., Kim, S. J., & Lee, Y. D. (2003). Vitellogenin synthesis via androgens in primary cultures of tilapia hepatocytes. *General and comparative endocrinology*, *132*(2), 248-255.

Kishida, M., & Specker, J. L. (1994). Vitellogenin in the surface mucus of tilapia (Oreochromis mossambicus): Possibility for uptake by the free-swimming embryos. *Journal of Experimental Zoology*, *268*(4), 259-268.

Knight, Z. A., Hannan, K. S., Greenberg, M. L., & Friedman, J. M. (2010). Hyperleptinemia is required for the development of leptin resistance. *PloS one*, *5*(6).

Kozłowski, J., & Wiegert, R. G. (1986). Optimal allocation of energy to growth and reproduction. *Theoretical Population Biology*, *29*(1), 16-37.

Kuzawa, C. W. (2007). Developmental origins of life history: growth, productivity, and reproduction. *American Journal of Human Biology*, *19*(5), 654-661.

Lardner, B., & Loman, J. (2003). Growth or reproduction? Resource allocation by female frogs Rana temporaria. *Oecologia*, *137*(4), 541-546.

Leal-Cerro, A., Soto, A., Martínez, M. A., Dieguez, C., & Casanueva, F. F. (2001). Influence of cortisol status on leptin secretion. *Pituitary*, *4*(1-2), 111-116.

Leong, G. M., Moverare, S., Brce, J., Doyle, N., Sjögren, K., Dahlman-Wright, K., & Leung, K. C. (2004). Estrogen up-regulates hepatic expression of suppressors of cytokine signaling-2 and-3 in vivo and in vitro. *Endocrinology*, *145*(12), 5525-5531.

Leung, K. C., Doyle, N., Ballesteros, M., Sjogren, K., Watts, C. K. W., Low, T. H., & Ho, K. K. Y. (2003). Estrogen inhibits GH signaling by suppressing GH-induced JAK2 phosphorylation, an effect mediated by SOCS-2. *Proceedings of the National Academy of Sciences*, *100*(3), 1016-1021.

Leung, K. C., Johannsson, G., Leong, G. M., & Ho, K. K. (2004). Estrogen regulation of growth hormone action. *Endocrine reviews*, *25*(5), 693-721.

López, M., & Tena-Sempere, M. (2017). Estradiol effects on hypothalamic AMPK and BAT thermogenesis: a gateway for obesity treatment?. *Pharmacology & therapeutics*, *178*, 109-122.

Ma, W., Wu, J., Zhang, J., He, Y., Gui, J., & Mei, J. (2016). Sex differences in the expression of GH/IGF axis genes underlie sexual size dimorphism in the yellow catfish (Pelteobagrus fulvidraco). *Science China. Life Sciences*, *59*(4), 431.

Malison, J. A., Kayes, T. B., Wentworth, B. C., & Amundson, C. H. (1988). Growth and feeding responses of male versus female yellow perch (Perca flavescens) treated with estradiol-17β. *Canadian Journal of Fisheries and Aquatic Sciences*, *45*(11), 1942-1948.

McCormick, S. D., O'Dea, M. F., Moeckel, A. M., Lerner, D. T., & Björnsson, B. T. (2005). Endocrine disruption of parr-smolt transformation and seawater tolerance of Atlantic salmon by 4-nonylphenol and 17β-estradiol. *General and comparative endocrinology*, *142*(3), 280-288.

Mrowka, W., & Schierwater, B. (1988). Energy expenditure for mouthbrooding in a cichlid fish. *Behavioral Ecology and Sociobiology*, *22*(3), 161-164.

Norbeck, L. A., & Sheridan, M. A. (2011). An in vitro model for evaluating peripheral regulation of growth in fish: effects of 17β-estradiol and testosterone on the expression of growth hormone receptors, insulin-like growth factors, and insulin-like growth factor type 1 receptors in rainbow trout (Oncorhynchus mykiss). *General and comparative endocrinology*, *173*(2), 270-280.

Paolucci, M., Coccia, E., Imperatore, R., & Varricchio, E. (2020). A cross-talk between leptin and 17β-estradiol in vitellogenin synthesis in rainbow trout Oncorhynchus mykiss liver. *Fish Physiology and Biochemistry*, *46*(1), 331-344.

Parent, A. S., Teilmann, G., Juul, A., Skakkebaek, N. E., Toppari, J., & Bourguignon, J. P. (2003). The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. *Endocrine reviews*, *24*(5), 668-693.

Peter, M. S., & Oommen, O. V. (1989). Oxidative metabolism in a teleost, Anabas testudineus Bloch: effect of testosterone and estradiol-17β on hepatic enzyme activities. *Fish physiology and biochemistry*, *6*(6), 377-385.

Piferrer, F., & Blázquez, M. (2005). Aromatase distribution and regulation in fish. *Fish physiology and biochemistry*, *31*(2-3), 215.

Porter, D. T., Roberts, D. A., & Maruska, K. P. (2017). Distribution and female reproductive state differences in orexigenic and anorexigenic neurons in the brain of the mouth brooding African cichlid fish, Astatotilapia burtoni. *Journal of Comparative Neurology*, *525*(14), 3126-3157.

Rljnsdorp, A. D., & Ibelings, B. (1989). Sexual dimorphism in the energetics of reproduction and growth of North Sea plaice, Pleuronectes platessa L. *Journal of Fish Biology*, *35*(3), 401-415.

Rosenbaum, M., & Leibel, R. L. (1998). Leptin: a molecule integrating somatic energy stores, energy expenditure and fertility. *Trends in Endocrinology & Metabolism*, *9*(3), 117-124.

Rottenberg, M. E., & Carow, B. (2014). SOCS3, a major regulator of infection and inflammation. *Frontiers in immunology*, *5*, 58.

