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The role of leptin in zebrafish (*Danio rerio*)

Novel insights into appetite regulation and reproduction

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Link to Zoom meeting: https://uu-se.zoom.us/j/61670031038

Abstract
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The hormone leptin is a peripheral metabolic signal and an important regulator of energy balance. In mammals, leptin acts on the appetite centers in the hypothalamus, causing anorexigenic functions by inhibiting food intake. It is also considered as a link between the nutritional status and the endocrine reproductive axis. However, the actions of leptin in teleosts are not fully understood. This thesis investigated the possible role of leptin in the regulation of appetite and reproduction in teleosts, using a loss of function leptin receptor zebrafish strain (*lepr* sa12953).

Under different feeding conditions (normal feeding, 7-day fasting, 2- and 6-hours post refeeding) the transcription of orexigenic and anorexigenic genes was influenced by leptin in the zebrafish brain. Leptin signaling inhibited the transcription of orexigenic genes, during short-term fasting and refeeding, and stimulated the transcription of anorexigenic genes under normal feeding in wild-types, indicating an anorexigenic role of leptin in appetite regulation in zebrafish. Moreover, a leptin-dependent gene regulatory network (GRN), involved in the behavioral and metabolic control of appetite was suggested in the brain, including the *cart*/*crhb*/*gnrh2* genes and their respective co-expressed modules, mediated by the transcription factor *sp3a*.

Furthermore, impaired leptin signaling resulted in severe reproductive deficiencies in female zebrafish. Folliculogenesis was not affected, but oocyte maturation and ovulation were disrupted in *lepr* mutant females, resulting in low number of ovulated eggs. Moreover, the transcripts of luteinizing hormone beta (*lhb*) in the pituitary were significantly lower in the mutant females. Analysis of candidate genes revealed differential expression of genes involved in steroidogenesis, oocyte maturation and ovulation in the ovaries of the *lepr* mutants. Transcriptomic analysis of isolated fully grown follicles linked the reproductive deficiencies to the suppression of essential metabolic pathways during oocyte maturation and ovulation in teleosts, such as estrogen regulation, ribosome biogenesis, mRNA translation and lipid metabolism.

Overall, the results from the present thesis provided, for the first time in zebrafish, evidence that leptin is involved in appetite regulation, by mediating the transcription of appetite-regulating genes and a GRN in the brain, as well as that leptin consists a critical regulator of female reproduction, especially during oocyte maturation and ovulation.

Keywords: zebrafish, leptin, appetite, GRN, brain, reproduction, oocyte maturation, ovulation

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In the memory of my father,
Apostolos G. Tsakoumis

Cover designed by Anna Sarigiannidou (@themakery_by_Anna)

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Ahi, E.P., Brunel, M., **Tsakoumis, E.**, Schmitz, M. (2019). Transcriptional study of appetite regulating genes in the brain of zebrafish (*Danio rerio*) with impaired leptin signalling. *Scientific reports*, 9(1), pp.1-14.

II Ahi, E.P., **Tsakoumis, E.**, Brunel, M., Schmitz, M. (2021). Transcriptional study reveals a potential leptin-dependent gene regulatory network in zebrafish brain. *Fish Physiology and Biochemistry*, pp.1-16.

III **Tsakoumis, E.**, Ahi, E.P., Schmitz, M. (2021). Impaired leptin signaling causes subfertility in female zebrafish. *Submitted Manuscript*

IV **Tsakoumis, E.**, Ahi, E.P., Schmitz, M. (2021). Transcriptomic analysis of fully grown follicles reveals metabolic pathways involved in leptin-dependent subfertility in zebrafish. *Manuscript*

Contents

Abbreviations

17,20bP, DHP	17a, 20b-dihydroxy-4-pregnen-3-one
agr2	anterior gradient 2
AgRP	agouti-related peptide
apln	apelin
ARC	arcuate nucleus
ATP	adenosine triphosphate
BMI	body mass index
bp	base pair
BPG	brain – pituitary – gonad
CART	cocaine and amphetamine regulated transcripts
CCK	cholecystokinin
cdh13	cadherin 13, H-cadherin
cdh30	cadherin 30
cDNA	complementary DNA
ckmt1	creatine kinase, mitochondrial 1
cnr1	cannabinoid receptor 1
cort	cortistatin
cpla2	phospholipase A2, group IVAa (cytosolic, calcium - dependent)
CRH	corticotropin-releasing hormone
crhb	corticotropin releasing hormone b
DA	dopamine
db	mammalian leptin receptor gene
dpf	days post fertilization
ERK/RSK	extracellular signal-regulated kinases/ribosomal protein S6 kinase
fancm	FA complementation group M
FSH	follicle-stimulating hormone
fshb	follicle-stimulating hormone beta
gadd45ab	growth arrest and DNA-damage-inducible, alpha, b
GAL	galanin
galr1a	galanin receptor 1a
GHRL	ghrelin
GIT	gastrointestinal tract
GnRH	gonadotropin-releasing hormone
gnrh2	gonadotropin-releasing hormone 2

GnRHRs	gonadotropin-releasing hormone receptors
GRNs	gene regulatory networks
HIS	hepato-somatic index
hpf	hours post fertilization
hsd3b1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
ICV	intra-cerebroventricular administration
INS	insulin
IP	intraperitoneal administration
IR	irisin
krox24	early growth response 1
LEP	leptin
lepa	leptin-a
lepa-I/II	leptin-a I/II
lepb	leptin-b
lepb-I/II	leptin-b I/II
lepr	leptin receptor
lepr-/-	leptin receptor mutant
LH	luteinizing hormone
lhb	luteinizing hormone beta
LHR	luteinizing hormone receptor
mc4r	melanocortin 4 receptor
MIH	maturation-inducing hormone
MPF	maturation-promoting factor
mprb	membrane progesterone receptor b
mRNA	messenger RNA
mTORC1	mechanistic target of rapamycin complex 1
mb	myoglobin
mmp15a	matrix metallopeptidase 15a
NE	norepinephrine
nppcl	natriuretic peptide C-like protein
NPY	neuropeptide Y
ob	obese gene
ORX	orexin
oxt	oxytocin
PGCs	primordial germ-cells
pgr	nuclear progesterone receptor
pgrmc2	progesterone receptor membrane component 2
pik3ip1	phosphoinositide-3-kinase interacting protein 1
pla2g4f.2	phospholipase A2, group IVF, tandem duplicate 2
pmchl	pro-melanin-concentrating hormone, like
POMC	proopiomelanocortin
ptger4b	prostaglandin E receptor 4 (subtype EP4) b
sat1a.2	spermidine/spermine N1-acetyltransferase 1a, duplicate 2

SER	serotonin
SNPs	single nucleotide polymorphisms
Sp1/3	specificity protein 1/3
sp3a	transcription factor sp3a
spx	spexin
star	steroidogenic acute regulatory protein
tcima	transcriptional and immune response regulator a
thbs4b	thrombospondin 4b
TRH, *trh*	thyrotropin-releasing hormone

Genes are written in italic and lowercase letters, while proteins and hormones in uppercase.

Introduction

The regulation of food intake is a vital mechanism for the development and survival of all living organisms, as it ensures the optimal allocation of energy resources to cover the energy requirements for several physiological and metabolic processes, including reproduction (Schwartz et al. 2000). Reproduction is an energetically costly event across vertebrates. Ectothermic vertebrates, such as teleosts, can spend almost half of their energy reserves for maintaining a normal reproductive function. The energy expenditure levels invested in reproduction are usually higher in females for the production of eggs and yolk, whereas in males, sperm production requires usually smaller amounts of energy (Hayward and Gillooly 2011). Therefore, the quality and quantity of food are essential factors for a normal reproductive function. Indeed, high amounts of food supply usually induce the reproductive system, while lower food supply might delay or even inhibit it (Schneider 2004).

Appetite and reproduction can be mediated by external (e.g. temperature, photoperiod) and internal (e.g. energy reserves, genetics) factors. Appetite regulation is also under the influence of several endocrine signals, originating either from the brain itself and/or peripheral organs, which act on the appetite centers in the brain (Parker and Bloom 2012; Sohn 2015; Rønnestad et al. 2017; Volkoff 2016, 2019; Blanco and Soengas 2021). Similarly, the onset of puberty, as well as a normal reproductive function during adulthood can also be controlled by similar metabolic cues, which act on the brain, the pituitary and/or the gonad (Fernandez-Fernandez et al. 2006; Hill et al. 2008; Shahjahan et al. 2014). Consequently, appetite and reproduction are two physiological processes, which are tightly linked to each other and their regulation depends on the nutritional status and the amount of stored energy reserves of the organism.

Background

Endocrine regulation of appetite

In teleosts, as in other vertebrates, the regulation of appetite and body weight consists a complex physiological process, mediated mostly centrally, in the hypothalamic region of the brain (Demski 1982; Timper and Brüning 2017; Soengas et al. 2018). In mammals, neurons in the arcuate nucleus (ARC) area of the hypothalamus are considered as the main regulators of appetite (Sobrino Crespo et al. 2014; Park and Ahima 2015; Sohn 2015), while in teleosts, these neurons are widely distributed, not only in the hypothalamus, but in the whole brain (Cerdá-Reverter and Canosa 2009). Appetite is also under the influence of environmental factors, such as photoperiod or temperature, as well as intrinsic physiological factors, such as the reproductive stage or energy reserves. Appetite is rigidly linked to the food intake of the organism, which can be regulated by two distinct mechanisms: the homeostatic and the non-homeostatic mechanism (Timper and Brüning 2017; Volkoff 2019).

The homeostatic food intake regulation mechanism maintains energy balance and arises in response to the nutritional status and the metabolic needs of the organism. It is mediated by the appetite-regulating neurons in the brain and the cognate neuropeptides they encode, which can be classified into two main categories (Fig. 1). The ones which stimulate food intake, such as the neuropeptide Y (NPY) or the agouti-related peptide (AgRP) are called orexigenic, while those suppressing food intake, such as the proopiomelanocortin (POMC) or the cocaine and amphetamine regulated transcripts (CART) are called anorexigenic. Furthermore, these neuronal populations are also able to integrate metabolic signals from peripheral organs, such as the gastrointestinal tract, the liver, the pancreas and the muscle, which can further mediate food intake and energy expenditure (Lin et al. 2000; Jensen 2001; Volkoff et al. 2005; Flik et al. 2006; Sobrino Crespo et al. 2014; Sohn 2015; Rønnestad et al. 2017; Timper and Brüning 2017; Volkoff 2016, 2019).

The non-homeostatic food intake regulation mechanism is related to the brain reward system and is under the influence of several neurotransmitters, such as dopamine (DA), norepinephrine (NE) or serotonin (SER) (Fig. 1) (Rossi and Stuber 2018; Volkoff 2019). These neurotransmitters act on the appetite centres in the brain and elicit positive emotions, which will be imprinted in the

memory of the brain and strengthen the desire to obtain again the same type of food in the future (Schultz 2015).

