Endocrine Control of Growth and Reproduction in Zebrafish

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1 LIST OF ABBREVIATIONS

AF	Atretic follicle
AGRP	Agouti-related peptide
BAC	Bacterial Artificial Chromosome
CART	Cocaine amphetamine-regulated transcript protein
CG	Compensatory growth
CGA	Glycoprotein hormone
CISH	Cytokine-inducible SH2 domain-containing protein
CNS	Central nervous system
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DHP	17a,20b-dihydroxy-4-pregnen-3-one
DIO	Diet-induced Obesity
dpf	Days post fertilization
dpp	Days post purging
ENU	N-ethyl-N-nitrosourea
ERK	Extracellular signal regulated kinases
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone-releasing hormone
GVBD	Germinal Vesicle Breakdown
GWAS	Genome-wide Association Studies
HPG	Hypothalamic-Pituitary-Gonadal
IGF	Insulin like growth factor
JAK	Janus Kinases
LEP	Leptin
LEPR	Leptin receptor
LH	Luteinizing hormone
LOF	Loss of function
MAPK	Mitogen-activated protein kinase
MC3R	Melanocortin receptor 3
MC4R	Melanocortin receptor 4
MIH	Maturation inducing hormone
MPF	Maturation promoting hormone
mRNA	messenger RNA
NMD	Nonsense-mediated mRNA decay
NPY	Neuropeptide Y
PGC	Primordial germ cell
PI3K	phosphatidylinositol 3-kinase
POMC	Pro-opiomelanocortin
PTP	Protein tyrosine phosphatase
PVN	Paraventricular nucleus of Hypothalamus

RNA	Ribonucleic acid
RMR	Routine metabolic rate
SAT	Sub-cutaneous adipose tissue
SH2	Src Homology 2
SMR	Standard metabolic rate
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
STDEV	standard deviation
TALEN	transcription activator-like effector nucleases
TCPTP	T cell protein tyrosine phosphatase
TILLING	Targeting induced local lesions in genomes
TSH	Thyroid stimulating hormone
TYK2	tyrosine-protein kinase 2
TYR	Tyrosine residues
UAS	Upstream activation sequence
VAT	Visceral adipose tissue
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WT	Wild type
α-MSH	Alpha-melanocyte stimulating hormone

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3 ABSTRACT

Energy is the basic currency required for all living organisms. In order to ensure homeostatic supply of energy, various neuro-endocrine systems have evolved in vertebrates, that control food intake, metabolism, and energy expenditure. Even minor discrepancies in the sensitive balance of these systems may result in severe pathologies. Obesity and metabolic disorders are often caused due to disturbances in the homeostasis of energy and is rapidly rising across the globe. Although diet and lifestyle certainly are one of the major factors influencing development of obesity, genetic and epigenetic factors also largely contribute. Monogenic mutations in leptin-melanocortin system have been associated with severe disruptions in the energy homeostasis pathway. In order to mitigate these diseases, better understanding of their basic biology and etiology is crucial.

Researches in last decade have relied on diverse animal models to unravel the complex interaction of environment and genetic factors. In this study, we used zebrafish as a model to study the metabolic endpoints caused as a result of long-term obesogenic diet, as well as early lifetime caloric restriction. Our results in publication 1 showed that long-term feeding in zebrafish lead to obesity hallmarks and metabolic phenotypes similar in mammals. While early caloric restriction did not result in exacerbated compensation of growth and other metabolic syndromes in adulthood, we observed phenotypes of catch-up growth in fish that were introduced to obesogenic diets, which caught up with growth and metabolic endpoints comparable to diet-induced obese fish.

The Leptin system plays an important role in maintenance of energy homeostasis. In fish, the role of leptin is controversial and is not considered as an adipostat. To understand the functional role of leptin in obesity and in fat regulation, we used three leptin receptor (*lepr)* and one leptin a (*lepa)* loss of function (LOF) lines. To validate the LOF alleles, we measured *socs3a* transcription levels after administration of recombinant mouse leptin and found impaired response in all the LOF alleles. Our results showed that none of the alleles led to obesity or altered growth phenotypes.

Given the absence of morbid obesity or drastic body growth phenotypes in *lepr* mutant strains, we were curious to investigate the role of leptin in regulation of reproduction using *lepr*^{sa1508} mutant zebrafish line in the publication 3. We found that mutations in the *lepr* gene caused dysregulation of gonadotropins in the pituitary. Despite an impaired HPG axis, these mutants did not show subfertility. However, they exhibited a delay in ovarian maturation and/or an increased rate of follicular atresia compared to their wild type siblings. In support of this, we found downregulation of candidate genes involved in the process of maturation and upregulation of genes involved in follicular atresia in the LOF line. Next, we elucidated the peripheral or direct role of leptin on the ovarian cells using an *in vitro* germinal vesicle breakdown (GVBD) assay. In cultured oocytes, leptin promoted GVBD and attenuated the rate of oocyte degradation. Additionally, we found *lepr* LOF abates the effect of maturation inducing hormone, 17α-20β-dihydroxy-4 pregnen-3-one (DHP), leading to reduced GVBD rates in ovarian cultures of mutants in comparison to wild type. In conclusion, we found that leptin has a central as well as peripheral role in the regulation of reproduction in zebrafish. On the long run, these findings will provide insights into the role of leptin in energy mobilisation in fish during reproduction.

Alongside, we also studied the central regulation of melanocortin system on body growth and reproduction in zebrafish. Previous research has shown that Agrp neurons are hypophysiotropic and regulate the expression of multiple endocrine axes. Therefore, in our study, we used the Agrp1 LOF model to investigate somatic growth and reproduction in adult zebrafish. We found, despite significant reduction in genes of growth and reproductive axes, adult zebrafish exhibited normal body growth phenotype and normal reproduction. Further we tested for compensation by candidate genes of neuroendocrine axis but found no changes. We conclude that some unknown central and peripheral compensatory mechanisms might allow for normal growth and reproductive function in Agrp1 LOF adult zebrafish, in spite of reduced levels of growth hormone (*gh)*, follicle stimulating hormone (*fsh*) and luteinising hormone (*lh*). Together these studies provide a systematic and functional analysis of the regulation of growth and reproduction across neuroendocrine axis in zebrafish.

4 INTRODUCTION

4.1 The obesity epidemic: its prevalence and causes

Obesity is an excessive accumulation of body fat, that has currently become a major global health concern due to its increasing incidence in both adults and children [1]. A combination of genetic and environmental factors influence the development of obesity. Although changes in the environment have indeed compelled the sharp rise in obesity, the genetic components accounting for 30-70% of the differences observed in obesity, plays a big role [2]. Body mass index (BMI) is a widely used estimate of total body fat and is calculated by dividing a person's weight (in kilograms) by the square of their height (in meters) [3, 4]. It is commonly accepted that an individual with a BMI of 30 kg/m^2 or higher is considered obese, and a BMI of 25 kg/m^2 or higher indicates being overweight [5, 6].