Sanchez-Garrido, M. A., & Tena-Sempere, M. (2013). Metabolic control of puberty: roles of leptin and kisspeptins. *Hormones and behavior*, *64*(2), 187-194.

Schreck, C. B., Contreras-Sanchez, W., & Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. In *Reproductive Biotechnology in Finfish Aquaculture* (pp. 3-24). Elsevier.

Shoelson, S. E., Herrero, L., & Naaz, A. (2007). Obesity, inflammation, and insulin resistance. *Gastroenterology*, *132*(6), 2169-2180.

Smith, C. J., & Haley, S. R. (1988). Steroid profiles of the female tilapia, Oreochromis mossambicus, and correlation with oocyte growth and mouthbrooding behavior. *General and comparative endocrinology*, *69*(1), 88-98.

Smith, C., & Wootton, R. J. (1994). The cost of parental care inHaplochromis ‘argens’(Cichlidae). *Environmental Biology of Fishes*, *40*(1), 99-104.

Tacon, P., Baroiller, J. F., Le Bail, P. Y., Prunet, P., & Jalabert, B. (2000). Effect of egg deprivation on sex steroids, gonadotropin, prolactin, and growth hormone profiles during the reproductive cycle of the mouthbrooding cichlid fish Oreochromis niloticus. *General and comparative endocrinology*, *117*(1), 54-65.

Tena-Sempere, M., Manna, P. R., Zhang, F. P., Pinilla, L., Gonzalez, L. C., Dieguez, C., & Aguilar, E. (2001). Molecular mechanisms of leptin action in adult rat testis: potential targets for

leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *Journal of Endocrinology*, *170*(2), 413-424.

Tena-Sempere, M., Pinilla, L., Gonzalez, L. C., Dieguez, C., Casanueva, F. F., & Aguilar, E. (1999). Leptin inhibits testosterone secretion from adult rat testis in vitro. *Journal of Endocrinology*, *161*, 211-218.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in fisheries science*, *11*(2), 107-184.

Tyler, C. R., & Sumpter, J. P. (1996). Oocyte growth and development in teleosts. *Reviews in fish biology and fisheries*, *6*(3), 287-318.

Verheyen, E., De Kegel, B., Helsen, P., & Adriaens, D. (2012). Dealing with food and eggs in mouthbrooding cichlids: structural and functional trade-offs in fitness related traits. *PLoS One*, *7*(2).

Vieira, M. N., Lyra e Silva, N. M., Ferreira, S. T., & De Felice, F. G. (2017). Protein tyrosine phosphatase 1B (PTP1B): a potential target for Alzheimer's therapy?. *Frontiers in aging neuroscience*, *9*, 7.

Volkoff, H. (2016). The neuroendocrine regulation of food intake in fish: a review of current knowledge. *Frontiers in neuroscience*, *10*, 540.

Von Schnurbein, J., Moss, A., Nagel, S. A., Muehleder, H., Debatin, K. M., Farooqi, I. S., & Wabitsch, M. (2012). Leptin substitution results in the induction of menstrual cycles in an adolescent with leptin deficiency and hypogonadotropic hypogonadism. *Hormone research in paediatrics*, *77*(2), 127-133.

Wang, H. P., Gao, Z., Beres, B., Ottobre, J., Wallat, G., Tiu, L., & Yao, H. (2008). Effects of estradiol-17β on survival, growth performance, sex reversal and gonadal structure of bluegill sunfish Lepomis macrochirus. *Aquaculture*, *285*(1-4), 216-223.

Wehrenberg, W. B., & Giustina, A. (1992). Basic counterpoint: mechanisms and pathways of gonadal steroid modulation of growth hormone secretion. *Endocrine Reviews*, *13*(2), 299-308.

Weigle, D. S., Duell, P. B., Connor, W. E., Steiner, R. A., Soules, M. R., & Kuijper, J. L. (1997). Effect of fasting, refeeding, and dietary fat restriction on plasma leptin levels. *The Journal of Clinical Endocrinology & Metabolism*, *82*(2), 561-565.

Won, E. T., Baltzegar, D. A., Picha, M. E., & Borski, R. J. (2012). Cloning and characterization of leptin in a Perciform fish, the striped bass (Morone saxatilis): control of feeding and regulation by nutritional state. *General and comparative endocrinology*, *178*(1), 98-107.

Woo, N. Y. S., Chung, A. S. B., & Ng, T. B. (1993). Influence of oral administration of estradiol-17β and testosterone on growth, digestion, food conversion and metabolism in the

underyearling red sea bream, Chrysophrys major. *Fish Physiology and Biochemistry*, *10*(5), 377-387.

Zhang, Y., & Scarpace, P. J. (2006). The role of leptin in leptin resistance and obesity. *Physiology & behavior*, *88*(3), 249-256.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*, *372*(6505), 425-432.