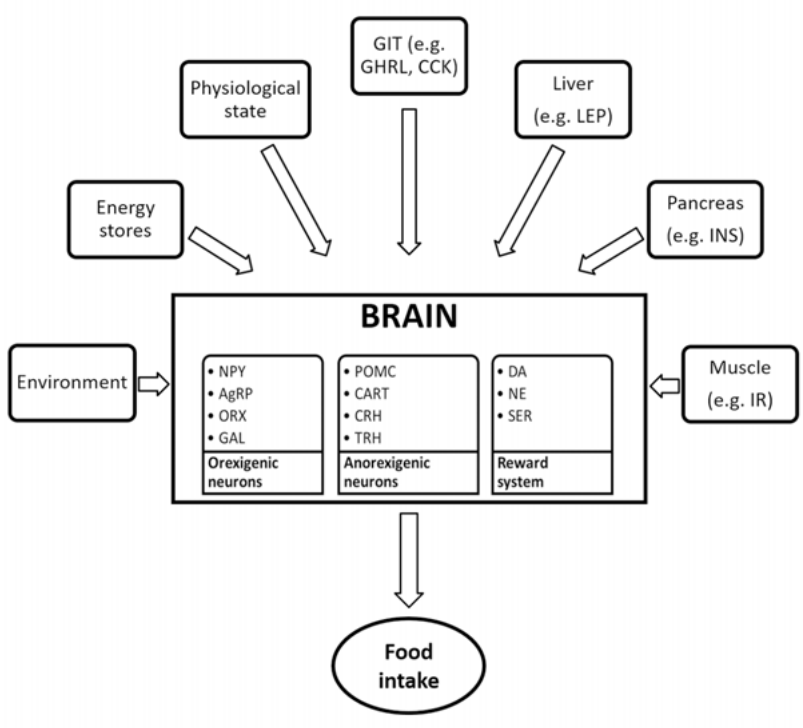

Figure 1. Schematic representation of the major external and internal factors involved in appetite regulation in teleosts. GIT: gastrointestinal tract; GHRL: ghrelin; CCK: cholecystokinin; LEP: leptin; INS: insulin; IR: irisin; NPY: neuropeptide Y; AgRP: agouti-related peptide; ORX: orexin; GAL: galanin; POMC: proopiomelanocortin; CART: cocaine- and amphetamine-regulated transcript; CRH: corticotropin-releasing hormone; TRH: thyrotropin-releasing hormone; DA: dopamine; NE: norepinephrine; SER: serotonin.

Endocrine regulation of reproduction

Reproduction in teleost fish, as in other vertebrates, is coordinated by the brain – pituitary – gonad (BPG) axis, which is also known as the reproductive axis. Brain, pituitary and gonad communicate and interact with each other, by positive or negative feedback mechanisms, regulating the reproductive cycle of the organism and leading the gonad to its final maturation, ovulation or spermiation and spawning (Fig. 2). During juvenile development, the reproductive axis is quiescent and is activated at the onset of puberty, when the organism acquires the capacity to reproduce for the first time, by producing

functional sperm or mature eggs and by synthesizing and secreting reproductive and steroidogenic hormones (Okuzawa 2002).

The reproductive axis can be activated and further controlled by external (e.g. photoperiod, temperature, food availability) and internal (e.g. hormones, neuropeptides, energy stores) factors (Bhattacharya 1992). Once the axis is activated, a cascade of events occurs (Fig. 2). Briefly, in the hypothalamic region of the brain, gonadotropin-releasing hormone (GnRH) is synthesized, a neuropeptide which regulates the activity of endocrine cells in the anterior pituitary for the release of the two gonadotropins, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) (Schally et al. 1971; Zohar et al. 2010). Two or three GnRH types have been characterized in teleosts, as well as multiple GnRH receptors (GnRHRs) and interestingly they all differ in their neuroanatomical localization and function (Muñoz-Cueto et al. 2020). The gonadotropins, FSH and LH, belong to the glycoprotein hormone family and they are both consisted of a common a-subunit and a hormone-specific b-subunit, which is the one determining their biological function (Pierce and Parsons 1981; Gharib et al. 1990). FSH and LH are then transferred through the bloodstream to the gonad (ovary or testis), where they bind to their cognate receptors and stimulate gonadal development, gametogenesis and steroidogenesis (Fig. 2). Several studies in a variety of teleost species have tried to investigate their specific biological functions. It is now generally accepted that FSH is involved in promoting early gonadal development and growth, whereas LH is mostly involved in the last stages of gonadal development, including the final maturation and release of the gametes: ovulation in females and spermiation in males (Swanson et al. 2003; Yaron et al. 2003).

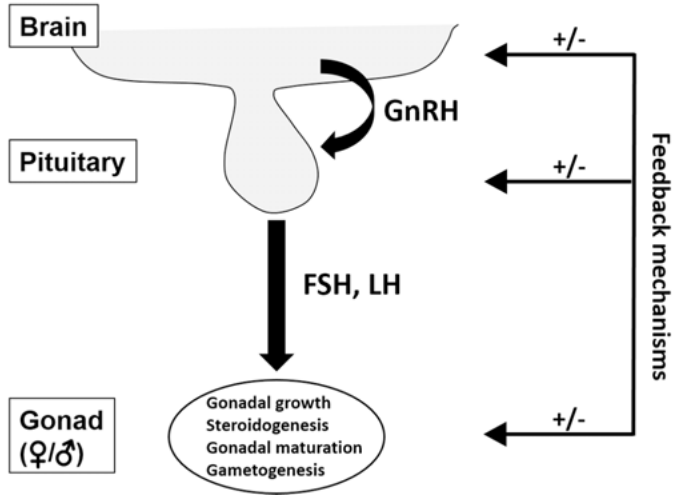

Figure 2. Schematic representation of the reproductive axis in female (♀) and male (♂) teleosts. GnRH: gonadotropin-releasing hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone. Stimulatory effects are indicated by thick arrows. Positive (+) and negative (-) feedback mechanisms between brain, pituitary and gonad are indicated by thin arrows.

Oocyte maturation and ovulation

Oocyte maturation and ovulation are two independent, but closely related processes during oogenesis. Briefly, in earlier stages of oogenesis, primordial germ-cells (PGCs) are transformed first into oogonia and then into primary oocytes, when they are arrested at their first meiotic prophase. Then, the primary oocytes grow massively in size, during the stage of vitellogenesis, whereby they accumulate nutritional reserves required for the development of the future embryo (Patiño and Sullivan 2002; Lubzens et al. 2010). During oocyte maturation, meiosis from the prophase I is resumed until metaphase II, when it is arrested again. During this arrestment period, various messenger RNAs (mRNAs) are translated into proteins, which ensure a good oocyte quality and at the same time the connections between the oocytes and the granulosa cells begin to break. During ovulation, proteolytic enzymes digest the layers of follicular cells, which surround the oocytes, resulting in follicular rapture and the release of the mature eggs (Nagahama and Yamashita 2008; Clelland and Peng 2009).

In teleosts, as in all vertebrates, LH is considered as the main regulator of oocyte maturation and ovulation (Patiño and Sullivan 2002; Nagahama and Yamashita 2008; Levavi-Sivan et al. 2010; Li and Cheng 2018). The specificity of LH actions during these two stages of oogenesis was also verified by

advanced, targeted, gene-knockout studies. In mammals, most of the information on the functions of LH has been obtained by gene-knockout mouse models (Kumar 2005, 2007). For instance, *lhb* deficiency disrupted the last stages of folliculogenesis and decreased the actions of steroids in both male and female mice, resulting in infertility (Ma et al. 2004). In teleosts, LH deficient fish lines have been generated in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), using transcription activator-like effector nucleases (TALEN). Similarly, as in mammals, LH deficient fish appear to have normal gonadal growth, but they fail to spawn and are therefore infertile (Chu et al. 2014; Zhang et al. 2015; Takahashi et al. 2016; Shang et al. 2019).

LH signaling initiates complex downstream physiological mechanisms. Briefly, LH exerts its actions by binding to its cognate receptor (LHR) on the granulosa cells of the fully grown follicles and triggers the production of the maturation-inducing hormone (MIH), called 17a, 20b-dihydroxy-4-pregnen-3-one (17,20bP or DHP). DHP binds then to its cognate receptors, located in the oocytes' membranes and stimulates the activation of the maturation-promoting factor (MPF), which releases the oocytes from their first meiotic prophase arrest and resumes maturation (Nagahama 1997; Nagahama and Yamashita 2008; Levavi-Sivan et al. 2010; Takahashi et al. 2019). Simultaneously, LH and DHP stimulate downstream factors and key-regulators for oocyte maturation and ovulation, including membrane (Tokumoto et al. 2006; Zhu et al. 2003, 2008; Thomas 2012; Wu et al. 2018, 2019, 2020) and nuclear progestin receptors (Hanna et al. 2010; Zhu et al. 2015; Tang et al. 2016; Wu and Zhu 2020), prostaglandins (Lister and Van Der Kraak 2009; Fujimori et al. 2012; Hagiwara et al. 2014; Takahashi et al. 2018; Tang et al. 2017, 2018) and matrix metalloproteinases (Ogiwara et al. 2005; Ogiwara and Takahashi 2017; Liu et al. 2018b, 2020).

The leptin system

The hormone leptin is a key regulator of body weight, appetite and metabolism. Leptin is a 16kD protein product, encoded in mammals by the obese gene (*ob*) and produced mainly in the white adipose tissue (Zhang et al. 1994). Under normal conditions, circulating leptin levels reflect body fat mass and thus leptin functions as an adipostat, signaling the size of the fat stores to the brain. However, leptin is also expressed in a variety of other peripheral organs, such as the pituitary gland, placenta, ovary, skeletal muscle and stomach (Margetic et al. 2002) and is involved in the regulation of a wide variety of physiological processes, including food intake, energy expenditure, lipid metabolism and reproduction (Friedman 2014, 2019).

In teleosts, leptin was first identified in pufferfish (*Takifugu rubripes*) by Kurokawa et al. (2005) and later in other species used frequently in biological research, such as common carp (*Cyprinus carpio*) (Huising et al. 2006), medaka (Kurokawa and Murashita 2009), zebrafish (Gorissen et al. 2009) and Atlantic salmon (*Salmo salar*) (Rønnestad et al. 2010). Today, orthologous leptin genes have been characterized in numerous teleost species, from almost all the currently known taxonomic orders (Blanco and Soengas 2021). Most teleosts have two leptin paralogues, leptin-a (*lepa*) and leptin-b (*lepb*), which were generated as a result of an ancient genome duplication event during the early evolution of teleosts (Taylor et al. 2003; Volff 2005). Furthermore, due to more recent genome duplication and tetraploidisation events (Londraville et al. 2014, 2017), some teleost species, including common carp and Atlantic salmon, carry four leptin paralogues, *lepa-I/II* and *lepb-I/II* (Huising et al. 2006; Rønnestad et al. 2010).

Unlike in mammals, the white adipose tissue of teleosts is not the major tissue for the production of the leptin paralogues: *lepa* is predominantly expressed in the liver, which is rich in fat droplets and consists an efficient adipose storage, while the tissue with the highest expression for *lepb* appears to vary between species (Gorissen and Flik 2014). The amino acid sequences of both leptin genes are not well conserved between mammals and teleosts, as well as within teleosts and show low homology (Gorrisen and Flik 2014). However, the secondary and tertiary structures of the protein products, as well as the gene arrangement and gene synteny seem to be highly conserved among vertebrates (Denver et al. 2011; Prokop et al. 2012; Londraville et al. 2014, 2017).

Leptin mediates its signal by binding to its cognate receptor, the leptin receptor (*lepr*), which is located in the cell membrane and belongs to the class I cytokine receptor family. In mammals, the *lepr* gene (*db*) produces several alternatively spliced variants, but only the long form of the receptor contains the full intracellular domain, which is needed to mediate the effects of leptin (Tartaglia 1997). Despite the low homology in the amino acid sequences between different species (Prokop et al. 2012), *lepr* orthologues have also been identified in teleosts. For instance, multiple paralogues of the *lepr* gene were found in Atlantic salmon (Rønnestad et al. 2010), European sea bass (*Dicentrarchus labrax*) (Escobar et al. 2016) and rainbow trout (*Oncorhynchus mykiss*) (Gong et al. 2013), while for other species only one isoform has been identified so far, such as in pufferfish (Kurokawa et al. 2008), medaka (Kurokawa and Murashita 2009) and zebrafish (Liu et al. 2010). In species with a single *lepr* gene, Prokop et al. (2012) suggested that the multiple leptin paralogues can all act as ligands and bind to the same, single receptor, through altered hydrophobic interactions in a temperature related manner.