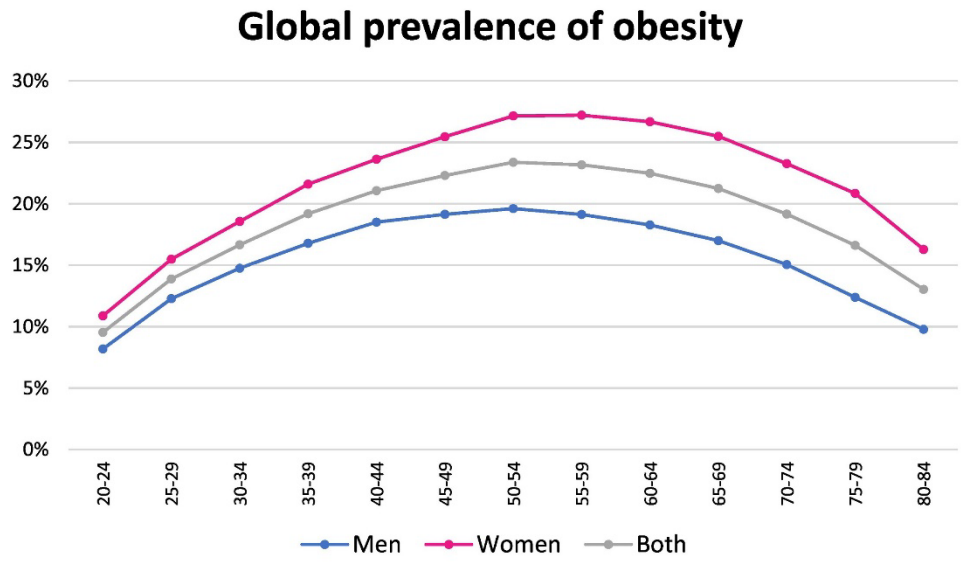

Figure 1: Global prevalence of obesity in adults.

The graph shows prevalence of obesity in adult (both males and females) over 20 years of age, according to the latest reports in 2019. Women in general show increased obesity rates, due to higher adipose depots in their body. Adapted from [7].

The global incidence of obesity varies between different population groups and is influenced by factors such as age, gender, race, financial status, education, dietary habits, physical activity, and mental health. The World Health Organization

(WHO) reported that the number of people with obesity has tripled globally since 1975 and considers obesity as one of today's most glaring but least addressed public health issues. In 2016, about 2 billion adults (39%) worldwide over the age of 18 were overweight, with 650 million (13%) considered obese [1, 8]. Additionally, an estimated 41 million children under 5 years old were either overweight or obese. Childhood obesity has increased tenfold from 1975 to 2016. Based on current trends, it is expected that 25% of children under 16 will be affected by obesity by 2050 [1, 9, 10]. Along with childhood obesity, the prevalence of maternal and gestational obesity is also on the rise globally. Pregnancy-related overnutrition and maternal body weight have been linked to an increased risk of metabolic syndrome in offspring [10, 11].

Obesity can result from a variety of genetic, behavioural, and environmental factors. The amount of body fat depends on the energy balance, the ratio between the amount of energy consumed and the amount of energy burned. It is generally accepted that obesity is the result of an imbalance between energy intake and energy expenditure caused by an increase in caloric intake and a decrease in energy output [3, 9]. This has undeniably been influenced by the modern era, which has led to the availability of high-calorie foods (high in fat and sugar) and a more sedentary lifestyle. [3, 12, 13]. However, obesity is not just influenced by diet, it is rather a combination of environmental factors and innate biological factors at interplay. It is crucial to note that a significant number of genetic factors account for the large fluctuations in body weight that individuals show in response to the obesogenic environment. The heritability factor of obesity, as estimated by twin and adoption studies is about 30 – 70% [14, 15]. Hence, genetic methods are important to understand the physiological and molecular mechanisms that govern body weight.

Monogenic mutations leading to severe obese phenotypes such as the LEPR mutations in people is a rare occurrence in the general population and cannot be solely responsible for the widespread obesity epidemic. Nevertheless, studying mutations in single genes in mouse models has enabled the discovery of key players involved in regulating energy homeostasis, such as the Melanocortin 4 receptor

(*Mc4r*), Proopiomelanocortin (*Pomc*), and Leptin (*Ob*) genes. And indeed, mutations in the *MC4R* are considerably more common with a prevalence of 0.5-1% in people with a BMI >30 and 5% in severe childhood obesity [16]. Multiple genetic determinants are said to play a role in common obesity, as evidenced by genome-wide association studies (GWAS). When combined with environmental factors that promote obesity, these genetic determinants are thought to contribute to the escalating prevalence of this disorder [15, 17].

4.1.1 Metabolic Comorbidities

Metabolic syndrome is a broad term used to describe a group of conditions that are linked to persistent obesity, such as type 2 diabetes, high cholesterol levels, insulin resistance, heart diseases, non-alcoholic fatty liver disease, chronic kidney diseases, and certain types of cancers [18, 19]. Metabolic syndromes are classified as non-communicable diseases and are now the leading cause of health problems globally. The occurrence of metabolic syndrome is often inter-dependent with the rise in obesity and type 2 diabetes, which are comorbid [20].

4.1.2 Diet-induced obesity (DIO)

One hypothesis to explain the pace of obesity epidemic is the profound availability of high-fat, high-density foods over the past 20–30 years, which have overwhelmed our regulatory mechanisms [21]. This particular type of obesity is known as diet-induced obesity (DIO). Many studies have attempted to characterize the responses of animals exposed to a high-fat diet in rodents [21, 22]. The common mice used for dissection of obesity are the C57BL/6J, also called B6 mice: these mice, when fed with a high-fat diet *ad libitum*, became obese as a result of significant increase in body fat percentage, adipocyte number and size, increased linear growth, compromised fertility, and various other metabolic anomalies. However, when fed with standard chow ad libitum, they remain lean [23, 24]. This DIO paradigm has been utilized in numerous research to investigate how food intake regulation functions in high-fat diets as a model of human obesity [25-27].