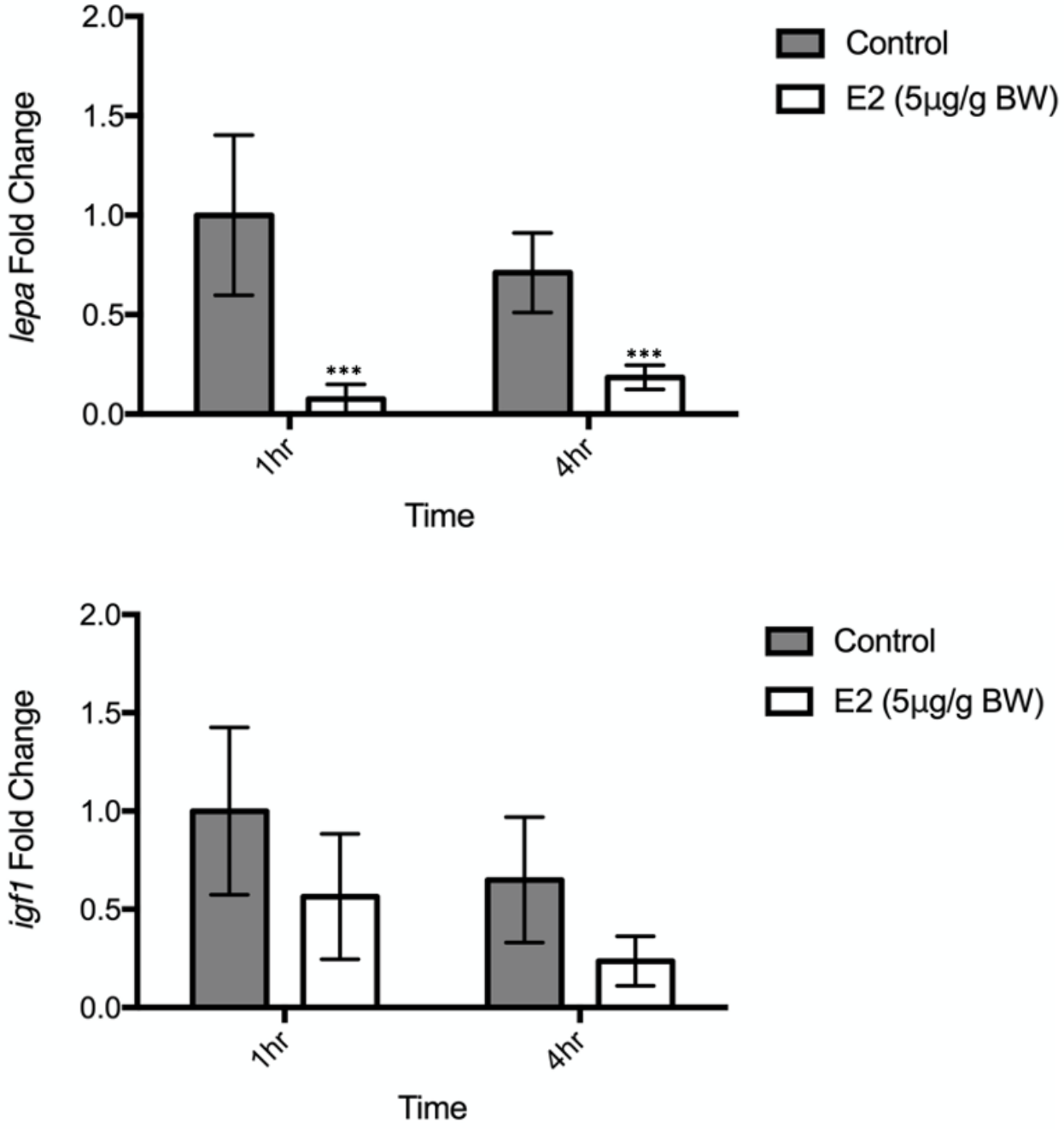

Figure 1. Effect of acute estradiol exposure on *lepa* and *igf1* mRNA levels (as fold change from control at 1 hr) following bolus injection at 1- and 4-hours. Values reported as means ± SEM. * denotes significant differences between treatments at each time point (n = 8/treatment; *** $P < 0.001$).