In fish, the role of leptin is still poorly understood. Recent studies indicate that leptin might play a pleiotropic role in the regulation of several physiological processes (Gorrisen and Flik 2014; van de Pol et al. 2017), including osmotic adaptation (Baltzegar et al. 2014; Douros et al. 2014), glucose homeostasis and metabolism (Won et al. 2012; Yu et al. 2012b; Michel et al. 2016; He et al. 2021), as well as stress regulation (Gorissen et al. 2012). However, so far, few studies have focused on the role of leptin in the regulation of appetite and reproduction.

Leptin and appetite

After the identification of leptin for the first time in teleosts, its possible role in appetite regulation was assessed by classic experiments in the field of fish physiology, using fasting or restricted feeding regimes. However, the results generated from these studies were not consistent between different teleost species. For instance, an upregulation of the circulating leptin levels after fasting was seen in fine flounder (*Paralichthys adspersus*) (Fuentes et al. 2012), rainbow trout (Kling et al. 2009) and tilapia (*Oreochromis mossambicus*) (Douros et al. 2017; Mankiewicz et al. 2021). Similarly, the transcripts of the leptin or leptin receptor paralogues after long- or short-term fasting were upregulated in Atlantic salmon (Rønnestad et al. 2010; Trombley et al. 2012), Arctic charr (*Salvelinus alpinus*) (Frøiland et al. 2010, 2012), orange-spotted grouper (*Epinephelus coioides*) (Zhang et al. 2013), tilapia (Douros et al. 2017), goldfish (*Carassius auratus*) (Li et al. 2019) and Northern snakehead (*Channa argus*) (Wen et al. 2020). On the other hand, in the green sunfish (*Lepomis cyanellus*) (Johnson et al. 2000) and burbot (*Lota lota*) (Nieminen et al. 2003), fasting resulted in lower plasma leptin levels, as well as in lower transcripts of genes belonging to the leptin system in crucian carp (*Carassius carassius*) (Cao et al. 2011), rainbow trout (Gong et al. 2016), striped bass (*Morone saxatilis*) (Won et al. 2012) and in the Cypriniformes species *Schizothorax prenanti* (Yuan et al. 2014). However, neither short- nor long-term fasting had any effect on the leptin transcripts in common carp (Huising et al. 2006), as well as on none of the genes from the leptin system in goldfish (Tinoco et al. 2012) and European eel (*Anguilla anguilla*) (Morini et al. 2015). To summarize, these results indicate species-specific responses of the leptin system in conditions of reduced food availability, questioning therefore the role of leptin as an adipostat in fish (Londraville et al. 2014).

In mammals, studies on mice and rats demonstrated that leptin exerts anorexigenic functions and inhibits food intake, by acting on the appetite-regulating neurons in the ARC area of the hypothalamus (Schwartz et al. 2000; Park and Ahima 2015; Friedman 2014, 2019). Leptin has the ability to suppress the orexigenic NPY and AgRP neurons (Stephens et al. 1995; Ahima et al. 1996;

Elias et al. 1998, 1999; Mizuno and Mobbs 1999; Korner et al. 2001; Morrison et al. 2005; Lee et al. 2013; Baver et al. 2014) and to stimulate the anorexigenic POMC and CART neurons (Schwartz et al. 1997; Kristensen et al. 1998; Mizuno et al. 1998; Elias et al. 1999; Cowley et al. 2001; Balthasar et al. 2004; Lee et al. 2013).

In accordance to studies in mammals, intraperitoneal (IP) or intra-cerebroventricular (ICV) administration of either homologous or heterologous leptin resulted in differential mRNA expression of orexigenic and anorexigenic neuropeptides in the brain of teleost fish as well. In fact, IP or ICV leptin administration downregulated the *npy* transcripts in goldfish (Volkoff et al. 2003; Yan et al. 2016), grass carp (*Ctenopharyngodon idella*) (Li et al. 2010), mandarin fish (*Siniperca chuatsi*) (Yuan et al. 2020), rainbow trout (Murashita et al. 2008; Aguilar et al. 2010) and Nile tilapia (*Oreochromis niloticus*) (Liu et al. 2018a). Similarly, decreased mRNA expression levels for *agrp* were reported in the goldfish (Yan et al. 2016) and the mandarin fish (Yuan et al. 2020). Regarding the anorexigenic neuropeptides, *pomca* levels were increased in goldfish (Yan et al. 2016) and rainbow trout (Murashita et al. 2008; Gong et al. 2016), but decreased in the mandarin fish (Yuan et al. 2020), after IP or ICV leptin administration. Similarly, an increase in the mRNA levels for *cart* genes was observed in goldfish (Volkoff and Peter 2001; Volkoff et al. 2003; Yan et al. 2016) and in rainbow trout (Gong et al. 2016), but *cart* mRNA expression was decreased in the mandarin fish (Yuan et al. 2020).

With the advent of more advanced molecular techniques, gene-targeted knockout studies in medaka and zebrafish supported further the findings that leptin can mediate the appetite centers in the fish brain. For instance, *lepr* deficient medaka had higher mRNA expression levels of *npya* and *agrp* than wild-types before and after feeding, as well as significantly lower levels of *pomc1* after feeding (Chisada et al. 2014). Similarly, *lepa* deficient zebrafish had higher hormonal levels of AgRP and DA, but lower levels of NE than wild-type fish under normal feeding conditions (Audira et al. 2018).

Altogether, the effects of leptin on appetite regulation and food intake in teleost fish do not seem to be as consistent as in mammals and reflect species-specific differences. The large diversity among teleosts, both in terms of dietary habits and habitats, the additional rounds of genome duplication events, as well as the low homology of the leptin genes within teleosts could partially explain the heterogeneity observed in the afore-mentioned studies (Denver et al. 2011; Prokop et al. 2012; Gorissen and Flik 2014; Londraville et al. 2014, 2017; Blanco and Soengas 2021). Therefore, further studies are required to uncover the precise mechanisms of leptin signaling in the regulation of appetite in teleosts.

Leptin and reproduction

The essential role of leptin in the regulation of reproduction in mammals has been clearly established for more than a decade (Tena-Sempere 2007; Roa et al. 2010). The first indications came by Zhang et al. (1994), who reported that the mutation of the *ob* gene resulted in complete infertility in male and female mice. A few years later, it was shown that leptin administration could stimulate the reproductive endocrine system in *ob/ob* mice of both sexes and rescue their infertility, verifying that leptin serves as critical regulator of the reproductive system (Barash et al. 1996). Moreover, leptin acts also as a permissive signal for the onset of mammalian puberty, allowing sexual maturation to proceed, once the organism has acquired adequate energy reserves to be invested for reproductive purposes (Cheung et al. 1997).

In mammals, studies have suggested a possible, regulatory connection between leptin and LH for the control of reproduction. Leptin administration enhances LH plasma levels in rodents (Donato et al. 2011) and *lepr* deficient female mice have both lower LH plasma levels and impaired synthesis of estrogens (Garris et al. 2005; Tu et al. 2018). In addition, a selected ablation of the *lepr* gene in the gonadotropes, the cells which synthesize and secrete the gonadotropins, resulted in subfertility in female mice, due to lower mRNA levels of activin, an important regulator of FSH (Bilezikjian and Vale 2011), as well as due to lower GnRHR protein levels in the pituitary (Akhter et al. 2014). Therefore, Odle et al. (2018) suggested that leptin mediates its actions also in the pituitary, either at the transcriptional level in the regulation of activin or at the post-transcriptional level in the regulation of the GnRHR protein, playing thus a central role in mammalian female fertility.

So far, similar studies in teleosts are still limited. The first indications that leptin can act as a possible regulator of the reproductive system in fish came from *in vitro* studies, using mammalian recombinant leptin. Peyon et al. (2001) reported a direct, positive action of mouse recombinant leptin on LH release *in vitro*, using pituitary cell cultures from pubertal and adult male European sea bass. Similarly, human recombinant leptin had a stimulatory effect on the secretion of LH and FSH in pituitary cells *in vitro* during early gametogenesis in male and female rainbow trout (Weil et al. 2003).

Several studies were conducted after the identification of the first leptin orthologue in teleosts and, interestingly, they concluded that leptin might play a critical role during sexual maturation. For instance, in ayu (*Plecoglossus altivelis*), circulating leptin levels were higher during and after spawning, compared to immature fish in both sexes (Nagasaka et al. 2006). In Arctic charr, hepatic leptin transcripts showed seasonal variation, reaching their highest levels in autumn, when the fish acquired also their sexual maturity

(Frøiland et al. 2010). In female chum salmon (*Oncorhynchus keta*), both mRNA and plasma leptin levels were positively correlated with increases in sex steroids during sexual maturation (Choi et al. 2014). In male Atlantic salmon parr, hepatic *lepa-I* transcripts were higher in mature than immature fish (Trombley and Schmitz 2013) and a significant upregulation of the *lepr* transcripts in the testis was observed throughout spermatogenesis (Trombley et al. 2014). In addition, steroid treatment resulted in upregulation of both *lepaI/II* paralogues in hepatocyte cultures from male and female Atlantic salmon *in vitro* (Trombley et al. 2015). In chub mackerel (*Scomber japonicus*), hepatic *lepa* transcripts were higher in mature than immature males, but similar differences were not evident in females (Ohga et al. 2015, 2017). However, in pre-pubertal female chub mackerel, administration of recombinant leptin induced FSH and LH secretion in primary pituitary cell cultures *in vitro* and ICV administration of recombinant leptin upregulated the transcripts of *fshb* and *lhb* in the pituitary (Ohga et al. 2020).

Overall, these studies indicate a possible role of leptin during puberty or later stages of sexual maturation in teleosts (Parker and Cheung 2020). However, the underlying mechanisms are still unclear and further studies are needed, in order to understand the interaction of the leptin system with the reproductive axis.

Zebrafish

The zebrafish is a tropical freshwater fish species and member of the Cyprinidae family (Froese and Pauly 2021), native in slow-moving waters or ponds in the floodplains of India and Bangladesh (McClure et al. 2006; Spence et al. 2006) and its diet is consisted mostly by zooplankton and insects (Spence et al. 2007). Zebrafish are asynchronous, batch spawners and once they reach sexual maturity and under optimal conditions (e.g. food availability and favorable water parameters) are able to spawn successfully frequently, even on a daily basis (Selman et al. 1993; Lawrence 2011; Aleström et al. 2020). The onset of puberty varies between the two sexes and is estimated at 45 days post fertilization (dpf) for females and slightly later for males (Chen and Ge 2012, 2013).

Zebrafish has emerged as a pioneer model organism in biological research. A number of favorable attributes, including its small size, short life cycle, rapid development and generation time, as well as its optical transparency during early development, have made zebrafish suitable for partial and full life cycle studies on development, ontogenetic differentiation and physiology (Meyers 2018; Choi et al. 2021).

Zebrafish has also been used in studies of ovarian and follicular development. With a variety of genome editing technologies, such as transcription activator-like effector nucleases (TALEN) or clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9), it is now possible to create loss of function mutant zebrafish lines and study directly the function of targeted genes, with essential known roles in ovarian physiology in other vertebrates (Li and Ge 2020). In these studies, particular focus has been given to the gene expression profile of fully grown follicles (Chu et al. 2014; Li et al. 2015; Zhu et al. 2015; Tang et al. 2016; Liu et al. 2018b, 2020). As the last developmental stage before maturation, fully grown follicles of zebrafish have also been used for transcriptomic studies of differentially expressed genes (DEGs) during ovarian follicle activation and ovulation in teleosts (Liu et al. 2017; Zhu et al. 2018).