4.1.3 Epigenetic effects

Recent studies have emphasized the importance of early growth patterns, which are associated with metabolic and cardiovascular disease in adulthood and are exacerbated by excessive weight gain and obesity [28]. This phenomenon was first explored by Barker and colleagues in their landmark papers, who put forward the “thrifty phenotype hypothesis”. They proposed that early malnutrition invoked a physiological compensation promoting survival at a young age, at the expense of health at an older age [29]. These theories paved the way for subsequent studies examining the changes in various organ systems due to both maternal undernutrition and overnutrition, leading to the foundation of the field of the developmental origins of health and disease [30-32]. The concept of "foetal programming" states that a stimulus or adverse experience during a crucial or sensitive stage of development can have a lasting or lifelong impact on an individual later in life [33, 34]. In conclusion, early postnatal growth rates are strongly influenced by attempts to compensate for prenatal limitations or the enhancement of foetal growth by maternal-uterine factors [35-37].

4.1.4 Catch-up growth vs Compensatory growth

The quality control of differentiating tissues restricts organism growth under normal circumstances. Optimal growth enhances fitness potential while minimizing the negative physiological impacts of cell damage brought on by fast expansion. The effectiveness of cell function, immunological function, and physiological stress resistance can all be impacted by rapid growth [38]. However, organisms often exhibit growth rates closer to their maximum when they have previously experienced a period of reduced growth. This phenomenon is called compensatory or catch-up growth [38, 39]. In animal development, both words are often used interchangeably to indicate faster than optimal growth after a period of dietary restriction [40]. After restoring normal feeding levels, restricted animals can reach the same size as controls (catch-up growth), or they may show accelerated development (compensatory growth) in response to early dietary restriction [40-43]. Early limitation affects later fitness traits. Infants who have had growth restriction *in utero* tend to gain or catch up on weight rapidly in the early postnatal period. In these

cases, the sequence of prenatal growth restriction and postnatal growth retardation may increase the risk of obesity [44].

In sum, the 21st century views obesity as a complex multifactorial disease resulting from an interaction of genetic and environmental factors. Understanding the factors contributing to obesity and related health issues is crucial [45]. To address this issue, two key actions must be taken: discovering the mechanisms behind obesity and developing effective prevention, education, and treatment methods. While genetics play a role in human obesity, the common obesity is considered to be polygenic, with numerous genes carrying small risk factors [14, 17]. The obesity phenotypes can be studied based on knockout genes, genetic background, and dietary protocols. Animal models of obesity can be categorized based on individual gene mutations or exposure to obesogenic environments [46-48]. Therefore, the development of animal models is vital in finding new treatments and preventative measures for obesity and its associated comorbidities [21, 47-49].

4.2 Energy homeostasis: Energy balance in mammals

4.2.1 Regulation of energy balance

Energy is required for all essential life functions and as such it is important to understand the body's total energy expenditure in order to understand nutritional needs and how the body invests in different activities [50]. To regulate energy, the body requires complex nutrient sensing and monitoring mechanisms, adaptable integrative system that can adapt to changing conditions, and powerful effector mechanisms for energy intake, regulation and metabolism [51]. The concept of a constant internal environment, or "homeostasis," was first introduced by Claude Bernard and further extended by Walter Cannon to explain how the body maintains set internal values despite varying external conditions [52, 53]. The key components of the energy balance are energy intake, energy expenditure and energy storage. Energy intake is the amounts of food consumed, which depends on the quality and quantity of the food. Energy expenditure includes, resting energy expenditure, which is the body's basal or normal metabolic rate, which represents the amount of energy needed to maintain essential physiological functions at rest. The thermic effect of food is also part of the energy expenditure, which takes about 10% of total daily

consumption to digest, absorb and store nutrients from food. Thermogenesis is another integral part of animal energy expenditure. Improved locomotion while foraging is a high priority additional energy expenditure. In addition to these essential energy requirements, an animal also uses its energy for additional tasks such as reproduction, physical expansion (growth), or non-foraging physical activity [3]. A recent study in mice showed that an animal is able to temporarily resist starvation, even against homeostatic pressure, as long as energy levels are not critically low to meet other important related demands such as social interactions, as well as competing needs such as searching for food fulfil mating chances [54]. Additionally, the body can store energy as body fat for usage during times of food restriction [3, 55].

4.2.2 The hypothalamic control of energy homeostasis

Research, some 70 years ago, identified the hypothalamus as essential for the regulation of food intake and body weight, and the most recent genome-wide association study found that a large majority of genes associated with body mass index are expressed in the central nervous system, many of them in the hypothalamus [56-58]. Positioned near the third ventricle and median eminence, the hypothalamic arcuate nucleus (ARC) is advantageously located in an area where the blood-brain barrier is relatively permeable, allowing nutrients and hormones to be readily absorbed from the bloodstream [59]. Consequently, the ARC is considered the central hub for nutrient acquisition in the hypothalamus [60]. It works in concert with the corticolimbic system and the brainstem to control and perform functions that regulate food intake [61]. The brainstem processes information from various sources to regulate ingestion, digestion, and absorption, while the corticolimbic system provides emotional, executive, and cognitive input. The hypothalamus has emerged as the primary integration point for variables emanating from both central and peripheral energy balance regulation systems [61, 62].

4.2.3 The melanocortin system: regulator of energy balance

The arcuate nucleus of the hypothalamus is responsible for regulating energy homeostasis in response to nutrient and hormonal stimuli [9, 63]. The melanocortin system within the ARC consists of two distinct neuronal populations with opposite

functions: orexigenic agouti-related peptide (AgRP) and neuropeptide Y (NPY)-expressing cells, and anorexigenic proopiomelanocortin (POMC) and cocaine amphetamine-regulated transcript protein (CART)-expressing neurons [64, 65]. AgRP neurons promote food intake and decrease energy expenditure during calorie deficit, while POMC neurons reduce food intake and increase energy expenditure during satiety states [66, 67]. The melanocortin system includes the downstream targets of these neurons, such as the melanocortin receptors. The most important of them are the melanocortin-3 (MC3R) and the melanocortin-4 (MC4R) receptors, which play a role in the regulation of energy metabolism [68, 69]. The MC4R receptor is activated by alpha-melanocyte stimulating hormone (α-MSH), released from POMC neurons during states of satiety, while AgRP released onto the MC4R during starvation states acts as an antagonist [70]. NPY released from AgRP neurons has an effect similar to AgRP in driving food intake and reducing energy expenditure [71]. AgRP neurons can also inhibit POMC neurons through synaptic release of gamma-aminobutyric acid (GABA) [70, 72].