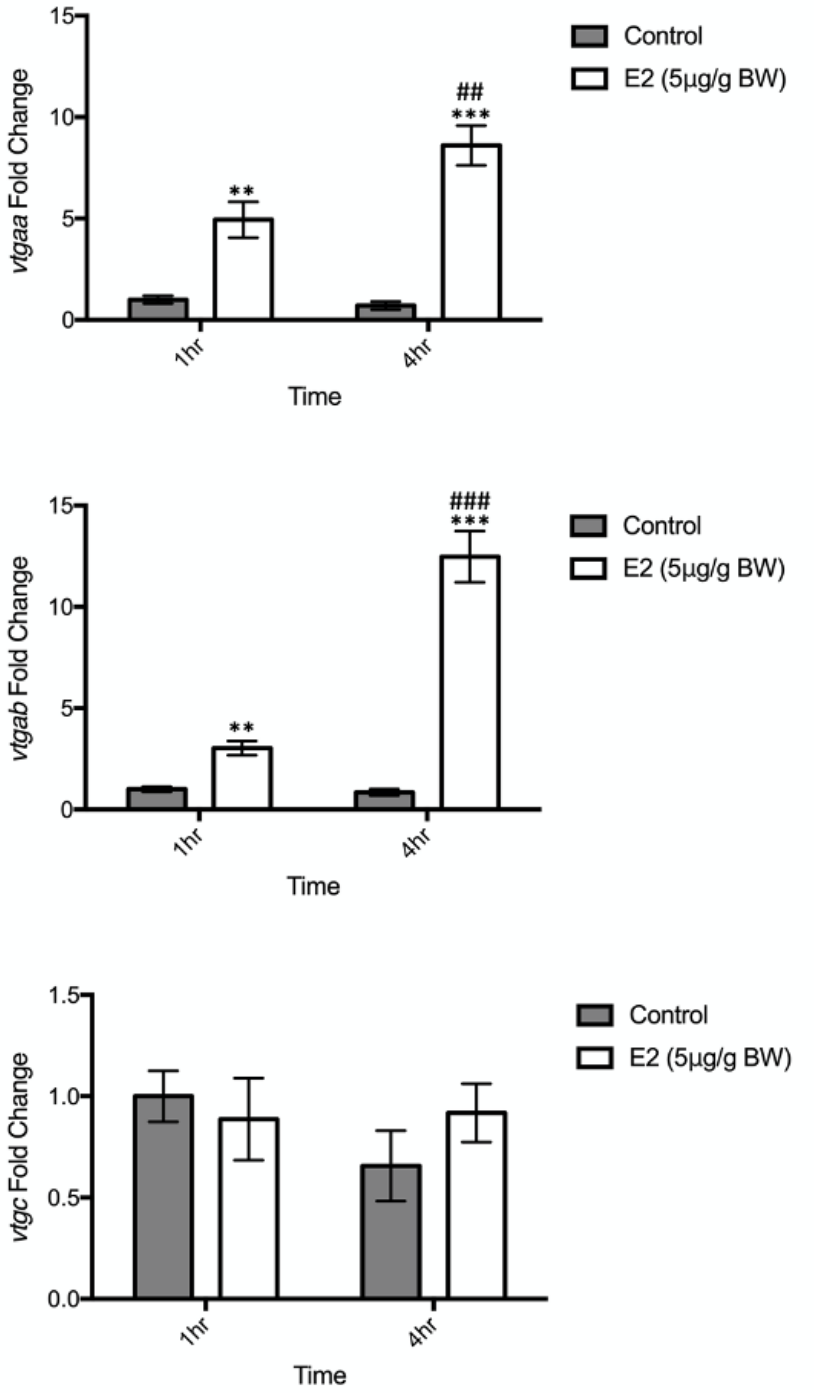

Figure 2. Effect of acute estradiol injection on *vtgaa, vtgab,* and *vtgc* mRNA levels (as fold change from control at 1 hr) at 1- and 4-hours following bolus injection. Values reported as means ± SEM. * denotes significant differences between treatments at each time point, while # denotes significant differences within treatments over time (n = 8/treatment; ** ## $P < 0.01$, *** ### $P < 0.001$).

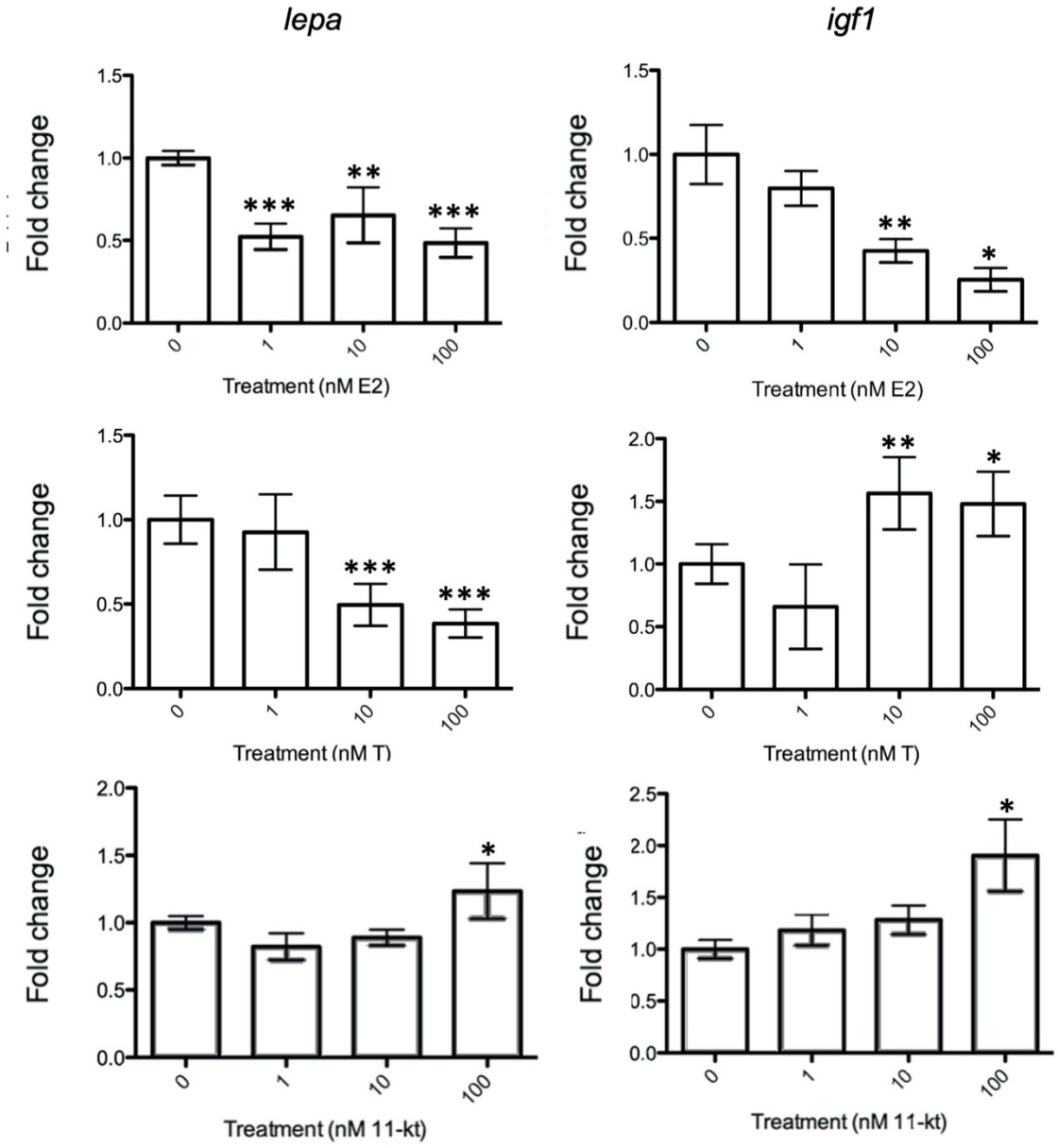

Figure 3. Effects of *in vitro* sex steroid treatment in primary male hepatocytes for 4-hours on *lepa* (left) and *igf1* (right) mRNA levels (shown as fold change from 0 nM treatment). Values reported as means ± SEM. * denotes significant difference from 0 nM treatment. (n = 6/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