Zebrafish as a model organism in leptin research

Zebrafish possesses duplicated leptin genes, *lepa* and *lepb*, which are mainly expressed in the liver and the gonads, respectively. The two leptin paralogues share a common 24% amino acid identity with each other and only 18% with their orthologous human leptin gene (Gorissen et al. 2009). Moreover, a single *lepr* gene has been identified so far in zebrafish, which shows also low homology not only to orthologues from other vertebrates, but also to those among teleosts (primary sequence identities: 20% and 32%, respectively) (Liu et al. 2010). The *lepr* gene is expressed during all stages of embryonic and larval development and in adults, its strongest expression is in the testes and the brain (Liu et al. 2010).

Studies applying genome editing technologies on one of the two leptin paralogues have suggested so far an essential role of leptin in zebrafish development and energy homeostasis. For instance, *lepa* knockdown embryos and larvae had body malformations and larger yolk sacs, compared to wild-type embryos or larvae in the same developmental stages (Liu et al. 2012). Furthermore, inhibition of *lepa* led to lower metabolic rates in larvae (Dalman et al. 2013), whereas treatment with heterologous human leptin increased their energy expenditure (Renquist et al. 2013). Leptin has also been associated with obesity, since *lepa* knockdown males and females displayed obese phenotype, with increased body weight and length (Audira et al. 2018). Similarly, *lepb* deficient adult zebrafish exhibited similar obese phenotypic characteristics, including significantly higher blood glucose levels, compared to their wild-type counterparts (He et al. 2021).

Concerning appetite regulation, short-term fasting resulted in downregulation of hepatic *lepb*, but not *lepa* transcript levels (Gorissen et al. 2009), while *lepa*

mRNA expression increased directly after feeding and decreased after fasting in the whole body of wild-type zebrafish (Tian et al. 2015). These results suggest that, similarly to mammals, leptin might also have anorexigenic functions in zebrafish. However, long-term overfeeding had no particular effect on the mRNA expression of leptin in the visceral adipose tissue of adult fish (Oka et al. 2010).

Recently, knockout studies, acting on the *lepr* gene, have investigated further the possible role of leptin signaling in appetite regulation in zebrafish, but their results were inconsistent. For example, adult fish with a point mutation on chromosome 6 (Chr 6: 31189497), resulting in a premature termination codon in the *lepr* gene and thus a truncated polypeptide, did not exhibit hyperphagia nor increased adiposity, but had higher insulin mRNA levels, as well as alterations in glucose homeostasis (Michel et al. 2016). However, when introducing a 17bp deletion in the *lepr* gene, adult zebrafish displayed increased food intake, weight and body fat accumulation, together with impaired glucose tolerance, during overfeeding regimes (Fei et al. 2017). Furthermore, a 16bp insertion in the *lepr* gene resulted in transcriptional differences of several genes related to food intake and digestion only during larval development, whereas no particular differences were observed in the metabolism, energy allocation or growth in adults (Del Vecchio et al. 2021).

Even though leptin research is nowadays one of the "hotspots" in biological research and zebrafish plays a fundamental role in the field, the role of leptin in the regulation of reproduction in zebrafish is still unknown. After long-term observations in our lab, we noticed that matings with *lepr* deficient male and female zebrafish, with a point mutation on chromosome 6 (Chr 6: 31219099), resulted usually in either none or low number of offsprings with low survival rates. Moreover, in a pilot experiment, we investigated the possible role of leptin in the regulation of puberty onset. Several genes, which are known to regulate puberty onset in mammals and teleosts, were analyzed in the main centers of the reproductive axis (brain, pituitary, gonads) of wild-type and *lepr* deficient male and female zebrafish. However, we did not observe any striking differences between the two genotypes in both sexes, suggesting that leptin is probably not essential for the onset of puberty in zebrafish (unpublished results). Michel et al. (2016) stated that adult zebrafish lacking a functional leptin receptor exhibited normal fertility. The authors reported that *lepr* deficiency had no effect, neither on the number of laid eggs by the *lepr* mutant females nor in the frequency of successful breedings, compared to wild-type zebrafish. So far, this is the only published study associating leptin and reproduction in zebrafish.

Aim of the thesis

The overall aim of the present thesis was to explore the possible role of leptin in the regulation of appetite and reproduction in teleost fish, using zebrafish as a model organism. To investigate how leptin can affect these physiological processes, we compared wild-type zebrafish with fish belonging to a loss of function *lepr* mutant (*lepr-/-*) strain (*lepr* sa12953). The specific aims were:

- To examine possible orexigenic or anorexigenic functions of leptin in appetite regulation in zebrafish **(Paper I)**.
- To explore the effects of impaired leptin signaling on the transcription of appetite-regulating genes in the brain, under different feeding conditions **(Paper I)**.
- To study potential leptin-dependent regulatory connections in the brain, which can regulate feeding and appetite **(Paper II)**.
- To analyze the effects of impaired leptin signaling on the main centers of the reproductive axis (brain, pituitary, ovaries) in female zebrafish **(Paper III)**.
- To investigate possible differential expression of genes involved in oocyte maturation and ovulation in fully grown follicles under impaired leptin signaling **(Papers III-IV)**.
- To identify metabolic pathways involved in oocyte maturation and ovulation, under the regulation of leptin signaling in fully grown follicles **(Paper IV)**.

Materials and methods

Experimental animals

The zebrafish used in the studies included in this thesis belonged to the strain *lepr* sa12953 and were obtained from the European Zebrafish Resource Centre. Knockdown zebrafish were created by the Sanger Institute for the Zebrafish Mutation Project. The *lepr* sa12953 strain has a point mutation on chromosome 6 (Chr 6: 31219099), where a thymine was replaced by an adenine (Fig. 3), resulting in a premature stop codon and thus to a shortened polypeptide (Busch-Nentwich et al. 2013).

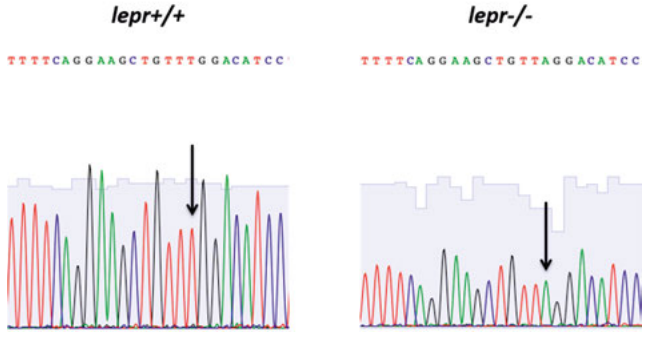

Figure 3. Verification of the single point mutation (black arrow), using wild-type (*lepr*+/+) and mutant (*lepr*-/-) zebrafish belonging to the *lepr* sa12953 strain, by sequencing. T: thymine (in red); C: cytosine (in blue); G: guanine (in black); A: adenine (in green).

The *lepr* sa12953 zebrafish lines were maintained by matings between heterozygote (*lepr*+/-) males and females. Zebrafish were kept at the Genome Engineering Zebrafish National Facility of Uppsala University, in accordance to the guidelines for zebrafish husbandry (Lawrence 2011; Aleström et al. 2020). Zebrafish were kept in 3-liter flow-through tanks under an artificial photoperiod (14:10 hours light:dark) and controlled water temperature (28.4°C). Zebrafish were fed three times daily: twice with dry pellets (zebrafeed by Sparos) in the mornings and afternoons and once with rotifers in the evenings. Water parameters were regularly monitored by the Facility staff. Water temperature (°C), pH and conductivity (µS/cm) were measured daily, while general hardness (°dGH) and carbonate hardness (°dKH), as well as the levels of

ammonia (NH4, mg/l), nitrites (NO2, mg/l) and nitrates (NO3, mg/l) were measured bi-weekly.

All experiments were conducted in accordance with the guidelines and the approval of the Swedish Ethical Committee on Animal Research in Uppsala (permit C10/16).

KASP genotyping assay

Offsprings were genotyped, using the KBioscience's Competitive Allele-Specific PCR (KASP) assay, a PCR-based assay, enabling highly accurate bi-allelic scoring of Single Nucleotide Polymorphisms (SNPs) (www.lgcgenomics.com). For the amplification of genomic DNA, KASP assay uses fluorescent allele-specific forward primers and a common reverse primer. Two 5′ fluor-labeled oligonucleotides are also included in the assay: one labeled with the fluorescent dye FAM and one with HEX, together with ROX, as the internal reference fluorescent dye. Altogether are designed to interact with the sequences of the tails of the allele-specific primers. Therefore, if the genotype at a given SNP is homozygous, only one of the two possible fluorescent signals will be generated (FAM or HEX). If the individual is heterozygous, it will result in a mixed signal (Fig. 4) (Dooley et al. 2013; He et al. 2014).

For the KASP genotyping assays, zebrafish were first anaesthetized, by immersion in Aquacalm (50 μg/ml) and then a small part from their caudal fin was clipped and kept in 50μl lysis buffer solution (10mM Tris-HCl; pH 8; 50mM KCl; 0.3% Tween-20; 0.3% NP40; 1mM EDTA; dH_20) at 4^oC. Fin samples were first incubated at 55^oC for 2 hours, followed by 15 minutes' incubation at 95^oC. Samples were later diluted to a final dilution volume of 1:100 and stored at 4^oC until further analysis. KASP PCR assays were performed on a MxPro-3000 PCR machine (Stratagene, La Jolla, CA), using the KASP Master mix (LGC genomics) and following the manufacturer's protocol.

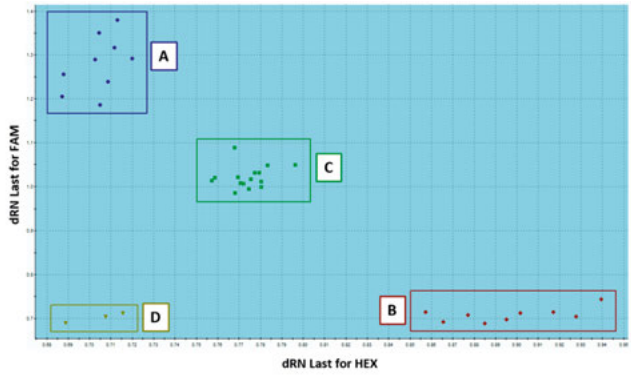

Figure 4. Representative result of a KASP genotyping assay of homozygote wild-type (A), homozygote mutant (B) and heterozygote (C) zebrafish belonging to the *lepr* sa12953 strain. D: negative control samples (dH_2O).

Experimental design: appetite experiments

For **Papers I and II**, wild-type and *lepr-/-* zebrafish of similar age were divided into groups and kept under four different feeding conditions: normal feeding; 7-day fasting; 7-day fasting and sampled 2 hours after refeeding; 7-day fasting and sampled 6 hours after refeeding. Each group was subdivided into two subgroups (wild-type and *lepr-/-* zebrafish), with similar sex ratios (1–2 females and 3–4 males). During samplings, their standard body length (SL, cm) and net weight (W, g) were recorded and their hepato-somatic index (HSI, %) was calculated. Fasting resulted in a weight loss of around 10% in both genotypes, however no significant differences were observed in other parameters. In order to avoid any possible bias between the biological samples from different feeding conditions, all samplings were performed at similar time of each sampling day.

Experimental design: reproduction experiments

For **Papers III and IV**, couples of adult wild-type and *lepr-/-* zebrafish were grouped according to the following combinations: wild-type males with wild-type females (control groups); *lepr-/-* males with wild-type females; wild-type males with *lepr-/-* females. Before all mating experiments, the zebrafish couples were first trained twice and then were mated frequently. The couples were placed in spawning tanks in the evenings and the next mornings the spawning events were recorded, the laid eggs were collected and their fertilization rate was calculated. The survival of the embryos and larvae was checked daily (24 hours post fertilization (hpf), 48 hpf, 72 hpf, 96 hpf, 120 hpf) and then all surviving larvae were euthanized. During samplings, the standard body length

(SL, cm) and net weight (W, g) of all fish were measured and their body mass index (BMI, g/cm^2) was calculated.