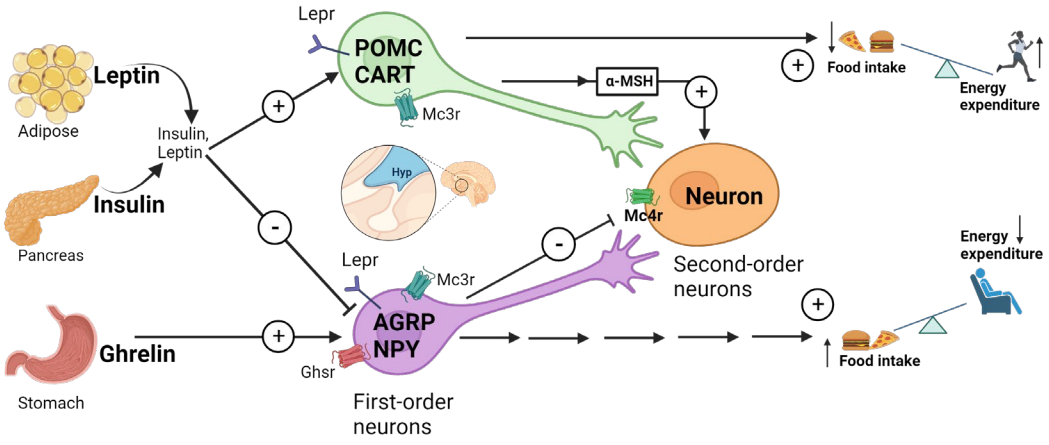

Figure 2: The melanocortin system in mammals

Peripheral hormones and metabolites convey the energy status of the body to the first-order neurons (POMC and AgRP) in the arcuate nucleus of the hypothalamus. In the case of calorie deficit, AgRP neurons are triggered, increasing food intake and energy saving. Conversely, POMC neurons respond to satiety, leading to the cessation of feeding and an increase in energy expenditure. The melanocortin system comprises five melanocortin receptors (MC1-5r), of which only MC3R and MC4R are present in the brain. During satiated states, α-MSH, released from POMC neurons, binds to MC4R, and stimulates downstream second-order neurons. During hunger, AgRP acts as an antagonist to MC4R. Both POMC and AgRP neurons can independently activate additional anorexigenic or orexigenic pathways. Figure adapted from **[73]**, modified and created with biorender.com.

Peripheral metabolic hormones such as insulin and leptin regulate the activity of both AgRP/NPY and POMC neurons (as indicated in Figure 2) and provide a mechanism for the brain to integrate information about energy status with neuronal activity in the hypothalamus [67, 74]. Insulin signalling on AgRP/NPY neurons reduces their firing rate, resulting in reduced AgRP and NPY release and subsequent suppression of food intake and increase in energy expenditure [75]. On the other hand, leptin receptor activation on AgRP/NPY neurons reduces their firing rate and suppress feeding, while loss of leptin signalling in these neurons leads to hyperphagia and obesity. Leptin also stimulates POMC neurons to increase α-MSH release and decrease AgRP expression [75-78]. In addition, leptin signalling in the brain increases sympathetic nervous system activity, resulting in increased energy expenditure [79]. Collectively, insulin and leptin act on POMC and AgRP/NPY neurons to regulate feeding behaviour, energy expenditure, glucose homeostasis, and adipose tissue metabolism [80]. Malfunction in insulin and leptin signalling in these neurons can lead to obesity, insulin resistance and metabolic disorders [72, 81].

4.3 The leptin system

4.3.1 Discovery, structure, and expression

Douglas Coleman and colleagues predicted the existence of leptin prior to its discovery based on Parabiosis experiments in which they linked the circulatory systems of homologous *ob/ob* and *db/db* mice. From these experiments, they inferred that *ob/ob* mice lacked a circulating factor that was abundant in db/db mice, and this factor could cure obesity in *ob/ob* mice, while *db/db* mice were unresponsive to it [82, 83]. Later, in 1994, the elusive obese gene (*Ob*) was located through positional cloning, and the factor causing the severe obesity observed in *ob/ob* mice was identified. This factor was found to be a 16kDa protein made up of 167 amino acids, which was named leptin, after the Greek word "leptos" meaning 'thin' or 'lean' [84].

Leptin belongs to the class 1 helix cytokine family and consists of four antiparallel helices connected by two long crossover links and a short loop, arranged in a left-handed helical bundle [85]. Formation of a disulphide bond between Cys96

and Cys146 is essential for structure folding and receptor binding [86]. The gene is located at 7q31.3 on the human chromosome, spans 20 kb and comprises 3 exons separated by 2 introns [87]. Leptin has a half-life of about 30 minutes in humans and about 80% of its clearance from the bloodstream is via the kidneys [88]. The similarity between mouse and human leptin is 83%, and both proteins show structural similarities with other helical cytokines, including interleukin-6 (IL-6) and growth hormone [89, 90].

In mammals, leptin is primarily expressed in white adipose tissue (WAT) [91]. However, recent studies have attributed its production to other tissues such as placenta, mammary gland, ovary, skeletal muscle, stomach, pituitary, and lymphatic tissue [92]. The level of circulating leptin in the body is directly proportional to the amount of body fat and thus indicates the state of long-term energy reserves [93]. Leptin levels also change in response to changes in caloric intake, with a significant decrease during periods of starvation [94, 95]. Leptin secretion is pulsatile and follows a circadian rhythm with lowest levels in the afternoon and highest levels at midnight. Although the pulse pattern is similar in obese and lean subjects, obese subjects have a higher pulse amplitude [96]. There are gender differences in leptin levels, with females having higher levels than males even when controlling for body fat mass, suggesting a role for sex hormones [97, 98]. Leptin levels are regulated by several factors, including insulin, glucocorticoids, catecholamines, and cytokines. Subcutaneous fat produces more leptin than visceral fat, which may contribute to gender differences in leptin levels [99, 100].

4.3.2 Leptin receptor isoforms and expression

Six different leptin receptor isoforms can be found in mammals due to variable splicing of the C terminal exon (LEPRa-LEPRf). The extracellular domain is shared by five of these isoforms, although their C-terminal sequences vary. The membrane-bound isoforms have three different domains: (I) a leptin-binding extracellular domain, (II) a transmembrane domain, and (III) an intracellular cytoplasmic domain [101]. The “long” LEPRb isoform is the primary mediator of leptin’s physiological effects, as seen in the morbidly obese *db/db* mice, which have a mutation that only affects expression of the LEPRb isoform: a G→T point mutation

causes a 106-nucleotide insertion that leads to premature termination of the intracellular domain [102]. The cytoplasmic domain of LEPRb consists of 302 amino acids and contains the binding sites for protein tyrosine kinase Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) [90]. LEPRb is the only isoform that contains complete intracellular tyrosine residues required for signalling. The Box1 motif of the LEPRb intracellular domain is a critical component for activation by JAK2. The Box1 motif contains a sequence found in all leptin receptor isoforms. LEPRb also has a cytokine box 2 that lacks the ability to activate JAK2. However, a 15 amino acid sequence downstream of box 1 can activate JAK2 [90, 103].