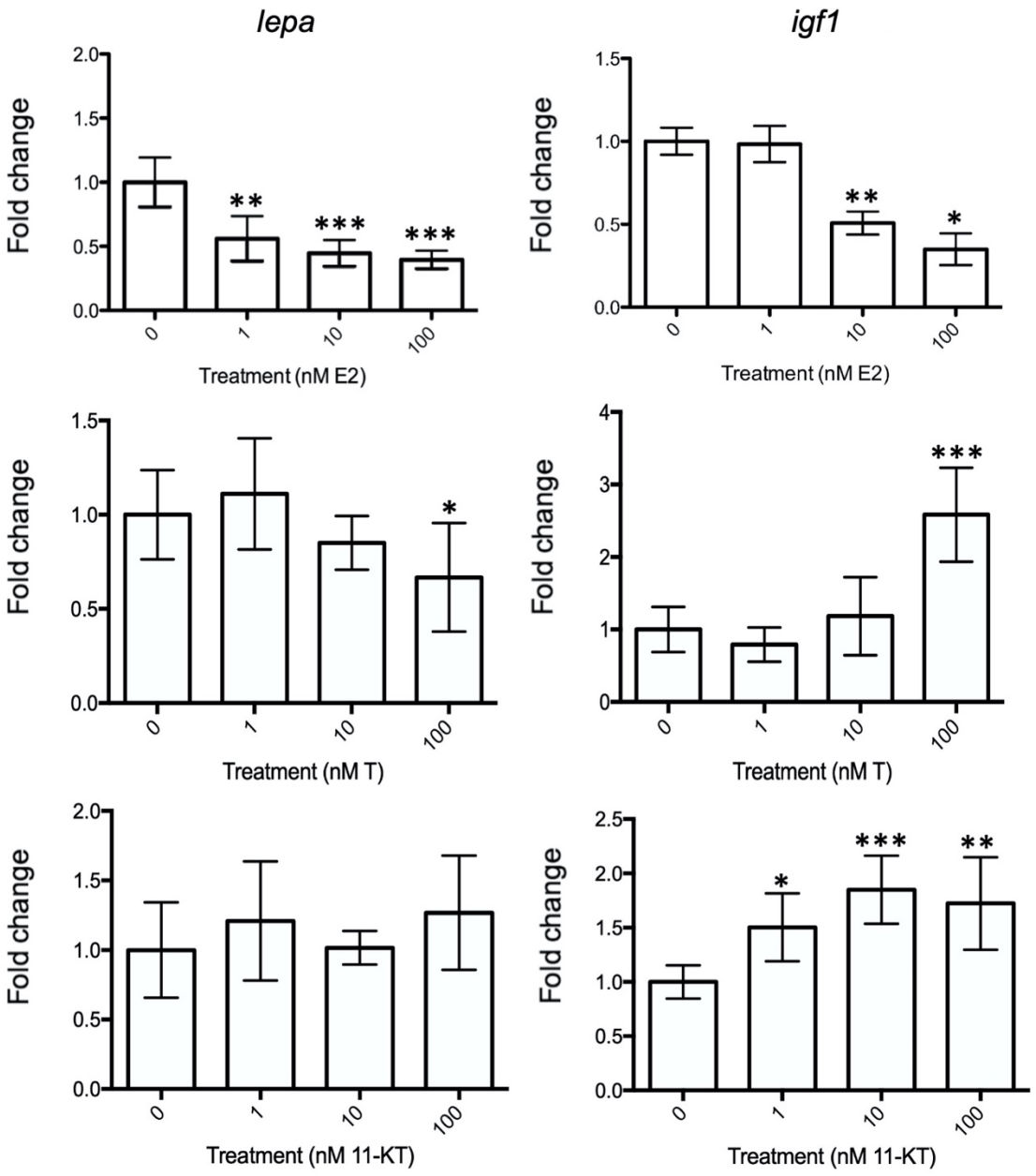

Figure 4. Effects of *in vitro* sex steroid treatment in primary female hepatocytes for 4-hours on *lepa* (left) and *igf1* (right) mRNA levels (shown as fold change from 0 nM treatment). Values reported as means ± SEM. * denotes significant difference from 0 nM treatment. (n = 6/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