Fish handling and dissections

During the samplings for the experiments included in all studies (**Papers I-IV**), zebrafish were anaesthetized by immersion in a 0.4 mg/ml tricaine solution (MS-222) and then euthanized by immersion in ice bath. Zebrafish were afterwards decapitated and the tissues of interest (brain, pituitary, gonads, liver) were dissected, as described by Gupta and Mullins (2010). Dissected tissues were first placed into tubes with 200μl of RNAlater (Ambion Inc, Austin Texas) at 4^{o}C for 24 hours and then stored at -20^{o}C.

Isolation of fully grown follicles

Additionally, in **Papers III and IV**, fully grown follicles were isolated from ovaries of wild-type and *lepr-/-* adult female zebrafish, following the methodology used by Li et al. (2015) and Tang et al. (2016). The staging system adopted for the developmental classification of the ovarian follicles was based on the original definition of Selman et al. (1993), as modified by Wang and Ge (2004) and Pang and Thomas (2009). After sampling, the fully grown follicles were washed twice with 500μl 60% Leibovitz L-15 medium, homogenized with a fine syringe needle in 200μl Trizol (Ambion) and stored at -80^{o}C.

Histological analysis

For the histological analysis performed in **Paper III**, ovaries from wild-type and *lepr-/-* adult female zebrafish were fixed in 4% formaldehyde solution (VWR Chemicals), dehydrated in a series of increasingly concentrated ethanol solutions, infiltrated and finally embedded in plastic (Technovit 7100), before sectioning. The sections were stained with Hematoxylin and Eosin (H&E) (Sigma). H&E are the most commonly used dyes in biological research; hematoxylin has a deep blue color and stains nucleic acids, while eosin is pink and stains the cytoplasm and the extracellular matrix (Fischer et al. 2008). The sections were observed under a Leica DFC550 microscope twice, aiming to minimize any possible bias in the classification of the follicles into different developmental stages. The staging system adopted for the histological analysis was based on Selman et al. (1993), as modified by Wang and Ge (2004) and Pang and Thomas (2009).

Relative gene quantification

In **Papers I-IV**, the total RNA of the samples was extracted, using Trizol (Ambion), according to the manufacturer's protocol. All RNA samples were DNAse treated for the removal of any genomic contamination, using the TURBO DNase-free kit (Ambion) and following the manufacturer´s instructions. The quantity and quality of the extracted RNA were measured spectrophotometrically, using NanoDrop (Thermo-Fisher Scientific) and cDNA synthesis was carried out by reverse transcription (SuperScript III Reverse Transcriptase, Invitrogen), according to the manufacturer's protocol.

Specific primers for each target and reference gene were designed, using the Primer Express 3.0 software (Applied Biosystems, CA, USA). Relative levels of gene expression were measured by real-time quantitative polymerase chain reaction (RT-qPCR), on a MxPro-3000 PCR machine (Stratagene, La Jolla, CA, USA), using the PowerUp SYBR Green Master mix (Applied Biosystems) and following the manufacturer's suggestions. All biological samples were tested in three technical replicates for each analyzed gene, aiming for an optimal experimental set-up, as suggested by Hellemans et al. (2007). Standard curves were generated from pooled cDNA of random samples obtained from the reverse transcription for each experiment and were tested also in three technical replicates. The efficiencies of the RT-qPCR assays for each target and reference gene were calculated from the slope of the standard curve automatically, by the MxPro™ QPCR software (Stratagene, La Jolla, CA, USA). Efficiencies and R^2 values were in all cases higher than 90.0% and 0.990, respectively. The mRNA expression levels of the target genes were normalized to those of reference gene(s) with stable expression among different experiments and tissues (Kubista et al. 2006). For each study, reference gene(s) were identified after validation, using three different algorithms: BestKeeper (Pfaffl et al. 2004), NormFinder (Andersen et al. 2004) and geNorm (Vandesompele et al. 2002).

Gene Regulatory Networks (GRNs)

Gene regulatory networks (GRNs) comprise sets of gene modules, which interact with each other and control several cellular functions (Karlebach and Shamir 2008). To investigate the existence of possible GRNs under the control of leptin signaling in the zebrafish brain (**Paper II**), we first selected genes with the highest co-expression values (supportability score > 1) with the genes of interest, using the online zebrafish database COXPRESdb (Obayashi et al. 2019). Those with expression profiles similar to the genes of interest were chosen for the next step of upstream regulator prediction, using the MEME algorithm (Bailey et al. 2009). Lastly, potential transcription factor binding

sites, as upstream regulators of the co-expression gene modules, were predicted by retrieving information from the TRANSFAC database (Matys et al. 2003) and using the STAMP web server (Mahony and Benos 2007).

RNA sequencing (RNA-seq)

RNA sequencing (RNA-seq) is a frequently used tool in molecular biology, especially for transcriptome-wide analysis of differential gene expression (Stark et al. 2019). The broad application of RNA-seq technology has started to unravel the complex physiological mechanisms occurring during oocyte maturation and ovulation in many vertebrate species, including zebrafish (Liu et al. 2017; Klangnurak et al. 2018; Xiong et al. 2020).

In **Paper IV**, RNA samples from isolated fully grown follicles from wild-type and *lepr-/-* females were used for differential gene expression analysis. Briefly, the concentration and integrity of the RNA samples were examined with Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and the RIN values for all samples were higher than 7.0, indicating good RNA integrity (Schroeder et al. 2006). For each sample, sequencing libraries were prepared from 500ng total RNA, using the TruSeqStranded mRNA sample preparation kit, according to the manufacturer's protocol (Illumina Inc., San Diego, CA) and their quality was evaluated using the TapeStation system (D1000 ScreenTape, Agilent Technologies). RNA sequencing for all samples was performed by the SNP&SEQ Technology Platform in Uppsala on the same lane of an Illumina NovaSeq 6000 Sequencing System, according to the manufacturer's instructions (Illumina, San Diego, CA). The quality of the RNA-seq data was assessed with the FastQC (v0.11.8) and RSeQC script packages (v3.0.1) (Wang et al. 2012). Trim Galore! (v0.6.4) was used for trimming the raw reads (Martin 2011), which were then aligned to the zebrafish reference sequence (GRCz10/danRer10), with Star (andTAR_2.6.1d) (Dobin et al. 2013). FeatureCounts (v1.6.4) was used to assign transcript counts (Liao et al. 2014). Raw counts were processed, using the R package DESeq2 (v1.30.1) (Love et al. 2014). Venn diagrams were prepared, using the R package VennDiagram (v1.6.20). Gene Set Enrichment Analysis (GSEA) was performed, based on the Gene Ontology (GO) (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) databases, using the R package clusterProfiler (v3.18.1) (Yu et al. 2012a).

Results

Leptin has anorexigenic functions in zebrafish (Paper I)

In **Paper I**, we analysed the expression patterns of 36 genes, which are already known to have appetite-regulating functions in Cypriniformes (12 orexigenic and 24 anorexigenic), in the brain of wild-type and *lepr-/-* zebrafish, under four feeding conditions (normal feeding, 7-day fasting, 2- and 6-hours post refeeding), by RT-qPCR. Comparisons were made first between feeding conditions within each genotype and then between the two genotypes for each feeding condition separately.

The results showed that both orexigenic and anorexigenic genes in the zebrafish brain were influenced by leptin signaling. While the transcript levels of the analysed orexigenic genes were not affected under normal feeding, a few genes were upregulated in the brain of *lepr-/-* zebrafish, during fasting (*agrp* and *galr1a*) and after refeeding (*apln*, *cnr1* and *trh*). These results suggest an inhibitory effect of leptin signaling on the transcription of orexigenic genes, during short-term fasting and refeeding in wild-type zebrafish. However, pronounced effects were found among the anorexigenic genes. Impaired leptin signaling resulted in reduced brain expression of a number of genes, including *cart1/2/3/4*, *crhb*, *gnrh2*, *mc4r*, *pomc* and *spx*, under normal feeding, suggesting a stimulatory effect of leptin signaling on the transcription of anorexigenic genes in wild-type zebrafish. In addition, using pairwise expression correlation analysis, we identified multiple cases of gain and loss of potential regulatory connections between the appetite-regulating genes in the brain samples of *lepr-/-* zebrafish. These results indicate the presence of possible regulatory connections and GRNs downstream of leptin signaling in the zebrafish brain.

The results from **Paper I** provided the first evidence that leptin can mediate the transcription of appetite-regulating genes in the zebrafish brain, under different feeding conditions. Overall, the results suggest an anorexigenic role for leptin in the regulation of appetite in zebrafish.

Leptin-dependent GRN in the zebrafish brain (Paper II)

In **Paper II**, the aim was to investigate the existence of potential leptin-dependent GRN(s) in the zebrafish brain, based on similar expression patterns of the *cart* genes, *crhb* and *gnrh2* observed in **Paper I**. Therefore, we followed a simple stepwise gene detection approach, using RT-qPCR on the same samples. Comparisons were again made both between feeding conditions within each genotype and between the two genotypes for each feeding condition (normal feeding, 7-day fasting, 2- and 6-hours post refeeding).

First, we selected the five genes with the highest probability of expression correlation with the genes of interest (*cart1/2/3/4*, *crhb* and *gnrh2*) and analysed their mRNA expression in each genotype and feeding condition. Similar expression patterns to those observed in the *cart* genes were found for *ckmt1*, *pik3ip1*, *sat1a*.2, *agr2* and *tcima*; with *crhb* for *cdh13*, *cort*, *nppcl* and *oxt* and with *gnrh2* only for *pmchl*. These results suggest the existence of possible regulatory connections among these gene modules in the zebrafish brain. As a next step, we predicted several transcription factors, as potential upstream regulators of these regulatory connections. However, only *sp3a* and *krox24* had similar expression patterns with the afore-mentioned gene modules. Moreover, *sp3a* was the only transcription factor with positive correlations between its mRNA expression and those of the genes of interest (*cart1/2/3/4*, *crhb* and *gnrh2*) in the wild-types, while in the *lepr-/-* zebrafish, all these positive correlations were lost.

These results suggest the existence of a regulatory connection between leptin and *sp3a* in the zebrafish brain. In fact, *sp3a* was also predicted to act as a transcriptional driver of a downstream GRN, including the genes *cart1,2,3,4*/*crhb*/*gnrh2* and their respective co-expressed modules. Altogether, the findings from **Paper II** provided, for the first time in a teleost species, evidence for the existence of a complex GRN in the brain, which is involved in the regulation of feeding and is under the influence of leptin signaling.

Leptin regulates reproduction in female zebrafish (Paper III)

Based on our observations that *lepr-/-* fish produced few eggs with low survival rates, we aimed to study in **Paper III** the possible role of leptin in the regulation of reproduction in adult zebrafish. Wild-type males were mated with wild-type females, *lepr-/-* males with wild-type females and wild-type males with *lepr-/-* females. The couples were mated frequently and their fecundity was estimated. The expression of important genes for reproduction in teleosts were studied, by RT-qPCR, in the main centers of the reproductive

axis (brain, pituitary, gonad), as well as in the liver and in fully grown follicles. Comparisons were made between wild-type and *lepr-/-* zebrafish.

The *lepr-/-* males were equally fertile to their wild-type siblings. However, *lepr-/-* females were subfertile and laid fewer eggs with low fertilization rates, compared to wild-types, indicating that their egg quality was also impaired. Histological analysis revealed that folliculogenesis was not affected, but the mRNA expression of LH beta (*lhb*) in the pituitary was significantly lower in the mutant females. In addition, several genes related to steroidogenesis, oocyte maturation and ovulation were differentially expressed in the *lepr-/-* females, implicating that leptin could be involved in the last steps of follicular development in zebrafish and specifically in oocyte maturation and ovulation. In particular, genes known also to be downstream targets of LH signaling in teleosts were either up- (*cpla2*, *hsd3b1*, *mmp15a*, *pgr*, *ptger4b*, *star*) or down-regulated (*mprb* and *pgrmc2*) in the *lepr-/-* ovaries. However, no differences were seen between the two genotypes, when these genes were analysed in isolated fully grown follicles.