LEPRb is primarily expressed in the hypothalamus, although low levels of expression have been detected in peripheral tissues such as liver and lungs [104]. LEPRa and LEPRc are highly expressed in the choroid plexus and microvessels, potentially facilitating leptin uptake or transport across the blood-brain barrier [105]. LEPRe may encode a soluble receptor, acting as a buffer for free circulating leptin [106]. Leptin's effects were originally thought to be restricted to the central nervous system, but the prevalence of LEPRa and LEPRb suggests the hormone's pleiotropic effects on multiple biological functions [107].

4.3.3 Intracellular leptin signalling

The JAK2-STAT3 pathway is a major signalling pathway that is activated by the binding of leptin to LepRb. When two leptin molecules bind to LEPRb, JAK2 is activated by autophosphorylation of specific tyrosine residues. In particular, the three tyrosine residues phosphorylated by Jak2 on LepRb—Y985, Y1077, and Y1138—each have distinct roles in LepRb signalling [108]. The Y985 residue appears to be involved in leptin signalling feedback suppression and acts as a binding site for the Suppressor of Cytokine Signalling 3 (SOCS3) [109]. A tyrosine to leucine mutation of Y985 protects mice from obesity induced by a high-fat diet, underscoring the importance of SOCS3 feedback in the development of obesity and leptin resistance [110, 111]. Y1077 plays a dominant role in STAT5 activation [112], and Y1138 is essential for initiating the downstream signalling cascade [113]. $Lep^{s/s}$ mice, who have a Y1138 mutation, exhibit an obese phenotype comparable to that

of $Lep^{db/db}$ mice, demonstrating the significance of Y1138 in the control of the adipostatic effects of leptin. Both glucose homeostasis and reproduction appear to be unaffected by Y1138-mediated JAK/STAT signalling [114]. $Lep^{db/db}$ animals are infertile and have severe glucose intolerance, but $Lep^{s/s}$ mice, although being morbidly obese, are fertile and have only minimally altered glucose homeostasis [115, 116]. Despite having intact reproductive function, mice with a LepRb mutant lacking Y1138 display hyperphagic obesity and dysregulation of the thyroid and adrenal axes. According to these results, signalling through Y1138 may only have a minor effect on energy balance, while Y1077 may be crucial to leptin's control of reproductive function [117].

In mammals, there are three other JAK proteins — JAK1, JAK3, and TYK2 (the non-receptor protein tyrosine kinase 2). All members possess a FERM domain that facilitates their attachment to the plasma membrane. They also have an SH2-related domain responsible for binding to the phosphorylated tyrosine residues found on LEPRb in the case of JAK2. Additionally, they contain a kinase domain that allows for phosphorylation of other proteins and a pseudo-kinase domain that helps regulate normal kinase activity but lacks enzymatic activity [118].

The LEPR belongs to the family of homodimeric cytokine receptors and exclusively binds JAK2. Once activated, JAK2 phosphorylates specific tyrosine residues on LEPRb and downstream STAT proteins [118]. Studies have shown that STAT3 is the only STAT protein required for leptin-mediated regulation of energy homeostasis. After phosphorylation of LEPRb tyrosine residues by JAK2, STAT3 binds to them through the SH2 domain, and JAK2 then tyrosine phosphorylates STAT3. This causes STAT3 to dissociate and migrate to the nucleus, where it induces transcription of multiple genes, including SOCS3, through a complex mechanism involving the core GTP-binding protein RAN, importins 3 and 6, and dimerized STAT3 [119]. Activation of JAK2-STAT3 signalling by leptin results in decreased expression of NPY and AgRP and increased expression of POMC and CART, resulting in decreased food intake and increased energy expenditure [113, 118].

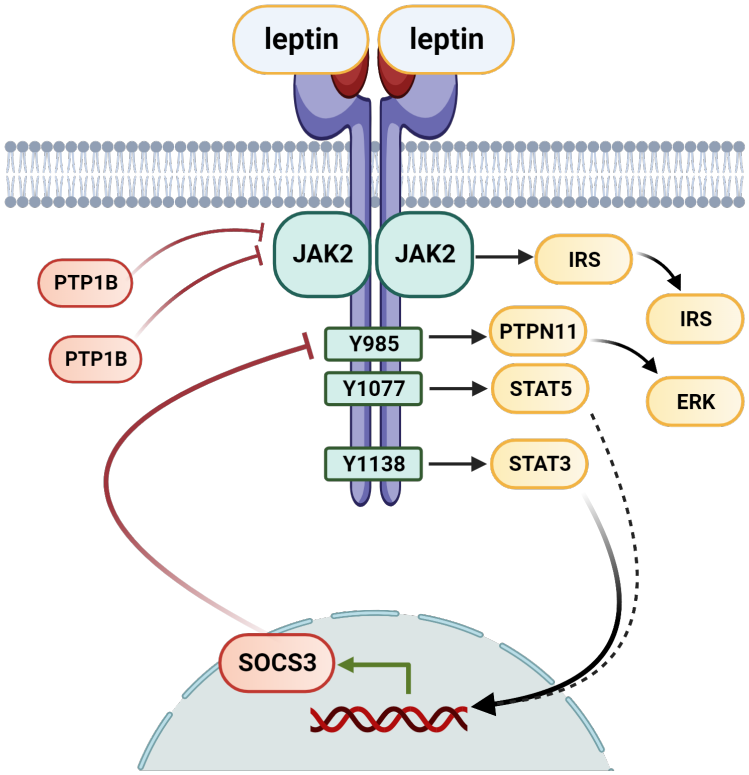

Figure 3: Schematic model showing the overview of intracellular leptin signalling in mammals
Binding of leptin to the extracellular domain of LEPRb activates the JAK2 tyrosine kinase and promotes phosphorylation of tyrosine residues 985, 1077, and 1138 of LEPRb. the phosphorylated TYR985 recruits PTPN11, which initiates the ERK signalling cascade. The phosphorylated TYR1077 and TYR1138 recruit STAT5 and STAT3, respectively, and allow them to migrate to the nucleus to regulate gene expression. Furthermore, SOCS3 can restrict LEPRb signalling by binding to phosphorylated TYR985, which is increased by LEP, and the tyrosine phosphatases PTP1B and TCPTP can dephosphorylate JAK2 and/or LEPRb to terminate LEP signalling. Adapted from **[120]**, modified and created with biorender.com.