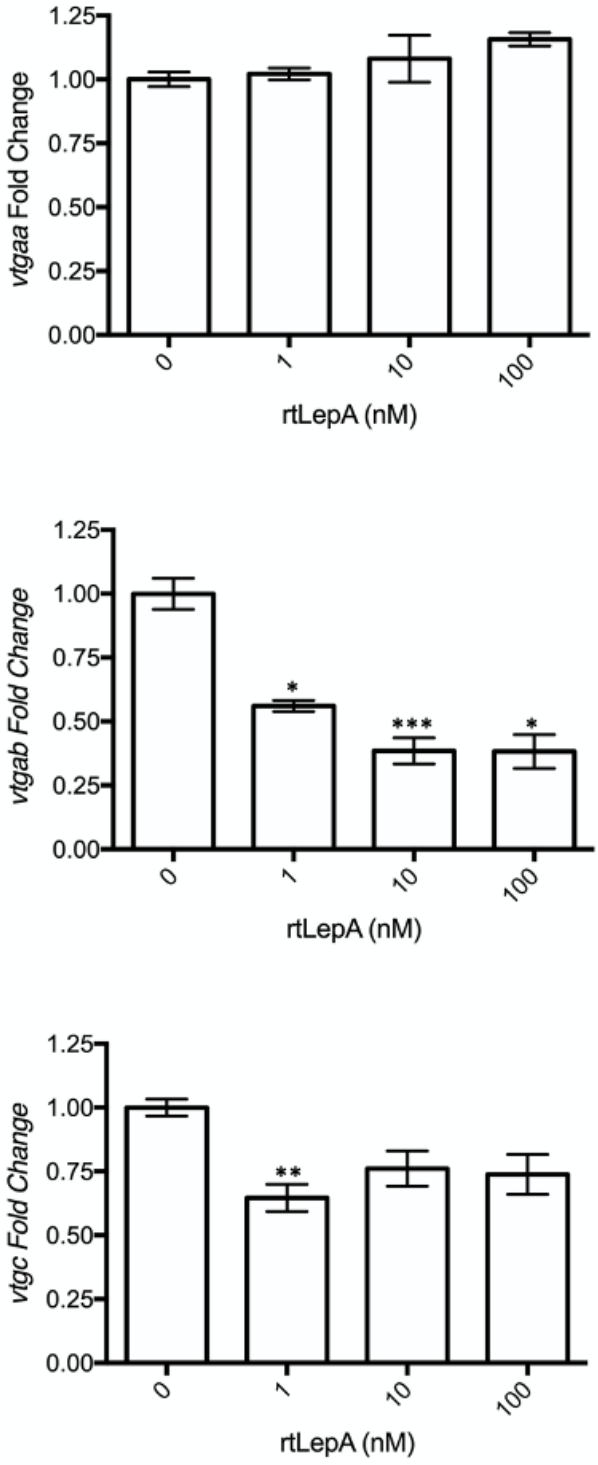

Figure 5. Effect of *in vitro* rtLepA treatment in primary female hepatocytes for 18-hours on *vtgaa, vtgab,* and *vtgc* mRNA levels (shown as fold change from 0 nM treatment). Values reported as means ± SEM. * denotes significant difference from 0 nM treatment. (n = 6/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

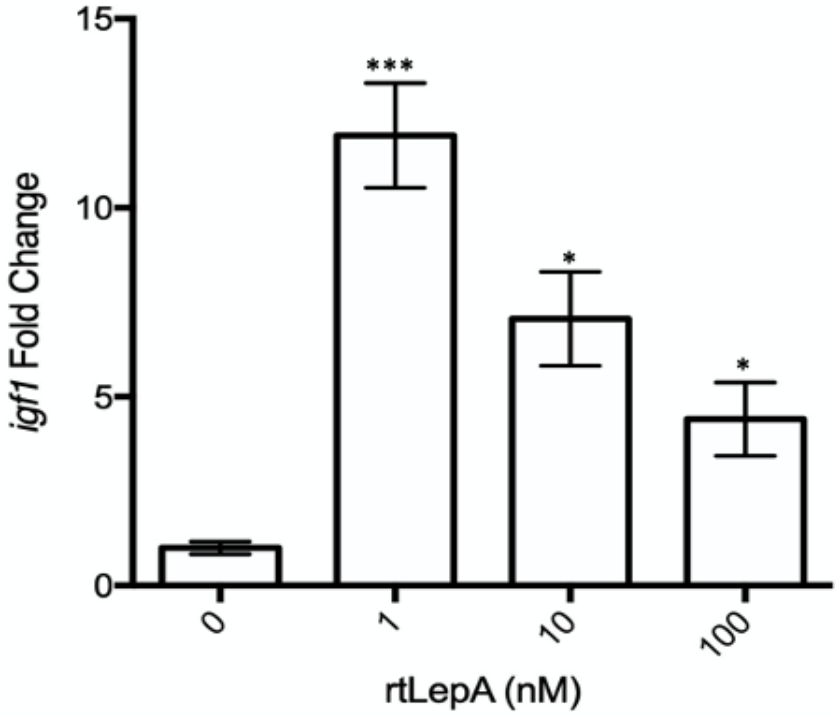

Figure 6. Effect of *in vitro* rtLepA treatment in primary female hepatocytes for 18-hours on *igf1* mRNA levels (shown as fold change from 0 nM treatment). Values reported as means ± SEM. * denotes significant difference from 0 nM treatment. (n = 6/treatment; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