Overall, the results from **Paper III** showed that impaired leptin signaling resulted in severe reproductive deficiencies only in female and not in male zebrafish. The results suggest that the *lepr* deficiency does not affect early stages of follicular development, but leptin might be essential in later steps, such as in oocyte maturation and ovulation. The finding that *lepr-/-* females had lower *lhb* expression may argue that leptin can regulate LH release at the pituitary level in zebrafish. To our knowledge, this is the first *in vivo* study, linking leptin to reproductive deficiencies in teleosts.

Metabolic deficiencies in response to impaired leptin signaling in isolated fully grown follicles (Paper IV)

In **Paper IV**, we performed RNA-seq in samples of isolated fully grown follicles from wild-type and *lepr-/-* female zebrafish, aiming to identify leptin-dependent factors, regulating oocyte maturation and ovulation. Before the sampling and similarly as in **Paper III**, couples of wild-type males and females, as well as couples of wild-type males and *lepr-/-* females were mated frequently and their fecundity was estimated.

Comparing to the wild-type females, the majority of the mutant females laid both in total and per spawning event significantly fewer eggs, with lower fertilization rates. However, some of the mutant females were laying more eggs and did not differ from the wild-types in none of the fecundity parameters analysed. This is why, in further analysis, the *lepr-/-* females were divided into

two subgroups: those laying few eggs and those laying more eggs. Several DEGs were identified in the samples of fully grown follicles. For instance, between wild-types and mutants laying few eggs, *gadd45ab*, *pla2g4f.2* and *thbsb4b*, which are crucial transcriptomic signatures for ovulation in zebrafish, were expressed at higher levels in the wild-types. Interestingly, 4 known genes (*cdh30*, *fancm*, *mb*, *si:ch211-269c21.2*) and one uncharacterized protein encoding gene (ENSDARG00000091793) were exclusively downregulated in the fully grown follicles from mutant females laying few eggs. Based on the results from the Gene Set Enrichment Analysis (GSEA), in the samples from mutants laying few eggs, genes related to estrogen regulation, ribosome biogenesis, mRNA translation and fatty acid beta-oxidation were downregulated, compared to the wild-types. However, only genes related to ribosome biogenesis and mRNA translation were downregulated, when these samples were compared to those from mutants laying more eggs.

Taken altogether, the results from **Paper IV** linked the reproductive deficiencies in *lepr-/-* female zebrafish mainly to downregulation of genes related to estrogen responses, mRNA translation, ribosome biogenesis and lipid metabolism. Genes related to these pathways are essential for ensuring a good egg quality in female teleosts. Therefore, the results from the present study supported further the indications of lower egg quality in the *lepr-/-* females.

Discussion

The role of leptin in appetite regulation in zebrafish

In mammals, the anorexigenic functions of leptin are well established. Leptin can mediate, among others, the transcription of appetite-regulating neuropeptides, by stimulating the anorexigenic and inhibiting the orexigenic neurons in the ARC area of the hypothalamus, controlling thus food intake (Sobrino Crespo et al. 2014; Park and Ahima 2015; Sohn 2015). However, in teleost fish, leptin seems to have a more heterogeneous role in appetite regulation, with species-specific actions (Blanco and Soengas 2021).

In **Paper I**, mRNA expression levels of orexigenic and anorexigenic genes in the brain of adult zebrafish were influenced by active leptin signaling. Among the orexigenic genes, increased transcripts of *agrp* were observed in the *lepr* mutant fish compared to wild-types during fasting, suggesting that leptin supresses *agrp* expression in wild-type zebrafish in conditions of reduced food availability, which is in agreement to results from other studies (Chisada et al. 2014; Yan et al. 2016; Audira et al. 2018; Yuan et al. 2020). Similarly, in rats and *ob/ob* mice, both brain mRNA expression and protein levels of AgRP are induced during fasting and they are downregulated after leptin administration (Hahn et al. 1998; Mizuno and Mobbs 1999; Korner et al. 2001; Morrison et al. 2005; Baver et al. 2014). However, no significant effects of leptin signaling in none of the feeding conditions were found for *npy* transcripts in the zebrafish brain, which is in contrast to what has been reported in rodents (Stephens et al. 1995; Ahima et al. 1996; Hahn et al. 1998; Elias et al. 1998, 1999; Korner et al. 2001; Morrison et al. 2005; Lee et al. 2013; Baver et al. 2014) and other teleost species (Volkoff et al. 2003; Murashita et al. 2008; Aguilar et al. 2010; Li et al. 2010; Chisada et al. 2014; Yan et al. 2016; Liu et al. 2018a; Yuan et al. 2020).

Leptin signaling had stronger actions on the transcription of the anorexigenic genes. Impaired leptin signaling resulted in decreased transcripts of several anorexigenic genes under normal feeding, including all the members of the *cart* gene family, *crhb*, *gnrh2*, *mc4r*, *pomc* and *spx* (**Paper I**). Similar regulatory connections between leptin and the afore-mentioned genes have been reported in other vertebrates. For instance, leptin regulates *cart* transcription in the brain of rodents (Kristensen et al. 1998; Lee et al. 2013) and teleosts

(Volkoff and Peter 2001; Volkoff et al. 2003; Gong et al. 2016; Yan et al. 2016; Yuan et al. 2020), *crh* mRNA levels in the brain of rats (Schwartz et al. 1996), *gnrh2* brain expression in pikeperch (*Sander lucioperca*) (Schaefer and Wuertz 2016), as well as *mc4r* transcripts in the brain of mice (Ghamari-Langroudi et al. 2011). Leptin is also a key regulator of POMC, both at the transcription and protein level in rodents (Schwartz et al. 1997; Thornton et al. 1997; Mizuno et al. 1998; Elias et al. 1999; Cowley et al. 2001; Balthasar et al. 2004) and teleosts (Murashita et al. 2008; Chisada et al. 2014; Gong et al. 2016; Yan et al. 2016; Yu et al. 2020b). Our results suggest that conserved regulatory connections between leptin and the afore-mentioned anorexigenic genes exist also in the zebrafish brain.

One of the most notable findings in **Paper I** was the similar expression patterns between the *cart* genes, *crhb* and *gnrh2* in the wild-type zebrafish, which were all lost in the *lepr* mutants. Except from their link to leptin, as described above, regulatory connections among these genes have also been reported in other vertebrates. Specifically, CRH is a downstream target of CART expression in the brain of rats (Sarkar et al. 2004; Smith et al. 2004) and CRH administration can stimulate *cart1* expression in the hypothalamus *in vivo*, as well as in cultured pituitary cells *in vitro* in chickens (Mo et al. 2015). Moreover, in goldfish, ICV administration of CRH induces *gnrh2* transcripts in the hypothalamus (Kang et al. 2011) and similar expression patterns between *crh* and *gnrh2* in response to feeding were recently reported in *Schizothorax davidi*, another Cypriniformes species (Yuan et al. 2021).

Analysing the regulatory connections between the *cart* genes, *crhb* and *gnrh2*, revealed a potential leptin-dependent GRN among these genes also in the zebrafish brain (**Paper II**). The *cart1/2/3/4* genes were predicted to form a network with *ckmt1*, *pik3ip1*, *sat1a*.2, *agr2* and *tcima*; *crhb* with *cdh13*, *cort*, *nppcl* and *oxt*; and *gnrh2* with *pmchl* (**Paper II**). Information about the functions of these gene modules in appetite regulation is limited. In mammals, *cdh13*, *cort* and *oxt* mediate locomotor activity and feeding behaviour (Spier and de Lecea 2000; Onaka et al. 2012; King et al. 2017; Kiser et al. 2019), however in teleosts, an association to appetite regulation has only been suggested for *ckmt1* and *pmchl*. In particular, *ckmt1* transcripts were stimulated in the muscle of zebrafish fed with a high-carbohydrate diet (Ma et al. 2020), whereas mRNA levels of *pmchl* were elevated after fasting in the brain of the starry flounder (*Platichthys stellatus*) (Kang and Kim 2013). However, studies linking all these genes to leptin are still lacking in teleosts.

Furthermore, the regulatory connections between the gene modules forming the suggested GRN in the zebrafish brain were also predicted to be mediated by the transcription factor *sp3a*, under leptin signaling (**Paper II**). In humans,

the orthologous proteins Sp1 and Sp3 control lipid metabolism and the pathogenesis of obesity in the adipose tissue (Barth et al. 2002; Hoffmann et al. 2013). Interestingly, leptin enhances the regulatory effects of Sp1 and Sp3 on the transcription of their downstream target genes, either by increasing their binding affinity to their regulatory elements on the promoters of their target genes or by direct induction of the Sp1 and Sp3 proteins (Lin et al. 2006; García-Ruiz et al. 2012). Hence, based on our results, a regulatory connection between leptin and *sp3a* seems to be conserved also in the zebrafish brain.

The role of leptin in reproductive physiology in zebrafish

In mammals, leptin is a key factor in the regulation of pubertal onset and adult fertility (Barash et al. 1996; Cheung et al. 1997; Tena-Sempere 2007; Roa et al. 2010). Yet, its role in the regulation of reproduction in teleosts is still by far understood (Parker and Cheung 2020).

Impaired leptin signaling resulted in severe reproductive deficiencies in female, but not in male zebrafish. *lepr* mutant female zebrafish were spawning fewer eggs with low fertilization rates and had also lower *lhb* pituitary expression, compared to their wild-type siblings (**Paper III**). In teleosts, LH is considered the key regulator of oocyte maturation and ovulation (Patiño and Sullivan 2002; Nagahama and Yamashita 2008; Levavi-Sivan et al. 2010; Li and Cheng 2018). Studies using *lhb* deficient zebrafish (Chu et al. 2014; Zhang et al. 2015; Shang et al. 2019) and medaka (Takahashi et al. 2016) reported the same phenotype, as observed in our mutant fish, in which folliculogenesis was normal, but oocytes failed to mature and be ovulated. These results suggest that the impaired fertility in the *lepr* mutant females could be linked to the low LH transcripts in the pituitary and that leptin might regulate LH release at the pituitary level in female zebrafish, similarly to what was observed in mammals (Garris et al. 2005; Donato et al. 2011; Akhter et al. 2014; Odle et al. 2018; Tu et al. 2018). In other teleosts, *in vitro* studies have shown that administration of recombinant leptin enhances LH release (Peyon et al. 2001; Weil et al. 2003; Ohga et al. 2020) and similar results were also recently reported after ICV leptin administration *in vivo* (Ohga et al. 2020). However, the regulatory mechanisms between leptin and LH remain unknown.

In addition, genes related to steroidogenesis, oocyte maturation and ovulation were either up- (*cpla2*, *hsd3b1*, *mmp15a*, *pgr*, *ptger4b*, *star*) or down-regulated (*mprb* and *pgrmc2*) in the ovaries of the *lepr* mutant females (**Paper III**). In mammals and teleosts, the transcription of most of these genes is under the regulation of LH signaling, as it was suggested for *cpla2* (Tang et al. 2016),

hsd3b1 (Shang et al. 2019), *mprb* (Yumnamcha et al. 2017), *pgr* (Kim et al. 2009; Akison and Robker 2012; Gal et al. 2016), *pgrmc2* (Vaitsopoulou et al. 2021), *ptger4b* (Tang et al. 2016) and *star* (Ings and Van Der Kraak 2006; Chu et al. 2014; Nakamura et al. 2016; Shang et al. 2019). Therefore, the differential expression of the afore-mentioned genes observed in the mutant ovaries could have occurred as a response to the lower LH transcript levels in the pituitary of these females.