However, regulatory proteins such as SOCS3 and protein-tyrosine phosphatase 1B (PTP1b), which operate as negative feedback regulators of leptin signalling, can decrease the effects of leptin [101]. The SOCS family consists of 8 members: cytokine inducible SH2 domain-containing protein (CISH) and SOCS1-7. Each of these proteins contains an SH2 domain and a specific site for interaction with JAK members called the SOCS box. The SOCS box can form a complex with the regulatory protein ubiquitin to target JAK2 for proteasomal degradation [121]. Through this mechanism, SOCS3 inhibits JAK2, thereby creating a negative feedback loop in the leptin signalling cascade. SOCS3 can also inhibit JAK2-STAT3

signalling by binding JAK2 using kinase inhibitor regions, thus preventing JAK2 from binding to other proteins [122]. Mutations in the SOCS3-binding region of the leptin receptor or deletion of SOCS3 elevates leptin sensitivity, resulting in decreased food intake and weight reduction [123]. PTP1b also suppresses leptin signalling by dephosphorylating the receptor and other downstream signalling molecules [101]. Furthermore, leptin-induced activation of the IRS-PI3K-AKT pathway plays a crucial role in regulating energy expenditure. Activation of the IRS-PI3K-AKT pathway by leptin plays a critical role in regulating energy expenditure. Activation of AKT leads to activation of downstream targets such as mTORC1 and FoxO1, which regulate protein synthesis, cell growth, and gluconeogenesis and fatty acid metabolism, respectively. AKT activation in peripheral tissues such as liver, muscle and adipose tissue promotes glucose uptake and utilization and improves glucose homeostasis. Thus, the IRS-PI3K-AKT signalling pathway is an essential mediator of leptin's metabolic effects, which regulates energy homeostasis, glucose homeostasis, and energy expenditure [124].

Leptin also exerts its effects on energy homeostasis via the MAPK/ERK pathway. Administration of leptin induces activation of ERK1/2, and food deprivation upregulates activated levels of ERK in the hypothalamus, which is reversed upon refeeding. LEPRb activates ERK1/2 via SHP2, a tyrosine-specific protein phosphatase, and SHP2 phosphatase activity is required for leptin-mediated ERK activation. While STAT3 activation occurs primarily through Tyr1138, ERK-mediated leptin action occurs through Tyr985 [125].

Although leptin is primarily known for its role in whole-body energy homeostasis, it also regulates energy homeostasis at the cellular level, primarily through adenosine monophosphate-activated protein kinase (AMPK) signalling. AMPK is an enzyme that activates the uptake and oxidation of glucose and fatty acids when cellular energy stores are low, e.g., in metabolic stress. Leptin has been shown to directly inhibit AMPK activity in the hypothalamus of mice, resulting in decreased food intake and reduced body weight. AMPK is also a mediator of leptin action in the periphery. Systemic administration of leptin enhances skeletal muscle AMPK activity and upregulates fatty acid oxidation [126].

4.4 Leptin as a regulator of endocrine function

Beyond its function in maintaining energy balance, leptin is also involved in the regulation of several neuroendocrine function. These include the release of growth hormone, the thyroid axis, the adrenal function, the release of prolactin, and the reproductive system. The link between leptin and reproduction was first shown by the infertility and atrophic reproductive organs observed in *Ob/Ob* mice lacking the leptin gene [127]. Leptin administration was shown to stimulate the reproductive endocrine system and restore fertility in these mice. Upon acquiring sufficient energy reserves for reproduction, leptin is also known to serve as a permissive signal for the beginning of puberty, allowing sexual maturation to occur [128, 129].

4.4.1 Central role of leptin in the regulation of HPG axis

It is known that leptin's role in regulating reproduction is via its effects on the secretion of gonadotropin-releasing hormone (GnRH), which is responsible for the regulation of puberty and ovulation. The majority of GnRH-producing cells in the brain are located in the preoptic area of the hypothalamus, and GnRH is secreted into the pituitary portal blood vessels to control the secretion of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The link between leptin and GnRH secretion has been suggested by various research findings [130]. Briefly, leptin acts on melanocortin neurons in the hypothalamus, which in turn stimulate the release of gonadotropin-releasing hormone (GnRH) from neurons in the hypothalamus. GnRH then stimulates the release of LH and FSH from the anterior pituitary gland, which in turn regulate the production of sex hormones in the gonads (ovaries in women and testicles in men) [131, 132].

Serum leptin levels are critical for linking adequate nutrition to hypothalamic-pituitary-gonadal (HPG) axis function. Under normal conditions, serum leptin levels correspond to fat mass, indicating optimal nutritional status [128, 133]. Conversely, the reduction in serum leptin in malnutrition acts as a metabolic signal for hunger, increased appetite, and foraging behaviour. At the same time, the low leptin signal inhibits energetically costly reproductive processes, such as pregnancy and lactation. Research suggests that there is a potential regulatory relationship between leptin and LH, which is important in controlling reproduction in mammals

[134, 135]. In rodents, leptin administration increases plasma LH levels, while mice lacking the leptin receptor have lower plasma LH levels and impaired estrogen synthesis. In addition, studies have shown that selective deletion of the leptin receptor gene in gonadotrophs leads to subfertility in female mice due to lower mRNA levels of activin, a key regulator of FSH, and lower levels of GnRHR protein in the pituitary gland. As a result, researchers have suggested that leptin may play a central role in female fertility, possibly by mediating its effects in the pituitary at the transcriptional or post-transcriptional level [136-138].

4.4.2 Peripheral role of leptin on the gonadal organs

Leptin plays a crucial part in the control of the Hypothalamic-Pituitary-Gonadal (HPG) axis, but it also has major effects on the peripheral gonadal organs. It is possible that leptin has endocrine and/or direct paracrine effects on the gonads because Leydig cells, granulosa, theca, and other ovarian follicular cells express leptin receptors. Leptin can decrease ovulation when administered systemically in vivo or in vitro into isolated perfused ovaries, according to studies. In the absence of growth factor augmentation, leptin does not appear to have any impact on steroid release or the number of preovulatory follicles [130, 139, 140].