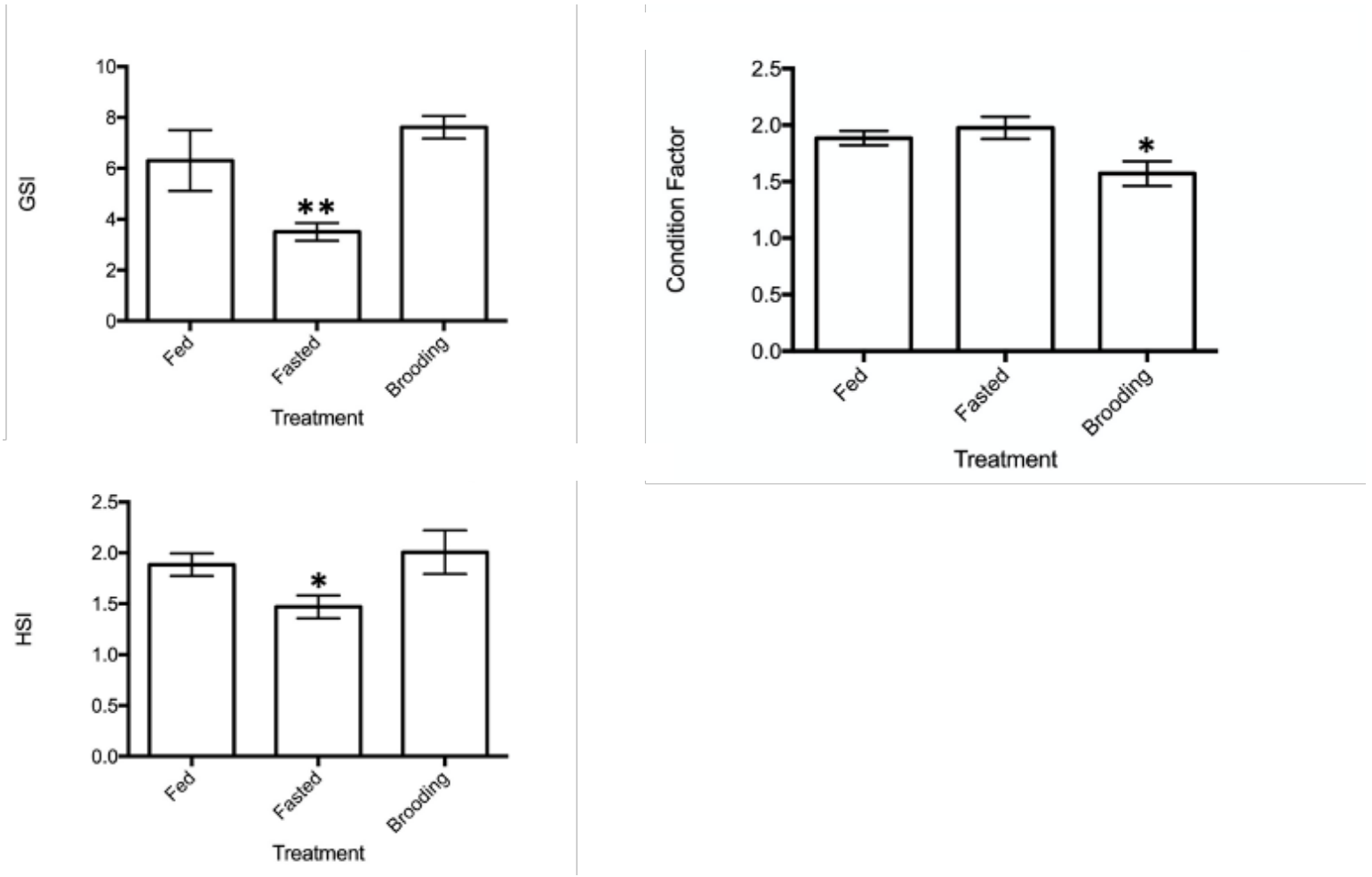

Figure 7. Average GSI, HSI, and condition factor of females under three reproductive conditions: fed-gravid, fasted, and mouthbrooding. Values reported as means ± SEM. * denotes significant difference from fed-gravid fish. (n = 10/group; * $P < 0.05$, ** $P < 0.01$).

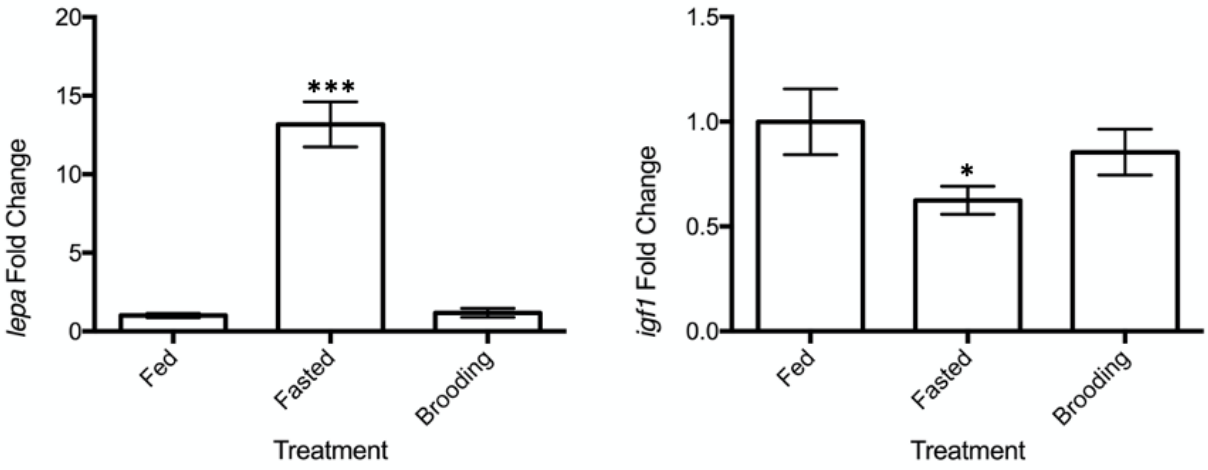

Figure 8. *lepa* and *igf1* mRNA levels of females (shown as fold change from fed-gravid fish) under three reproductive conditions: fed-gravid, fasted, and brooding. Values reported as means ± SEM. * denotes significant difference from fed-gravid fish. (n = 10/group; * $P < 0.05$, *** $P < 0.001$).