Nonetheless, the differential expression of these genes could also indicate that leptin has direct actions on the gonadal level in female zebrafish, similarly as in mammals (Tena-Sempere and Barreiro 2002; Kendall et al. 2004). Yet, there are currently no studies in teleosts supporting this hypothesis or linking these genes to leptin. Recently, it was reported that short-term leptin administration stimulated the transcription of *pgr* in the uterine tissue of ovariectomized mice (Shetty et al. 2020). However, a direct link between leptin and *pgr* or any other of these genes is still unknown, both in mammals and teleosts.

In **Paper III**, no differences were found in the mRNA expression of any of the afore-mentioned genes between the two genotypes, when they were analysed in isolated fully grown follicles. In species like zebrafish, which are asynchronous spawners (Selman et al. 1993), gene expression analysis in the entire ovary reflects changes related to a mixture of follicles from different developmental stages and depends on the number of follicles from each stage present in the ovary at the time of sampling. Therefore, in order to get a better understanding of possible leptin-dependent factors resulting in impaired oocyte maturation and ovulation in the *lepr* mutant females, we performed RNA-seq in samples of isolated fully grown follicles from wild-type and *lepr* mutant females (**Paper IV**).

The RNA-seq data revealed a large number of DEGs in the samples of fully grown follicles, linking the reproductive deficiencies in the *lepr* mutant females to the downregulation of genes related to essential metabolic pathways for female reproduction, such as estrogen regulation, mRNA translation, ribosome biogenesis and lipid metabolism (**Paper IV**). Similar results were reported in transcriptomic studies in zebrafish (Zheng et al. 2013; Liu et al. 2017) and common carp (Liu et al. 2021), in which the important role of these pathways in the regulation of oocyte maturation and ovulation was also identified. In mammals, estrogens can also regulate ribosomal function and biogenesis, as well as lipid metabolism and beta-oxidation (Zhou et al. 2012; Ray et al. 2013; Bicker et al. 2015). Therefore, our results suggest the existence of conserved interactions between the afore-mentioned metabolic pathways also in zebrafish.

The most affected biological processes in the *lepr* mutant females laying few eggs were related to cellular responses to estrogens (**Paper IV**). A crosstalk between the leptin and estrogen systems has already been suggested in mammals for the regulation of several physiological aspects, including reproduction (Catalano et al. 2004; Gao and Horvath 2008; Fusco et al. 2010; Nestor et al. 2014). However, information in teleosts is still scarce. For instance, estrogen treatment had stimulatory effect on the transcription of leptin paralogues in Atlantic salmon (Trombley et al. 2015) and the white-clouds mountain minnow (*Tanichthys albonubes*) (Chen et al. 2016). Moreover, in ayu, the plasma levels of estradiol and leptin showed similar expression profiles during the reproductive cycle (Nagasaka et al. 2006). Therefore, the low enrichment scores of genes related to these biological processes could have occurred due to the impaired leptin signaling.

In subfertile *lepr* mutant females, genes related to mRNA translation, as well as to ribosome functions and biogenesis were also downregulated (**Paper IV**). The activation of leptin signaling initiates an evolutionary conserved cascade of molecular processes, by which ribosomal function is regulated through the ERK/RSK (Gong et al. 2007; Villanueva and Myers 2008; Denver et al. 2011; Londraville et al. 2017) and mTORC1 pathways (Fingar and Blenis 2004; Lynch et al. 2006; Villanueva and Myers 2008). Thus, the lower enrichment scores of genes involved in ribosome biogenesis and protein translation in the *lepr* mutants laying few eggs could be affiliated to the impaired leptin signaling.

Lastly, genes related to fatty acid metabolism and beta-oxidation were downregulated in both *lepr* mutant subgroups, regardless the number of laid eggs (**Paper IV**), suggesting that leptin mediates beta-oxidation also in zebrafish, as observed in rodents (Muoio et al. 1997; Steinberg et al. 2002) and the yellow catfish (*Pelteobagrus fulvidraco*) (Song et al. 2018). In mammals, beta-oxidation is stimulated during oocyte maturation and ovulation (Downs et al. 2009; Dunning et al. 2010, 2011; Valsangkar and Downs 2013) and treatment with fatty acids promotes ovulation (Broughton et al. 2010; Dunning et al. 2011). However, similar studies in fish are still limited (Manor et al. 2015a,b; Song et al. 2018). Furthermore, in teleosts, lipids and fatty acids are stored within the yolk (Wiegand 1996; Fraher et al. 2016) and represent an important energy source for the oocytes, because they generate high ATP yields when they are metabolized during beta-oxidation (Dunning et al. 2014; Sant and Timme-Laragy 2018). They are also utilized for the synthesis of prostaglandins, which are key regulatory molecules for ovulation (Takahashi et al. 2018). Consequently, the downregulation of genes related to these pathways in the mutant females could also explain the lower egg quality and impaired ovulation among the *lepr* mutant females observed in **Paper III**.

Conclusions and future perspectives

The role of leptin in appetite regulation in zebrafish

In conclusion, the results from **Papers I and II** suggested that, similarly to mammals, leptin has anorexigenic functions in zebrafish. Moreover, we provided for the first time in zebrafish, evidence for the effect of leptin signaling on the transcription of appetite-regulating genes, as well as on the control of a potential GRN in the brain, which is involved in the behavioural and metabolic regulation of feeding (Fig. 5). However, additional studies are required to clarify the exact role of leptin in appetite regulation in zebrafish.

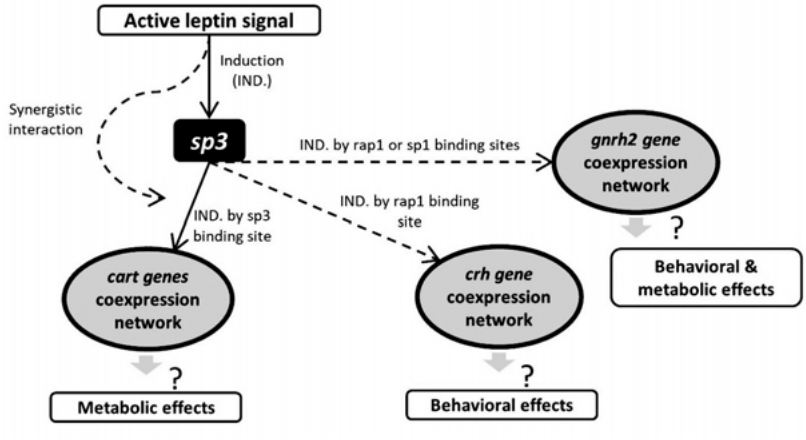

Figure 5. Potential regulatory interactions between leptin signaling and *cart-*/*crhb-*/*gnrh2-* co-expression modules, mediated by *sp3a* in the zebrafish brain (from Ahi et al. 2021).

Studies using overfeeding conditions could help towards a more comprehensive understanding of leptin-dependent regulation of feeding. Female zebrafish grow larger in size and weight compared to male fish, but sex-dependent differences in feeding and the possible role of leptin in this process are not known yet. The use of high-throughput methods, such as transcriptomics or proteomics, could also provide insights into more leptin-dependent factors in the brain controlling appetite. Furthermore, studies in peripheral tissues with crucial role in feeding regulation, such as the intestine, could reveal feedback mechanisms and crosstalk between these tissues and the brain. Structural

and functional characterization of the gut microbial communities in *lepr* mutants could also suggest possible associations between leptin and the microflora for the regulation of several physiological aspects, including appetite and reproduction.

The role of leptin in reproductive physiology in zebrafish

In conclusion, **Papers III and IV** provided, for the first time, evidence that leptin can regulate female reproduction in a fish model *in vivo*, possibly acting on the pituitary level, by mediating LH release and/or on the gonadal level, by stimulating important metabolic pathways for the maturation and ovulation of the growing oocytes (Fig. 6). However, further studies are needed, in order to interpret the role of leptin in female reproduction in zebrafish.

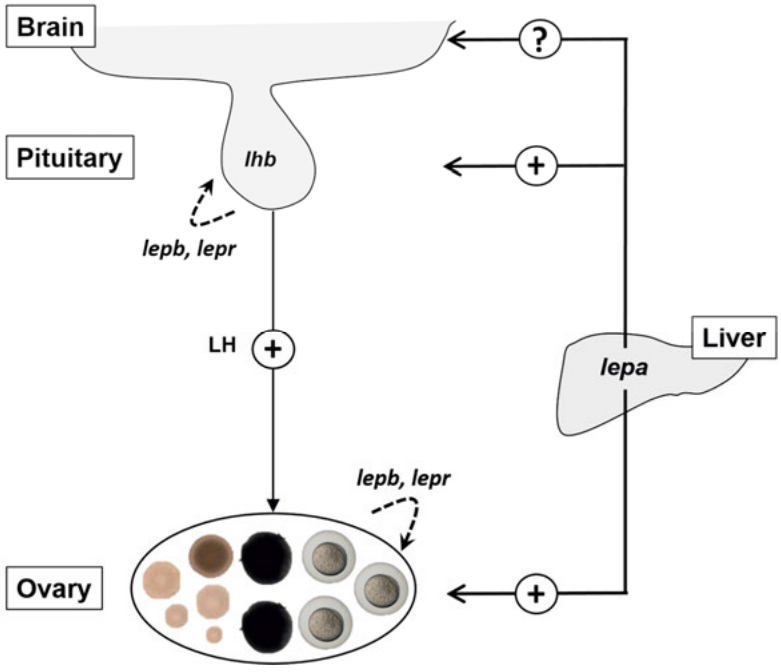

Figure 6. Putative model of the stimulatory effects (+) of leptin signaling on the different centers of the reproductive axis (brain, pituitary, ovary) in female zebrafish. Possible paracrine actions are indicated with dashed lines, while unknown effects with question marks (?).

So far, it is still unknown in which cell types the genes of the leptin system are expressed in the ovaries, so knowledge about their location could give further insights into possible regulatory mechanisms, including also paracrine

regulation. An investigation of possible GRNs in the pituitary could also contribute to an understanding of the leptin-dependent molecular mechanisms regulating LH transcription. Furthermore, a comprehensive transcriptome analysis of all the follicular developmental stages could point out stages, which are mostly affected by the *lepr* deficiency and thus, better track the actions of leptin during follicular development. The identification and quantification of yolk lipids in fully grown follicles and/or mature oocytes could also point out leptin-dependent factors mediating oocyte quality.

Swedish summary/Svensk sammanfattning

Hos däggdjur är leptin en perifer metabolisk signal och en kritisk regulator för energibalansen. Leptin påverkar aptitcentrum i hypotalamus i hjärnan och orsakar anorexigena effekter, detta eftersom det undertrycker matintaget. Dessutom spelar leptin en viktig roll i regleringen av reproduktion. Detta genom att skicka signaler till hjärnan för att stimulera reproduktionsaxeln om organismen har förvärvat tillräckliga energireserver som ska investeras för reproduktionsändamål. Dock är leptins roll i fiskens fysiologi fortfarande inte välstuderad. Denna doktorsavhandling undersökte den möjliga rollen som leptin har vid reglering av aptit och reproduktion hos äkta benfiskar genom användning av en zebrafisk mutant (*lepr* sa12953), där leptinreceptorn är inaktiverad.