In addition, leptin can have different effects on different cell types in the ovaries or testicles. Leptin plays a role in follicular atresia. Atresia is a degenerative process that eliminates non-ovulating oocytes from mammalian ovaries through mechanisms of autophagy and apoptosis. Studies have shown that loss of function of the leptin gene in mice leads to an increase in granulosa cell apoptosis, which contributes to follicular atresia [141]. On the other hand, leptin injection in rats attenuated follicular atresia [142]. These findings suggest that leptin has a dual role in aiding oocyte maturation and preventing oocyte breakdown [143]. It is important to note that the effects of leptin on gonadal function can vary depending on the specific dose and context in which it is acting [133, 144]. While moderate physiological doses of leptin may antagonize the enhancing effects of certain growth factors (insulin-like growth factor I [IGF-I], transforming growth factor [TGF-]) and hormones (insulin, glucocorticoids) on gonadotropins and thereby suppress

steroidogenesis, lower doses of leptin may in actually enhance steroidogenesis in some contexts [139, 145].

Both low and high body-mass index (BMI) levels are associated with infertility, indicating that the role of leptin in reproduction is complex and may be influenced by various factors [146]. Leptin affects reproduction, but the underlying mechanisms are not well known, necessitating additional study. The confounding effect of morbid obesity in leptin loss-of-function animals, which is challenging to distinguish from leptin's direct impact, is one obstacle to understanding the role of leptin in reproductive processes [147]. Therefore, it is still unclear how exactly leptin regulates reproductive processes in the female reproductive system, but further investigation may provide insight into the relationship between leptin and gonadal functions.

4.4.3 Gender differences in circulating leptin levels

Women have been found to have higher serum leptin concentrations than men, even after adjusting for variations in body weight and fat mass. This sexual dimorphism is thought to be caused by a number of different reasons. Although leptin pulse frequency is similar in both sexes, the initial amplitude of leptin secretion from adipose tissue is larger in females than in males. This difference in leptin secretion may be brought about by women having more visceral and subcutaneous fat than males do. Second, whereas leptin-binding protein levels are lower in women than in males, overall serum leptin levels are higher in women. According to this, men and women have different blood levels of free leptin. Thirdly, female adipose tissue may be more sensitive to substances that stimulate the creation of leptin as well as hormones like insulin and glucocorticoids. For instance, it has been shown that androgens lower leptin levels while estrogens enhance them. These elements might work together to explain why women's serum leptin levels are higher than those of men [139, 148, 149].

4.5 Hormonal regulation of growth in mammals

Mammals exhibit rapid somatic growth during embryonic and early postnatal development that slows with age and approaches zero once adult height is reached. The rate of growth slowdown varies between species, and the rate of slowdown determines a species' adult body size. Somatic growth is the result of both cell proliferation and cell expansion, with loss of somatic growth rate with age primarily reflecting a reduction in attributed to cell proliferation. In mammals, energy metabolism and somatic growth are closely synchronized, with GH synchronizing growth and metabolism. Somatotrophs in the pituitary secrete GH. PVN-Sst-expressing neurons limit growth hormone (GH) expression and release, while hypothalamic GH-releasing hormone (GhRH)-expressing neurons have the opposite effect on GH [150]. In mammals, GH insufficiency is linked to delayed somatic growth and increased fat tissue. All of GH's known actions are mediated through the transmembrane GH receptor (GHR). Mammalian signalling downstream of GHR is mediated by the GHR-associated tyrosine kinase Jak2 and Stat5B's transcriptional stimulation of IGF. In humans, mutations in GHR, JAK2, STAT5B, and IGF result in severe diseases marked by GH insensitivity, significant growth failure, short height, obesity, and elevations in specific adipose regions [151-153].

4.6 Regulation of energy homeostasis across vertebrates

Energy allocation is a bargain all organisms face, in which they must decide when to grow, reproduce, or store energy for future use. In order to make favourable decisions about these trade-offs, animals must integrate signals between the systems responsible for energy acquisition, storage, and demand. Research into the genetic basis of energy homeostasis in mice led to the discovery of key genes involved in mammalian energy homeostasis, such as the leptin system and the melanocortin-4 receptor system. These genes have been found to be conserved among mammals and all vertebrates, but the anatomical and functional data on these genes vary between species [154]. While the fundamental components of energy homeostasis are essentially preserved, the core regulation of these processes is likely species-specific and depending on the ecological niche and life history of a given species. Understanding what defines a conserved building block

in energy homeostasis and how these are centrally regulated is essential for elucidating the development of biological diversity and treating aberrant homeostasis patterns in people [154, 155].

The melanocortin system comprises melanocortin peptides produced from the Pomc gene, five G protein-coupled MCRs, and the endogenous antagonists agouti and AgRP. It is implicated in the regulation of locomotion, food intake, hunger, and other anticipatory behaviours in animals. Recent research indicates that the HPG axis is also involved in the regulation of somatic development and reproduction. The system is derived from the pro-opiomelanocortin (Pomc) gene, which undergoes tissue-specific post-translational processing to generate numerous POMC peptides with diverse physiological roles. In contrast to mammals and birds, which have a single copy of the Pomc gene, the majority of bony fish have two to three distinct transcripts due to genome duplication. Three copies of the *pomc* gene and a splice variation are found in Atlantic salmon and rainbow trout [154]. The affinity of vertebrate MCRs for different melanocortins, agouti, and AgRP varies. MC3R and MC4R perform crucial functions in energy homeostasis and are widely expressed in the central nervous system of vertebrates. Compared to mammals, fish Mcr ligands are expressed in a highly conserved manner, and the neuronal circuits involved in the hypothalamic control of energy balance are evolutionary conserved [156]. Agouti and agRP are two endogenous antagonists, with AgRP serving as a potent orexigenic factor antagonistic to MC3R and MC4R in the hypothalamus. In vertebrates, AgRPs have a conserved role in energy balance and their relationship to the melanocortin system. *agrp* genes have been identified in several fish species, with hypothalamic Agrp expression upregulated during fasting conditions [154].

Leptin plays a critical role in regulating energy homeostasis in animals by integrating signals between systems responsible for energy intake, storage, and demand. Since most research has focused on the role of leptin in mammalian obesity, there is a growing need to investigate its role in the neuroendocrine systems in other vertebrates. This could tell us something about its general role as an anorexic hormone and the mechanism behind its mode of action in different species

depending on their life history and ecological niche [157]. Additionally, a better understanding of energy homeostasis and the role of leptin in different species may provide insight into the evolution of leptin biodiversity and physiology [96]. Using the principle of gene synteny and conservation of order, non-mammalian leptin was positionally cloned in 2005 in the Japanese Pufferfish [158]. Today, leptin orthologues have been described in all classes of vertebrates. Although they represent only a tiny fraction of all research in this area, studies of leptin outside of mammals are important not only to answer comparative questions, but also to uncover the evolutionary origins of leptin function and how the hormone regulates species-specific energy homeostasis [159-161].