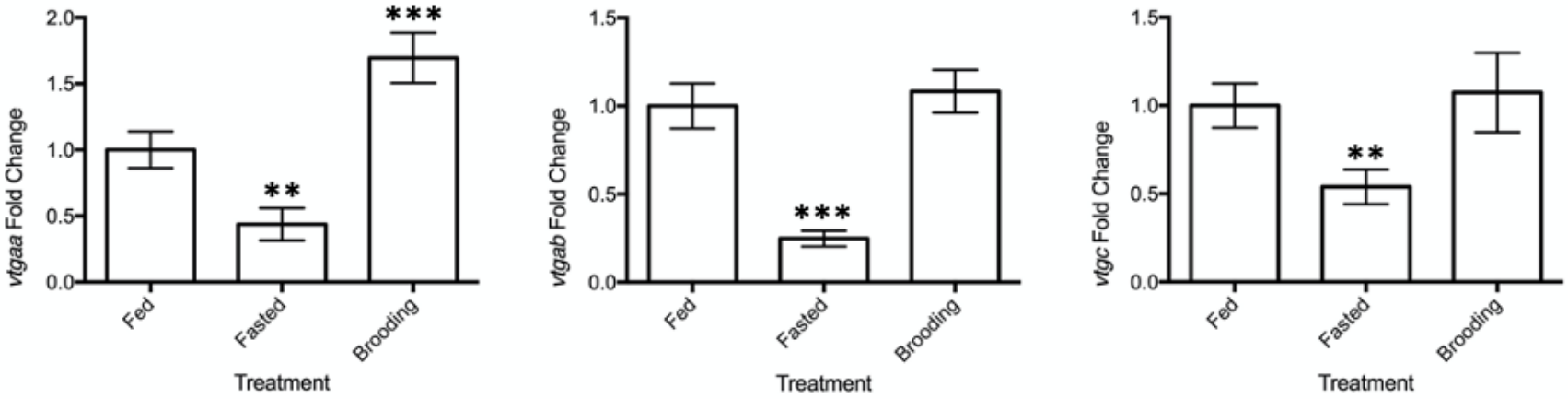

Figure 9. *vtgaa, vtgab,* and *vtgc* mRNA levels of females (shown as fold change from fed-gravid fish) under three reproductive conditions: fed-gravid, fasted, and brooding. Values reported as means ± SEM. * denotes significant difference from fed-gravid fish. (n = 10/group; ** $P < 0.01$, *** $P < 0.001$).

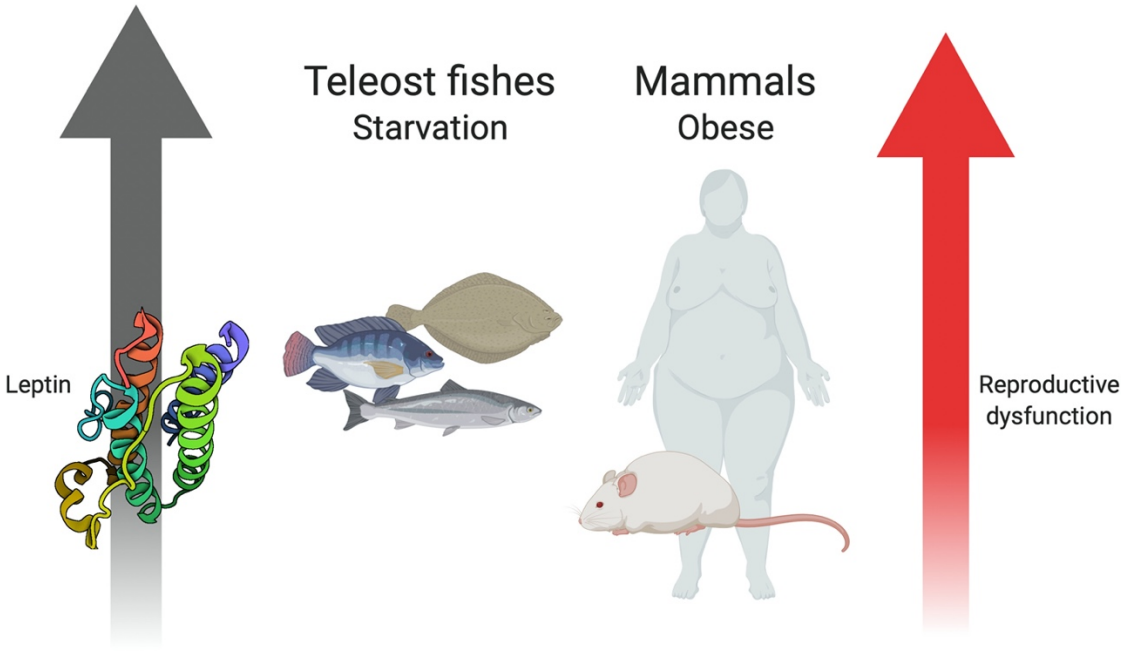

Figure 10. Conceptual diagram outlining the deleterious effects of chronically elevated leptin levels on reproductive function in teleost fishes and mammals. This diagram does not preclude the permissive properties of leptin (i.e., for onset of puberty in mammals), but rather, describes the impact of metabolic abnormalities/extremes.

APPENDIX

Appendix A

Gene	Forward	Reverse	Accession Number
agrp2	CAGTTTGGAGCCAAGCGAAG	CGACTTGTGCTTAGGACTGACA	NM_001279579.1
b-actin	CCTGACAGAGCGTGGCTACTC	TCTCTTTGATGTCACGCACGAT	EU887951
cart	CCCCTCTTGTGATCTTGGGG	GACAGTCGCACATCTTCCCA	XM_003455220.5
crf	GGAGAGGCTTGGGGAGGA	TGCCCTGTAAAAGACGCCG	AJ011835.1
gnih	GGTTCTATCCGCCCACTGTC	TGGTTCGGTGCTCGTCTGA	KF444208.1
gnrh1	CTCGCAGGGACGGTGTTT	TCTTCCCTCCTGGGCTCAGT	XM_013271546.3
igf1	ATGTCTAGCGCTCTTTCCTT	CTACATTCTGTAGTTTCT	NM_001279503.1
kiss2	CGCTTTGGGAAACGCTACA	CGCAAGAACAGAGAGAAGGGTG	NM_001279468.1
lepa	GGGTCTCCCAGATCAAGTACGA	TGCCGCCACAGATGAATG	KC354702.1
npya	ATGCATCCTAACTTGGTGAG	GTCTTGTGATGAGGTTGATG	XM_003448854.5
pomc	TGTGTGGCTTTTGGTGGCAT	GATGCACTCCATCATGTTGCTC	XM_003457943.5
vtgaa	GAATGTGAATGGGCTGGAAATAC	TTTGTTTGATCTGGATGTCAGCTT	EF408235
vtgab	AAGTTGCAGACTGGATGAAAGGA	GCGGTACTCGTCTCCGACAT	EF408236
vtgc	GGACCTTGCAGAACCCAAAG	CATCGTTTCTTGCCAGTTCCA	EF408237

Table 1: Alphabetically-listed forward and reverse primer sequences with affiliated NCBI accession numbers used for qPCR throughout this dissertation.