För att studera aptitreglering analyserade vi genuttrycket av 36 gener vilka är kända för att ha aptitreglerande funktioner hos fiskar (12 orexigena och 24 anorexigena). Dessa studerades i hjärnan hos vildtyp och *lepr* mutanter under fyra utfodringsförhållanden (normal utfodring, 7-dagars fasta samt matning efter 2 och 6 timmar efter en 7-dagars fasta). Transkriptionen av orexigena och anorexigena gener påverkades av leptinsignalering i zebrafiskhjärnan under dessa utfodringsförhållanden. Medan transkriptionsnivåerna för de analyserade orexigena generna inte påverkades under normal utfodring uppreglerades några gener i hjärnan hos mutanterna under fasta (*agrp* och *galr1a*) och vid matning efter fasta (*apln*, *cnr1* och *trh*). Dessa resultat tyder på en hämmande effekt av aktiv leptinsignalering på transkriptionen av orexigena gener under kortvarig fasta och matning efter fasta i vildtypszebrafiskar. Emellertid hittades tydligare effekter bland de anorexigena generna. Nedsatt leptinsignalering resulterade i reducerat hjärnuttryck av flera gener (*cart1/2/3/4*, *crhb*, *gnrh2*, *mc4r*, *pomc* samt *spx*) under normala utfodringsförhållanden, vilket tyder på en stimulerande effekt av aktiv leptinsignalering i vildtyp zebrafiskar.

Dessutom föreslogs ett leptinberoende genreglerande nätverk involverat i beteendemässig och metabolisk aptitkontroll hos zebrafiskhjärnan. Det inräknar *cart1,2,3,4*/*crhb*/*gnrh2* generna och deras respektive samuttryckta gener (*ckmt1*, *pik3ip1*, *sat1a.2*, *agr2*, *tcima* med *cart* generna; *cdh13*, *cort*, *nppcl*, *oxt* med *crhb*; *pmchl* med *gnrh2*). Dessa resultat tyder på möjliga reglerande kopplingar mellan dessa genmoduler i zebrafiskhjärnan. Vi förutspådde också

flera transkriptionsfaktorer som potentiella uppströmsregulatorer av dessa reglerande kopplingar, men endast *sp3a* och *krox24* hade liknande uttrycksmönster med de ovannämnda genmodulerna. Emellertid var *sp3a* den enda transkriptionsfaktorn med positiva korrelationer med alla dessa gener.

När det kommer till regleringen av reproduktion visade det sig att nedsatt leptinsignalering ledde till tydliga försämringar hos honorna men inte hos hanarna. Histologisk analys avslöjade att follikulogenes inte påverkades medan oocytmognad och ägglossning störtes hos *lepr* mutanter vilket resulterade i färre lagda ägg. Dessutom var transkription av luteiniserande hormon beta (*lhb*) i hypofysen lägre hos mutanterna. Analys av kandidatgener avslöjade också differentiellt uttryck av gener i äggstockarna hos *lepr* mutanterna, vilka är involverade i steroidogenes, oocytmognad och ägglossning. Detta innebär att leptin kanske medverkar i de sista stegen av follikulär utveckling hos zebrafisk. Alla dessa gener som var upp- (*cpla2*, *hsd3b1*, *mmp15a*, *pgr*, *ptger4b*, *star*) eller ned-reglerade (*mprb*, *pgrmc2*) är också nedströms mål för LH-signalering hos äkta benfiskar. Emellertid sågs inga skillnader mellan de två genotyperna när dessa gener analyserades i isolerade fullvuxna folliklar.

Därtill utförde vi transkriptomanalys i isolerade fullvuxna folliklar från vildtyp och *lepr* mutants honor i syfte att identifiera mer leptinberoende faktorer som reglerar äggmognad och ägglossning. Flera differentiellt uttryckta gener identifierades mellan fullvuxna folliklar. Till exempel, *gadd45ab*, *pla2g4f.2* och *thbsb4b*, som är grundläggande faktorer under ägglossning, var högre uttryckt i vildtypsgruppen än i mutanterna. Intressant nog var 4 kända gener (*cdh30*, *fancm*, *mb*, *si:ch211-269c21.2*) och en okarakteriserad proteinkodande gen (ENSDARG00000091793) uteslutande nedreglerad enbart i de fullvuxna folliklarna från de mutanter som lade få ägg. Dessutom kopplades reproduktionsbrister till nedreglering av gener relaterade till viktiga metaboliska vägar för oocytmognad och ägglossning, såsom östrogenreglering, fettsyraoxidation, ribosombiogenes och mRNA-translation i ribosomer jämfört med vildtypen.

Sammanfattningsvis gav resultaten från denna doktorsavhandling för första gången bevis för att leptin är involverat i zebrafisk aptitreglering genom att förmedla transkriptionen av aptitreglerande gener och ett GRN hos zebrafiskhjärnan. Likväl visades det att leptin utgör en kritisk regulator för honlig reproduktion, särskilt vid oocytmognad och ägglossning.

Greek summary/Περίληψη στα ελληνικά

Στα θηλαστικά, η λεπτίνη αποτελεί έναν κρίσιμο ρυθμιστή της ενεργειακής ισορροπίας. Παράγεται στο λιπώδη ιστό, έπειτα εκκρίνεται στο κυκλοφορικό σύστημα και καταλήγει στον υποθάλαμο του εγκεφάλου. Εκεί, δεσμεύεται στον υποδοχέα λεπτίνης και επηρεάζει τα κέντρα όρεξης, καταστέλλοντας την πρόσληψη τροφής και προκαλώντας ανορεξιγόνες αποκρίσεις. Επίσης, κατέχει σημαντικό ρόλο στη ρύθμιση της αναπαραγωγής, διαβιβάζοντας σήματα στον εγκέφαλο, ώστε να διεγείρει το αναπαραγωγικό σύστημα, εφόσον ο οργανισμός αποκτήσει επαρκή ενεργειακά αποθέματα για αναπαραγωγική επένδυση. Ωστόσο, ο ρόλος της στη φυσιολογία των ψαριών δεν είναι ακόμη γνωστός. Η παρούσα διδακτορική διατριβή διερεύνησε τον πιθανό ρόλο της λεπτίνης στη ρύθμιση της όρεξης και της αναπαραγωγής στους τελεόστεους, χρησιμοποιώντας ένα μεταλλαγμένο στέλεχος του ψαριού-ζέβρα με μειωμένη λειτουργία του υποδοχέα λεπτίνης (*lepr* sa12953).

Σχετικά με τη ρύθμιση της όρεξης, μελετήθηκε η έκφραση 36 γονιδίων (12 ορεξιογόνων και 24 ανορεξιογόνων) στον εγκέφαλο ψαριών-ζέβρα με φυσιολογική και μειωμένη λειτουργία υποδοχέα λεπτίνης, υπό τέσσερεις διατροφικές καταστάσεις (κανονική σίτιση, ασιτία 7 ημερών, 2 και 6 ώρες έπειτα από ασιτία 7 ημερών και ανατροφοδότηση). Παρότι τα επίπεδα μεταγραφής των ορεξιογόνων γονιδίων δεν επηρεάστηκαν υπό φυσιολογική σίτιση, ορισμένα γονίδια παρουσίασαν αυξημένα επίπεδα μεταγραφής έπειτα από ασιτία (*agrp*, *galr1a*) και επανατροφοδότηση (*apln*, *cnr1*, *trh*). Ωστόσο, πιο έντονες διαφορές παρατηρήθηκαν μεταξύ των ανορεξιογόνων γονιδίων, καθώς το μειωμένο σήμα λεπτίνης είχε ως αποτέλεσμα τη μειορρύθμιση περισσότερων γονιδίων (*cart1/2/3/4*, *crhb*, *gnrh2*, *mc4r*, *pomc*, *spx*). Τα συγκεκριμένα αποτελέσματα υποδηλώνουν ανασταλτική επίδραση του ενεργού σήματος λεπτίνης στη μεταγραφή ορεξιογόνων και διεγερτική επίδραση στη μεταγραφή ανορεξιογόνων γονιδίων στο ψάρι-ζέβρα.

Επιπλέον, ένα γενετικό ρυθμιστικό δίκτυο, εξαρτώμενο από τη λεπτίνη και εμπλεκόμενο στον έλεγχο της όρεξης, βρέθηκε στον εγκέφαλο του ψάριού-ζέβρα, περιλαμβάνοντας τα γονίδια *cart1,2,3,4*/*crhb*/*gnrh2* και τα συνεκφραζόμενα γονίδιά τους (*ckmt1*, *pik3ip1*, *sat1a.2*, *agr2*, *tcima*; *cdh13*, *cort*, *nppcl*, *ox*; *pmchl*, αντιστοίχως). Επίσης, προβλέφθηκανμεταγραφικοί παράγοντες, οι οποίοι ελέγχουν τις ανωτέρω ρυθμιστικές σχέσεις, αλλά μόνο οι *sp3a* και

krox24 εμφάνισαν παρόμοια πρότυπα έκφρασης με τα προαναφερθέντα γονίδια. Ωστόσο, ο *sp3a* αποτελεί το μόνο μεταγραφικό παράγοντα, του οποίου η έκφραση συσχετίστηκε θετικά με την έκφραση των ανωτέρω γονιδίων.

Σχετικά με τη ρύθμιση της αναπαραγωγής, το μειωμένο σήμα λεπτίνης οδήγησε σε έντονες αναπαραγωγικές ανεπάρκειες μόνο στα θηλυκά και όχι στα αρσενικά ψάρια-ζέβρα. Η ιστολογική ανάλυση υπέδειξε ότι η ωοθυλακιογένεση δεν επηρεάστηκε στα μεταλλαγμένα θηλυκά ψάρια, τα οποία όμως παρουσίασαν χαμηλότερα επίπεδα ωορρηξίας, καθώς και μειωμένα αντίγραφα του γονιδίου της ωχρινοτρόπου ορμόνης (*lhb*) στην υπόφυση. Επιπρόσθετα, στις ωοθήκες των συγκεκριμένων ψαριών, παρατηρήθηκε διαφορική έκφραση γονιδίων, τα οποία εμπλέκονται στην παραγωγή των στεροειδών, την ωρίμανση των ωαρίων και την ωορρηξία και είναι επίσης κατάντη στόχοι της ωχρινοτρόπου ορμόνης (*cpla2*, *hsd3b1*, *mmp15a*, *mprb*, *pgr*, *pgrmc2*, *ptger4b*, *star*). Ωστόσο, παρόμοιες διαφορές δεν παρατηρήθηκαν, όταν τα ίδια γονίδια αναλύθηκαν σε απομονωμένα ανεπτυγμένα ωοθυλάκια.

Προκειμένου να εντοπιστούν περισσότεροι παράγοντες που εξαρτώνται από τη λεπτίνη και ρυθμίζουν την ωρίμανση των ωαρίων και την ωορρηξία, πραγματοποιήθηκε μεταγραφική ανάλυση σε απομονωμένα ανεπτυγμένα ωοθυλάκια ψαριών-ζέβρα με φυσιολογικό και μειωμένο σήμα λεπτίνης. Διαφορική έκφραση ταυτοποιήθηκε για εκατοντάδες γονίδια μεταξύ των δύο γονοτύπων. Για παράδειγμα, τα γονίδια *gadd45ab*, *pla2g4f.2* και *thbsb4b*, που είναι θεμελιώδεις παράγοντες ωορρηξίας, εμφάνισαν χαμηλότερα επίπεδα έκφρασης στα μεταλλαγμένα ψάρια. Ακόμη, 4 γνωστά γονίδια (*cdh30*, *fancm*, *mb*, *si:ch211-269c21.2*) και ένα γονίδιο που κωδικοποιεί μία άγνωστη πρωτεΐνη (ENSDARG00000091793) εμφάνισαν χαμηλότερα επίπεδα έκφρασης αποκλειστικά και μόνο στα ωοθυλάκια μεταλλαγμένων ψαριών με αναπαραγωγικές ανεπάρκειες. Επίσης, τα αποτελέσματα της μεταγραφικής ανάλυσης συνέδεσαν τις αναπαραγωγικές ανεπάρκειες με δυσλειτουργίες σημαντικών μεταβολικών μονοπατιών κατά την ωρίμανση και ωορρηξία ωοκυττάρων, όπως τη ρύθμιση της δράσης των οιστρογόνων, την οξείδωση λιπαρών οξέων, τη μετάφραση και παραγωγή πρωτεϊνών, καθώς και τη γένεση και λειτουργία ριβοσωμάτων.

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