In recent years, a rather different leptin physiology has emerged in teleost compared to mammals. Fish and birds do not express leptin in adipose tissue, and in most species hepatic leptin expression increases after fasting instead of increasing in adipose tissue after feeding. The reasons for this could be the higher starvation resistance and lower fat stores of fish and arthropods than ectotherms, which may influence their energy storage responses compared to endotherms [160, 162]. While the most important body composition changes observed in mammals with leptin loss-of-function models are morbid obesity, in fish with Leptin loss-of-function models it is either mild or absent. Studies in fish have shown that loss of function of leptin receptors in fish, such as Lepr in medaka and zebrafish and Lepa and Lepb in zebrafish, does not lead to morbid obesity. These results suggest that fish can serve as a useful model to study the role of leptin in the body without the confounding factor of obesity [157, 159, 163].

4.7 Zebrafish as a vertebrate model

Zebrafish have gained popularity as a model organism in research, due to many favourable characteristics. They were established as a model organism in 1970, and they have since emerged as a pioneer in biological research. Zebrafish are tropical freshwater fish belonging to the Cyprinidae family. They are native to the floodplains of India, Pakistan, Burma, and Bangladesh, where they inhabit slow-moving waters or ponds [164]. Zebrafish are asynchronous, batch spawners, meaning that they are able to spawn frequently, even on a daily basis, under optimal

conditions such as food availability and favourable water parameters. Females reach puberty approximately 45 days after fertilization (dpf), around 20-30 mm Standard length, whereas males reach puberty slightly later [165]. Zebrafish are small, have a short life cycle, and rapid development and generation times, making them ideal for imaging and manipulating developing organs or single genes in vivo. Their optical transparency during early development is an added benefit for imaging purposes [166, 167]. In addition, genetic knockdown utilizing morpholino technology permits rapid analysis of gene function and is suitable for forward genetic screening due to the high number of offspring and ease of chemical mutagenesis. Historically, positional cloning was used to identify the mutated gene responsible for a peculiar phenotype. However, new methods, such as whole genome sequencing (WGS) and whole exome sequencing (WES), have been developed that expedite the process of identifying mutations and thus facilitate the evaluation of large-scale genetic screens [168, 169].

In addition to forward genetics, several technologies enable the screening of zebrafish using reverse genetics. One such technology is "Targeting induced local lesions in genomes" (TILLING), which involves analysing the genomic DNA of zebrafish treated with ethylnitrosourea (ENU) for mutations [170]. Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and the clustered, regularly interspaced, short palindromic repeats (CRISPR) and the CRISPR associated system (Cas9) can also be used for targeted mutagenesis in zebrafish [171-173]. TALEN and CRISPR-Cas can also be used to modify the sequence in the region of interest [174]. Transgenesis is another common technique used in zebrafish research, as it permits the genomic integration of plasmids or bacterial artificial chromosomes (BACs) containing the template sequence [175, 176]. This technique allows for overexpression studies and labelling of specific cells *in vivo* [177]. Finally, systems such as Cre-loxP or GAL4-UAS can be used to control the temporal and spatial expression of transgenes in zebrafish [178, 179]. Overall, the combination of these techniques has significantly broadened the scope of genetic studies that can be conducted on zebrafish, making it a potent model organism for both forward and reverse genetics research [180, 181].

4.7.1 Energy homeostasis in Zebrafish

For a very long time, rodent models have been the method of choice for investigating the underlying mechanisms of human obesity. However, the zebrafish has recently gained popularity as an alternative vertebrate model for studying human physiology, including energy homeostasis and metabolic diseases. One reason for this is that zebrafish tissues and organs are functionally and structurally comparable to those of mammals [182]. In addition, the neural and endocrine signals involved in regulating energy homeostasis, such as the leptin and melanocortin systems, are conserved between zebrafish and mammals. Using zebrafish as a model for DIO research has enabled the identification of new regulators of energy homeostasis and potential obesity-treating drugs via genetic and chemical screens. Furthermore, research on the development and metabolism of adipocytes reveals striking similarities between mammals and zebrafish [183, 184].

However, because fish have a very different life history and ecology, there are many differences in their energy regulatory systems compared to mammals. First, fish that are poikilotherms usually have a lower body temperature than mammals and therefore expend very little energy on thermoregulation [185]. Unlike mammals, the liver is one of the primary stores of energy (glycogen and fat) in fish, and in many fish species, lipid metabolism is the primary source of energy [186]. Evolutionarily, teleost have undergone an additional whole genome duplication compared to mammals, resulting in multiple protein-encoding genes and making genetic studies more complex to conduct [187]. Because of this evolutionary difference from mammals, although some appetite-regulating peptides in mature forms can show close amino acid similarities between fish and mammals, this is not always the case for all peptides, such as Leptin. Despite these differences, the zebrafish model offers a valuable tool for studying energy homeostasis and metabolic diseases. Comparative research across species can be helpful in elucidating the general principles and variabilities of the endocrine system that regulates energy homeostasis [188].

4.7.2 Regulation of reproduction

Reproduction in vertebrates is an energy-intensive process closely linked to nutritional status and energy reserves. The reproductive process in teleosts is controlled by the hypothalamic-pituitary-gonadal (HPG) axis, also referred to as the reproductive axis. The hypothalamus, pituitary, and gonads interact and communicate using positive and negative feedback mechanisms to regulate the reproductive cycle of an organism, resulting in the maturation of the gonads and the release of mature eggs or sperm [189]. The reproductive axis is inactive during adolescence and activates during puberty [190]. External factors such as photoperiod, temperature, and food availability, as well as internal factors such as hormones, neuropeptides, and energy stores can further regulate reproduction [191].

The neuropeptide gonadotropin-releasing hormone (Gnrh) is synthesized in the hypothalamus and is regarded as the primary reproductive regulator. Gnrh controls the release of two gonadotropins, FSH and LH, by regulating the activity of endocrine cells in the anterior pituitary gland [192, 193]. Several types of Gnrh and Gnrh receptors (gnrhr) have been identified in teleost, each with distinct neuroanatomical localization and function. Fsh and Lh belong to the glycoprotein hormone family and consist of a common alpha subunit and a hormone-specific beta subunit that determines their biological function [194]. The gonadotropins are transported through the bloodstream to the gonads (ovary or testes), where they bind to their respective receptors and stimulate gonad development, gametogenesis, and steroidogenesis [195]. Fsh is responsible for promoting early gonad development and growth, whereas Lh is primarily involved in the final stages of gonad development, including maturation and gamete release [194, 196].

Despite attempts to genetically manipulate the hypophysiotropic neuropeptide systems, results have been less successful in fish compared to mammals. Studies found knockout of critical genes results in only subtle phenotypes [197]. In particular, knockout of Gnrh2, Gnrh3, or double knockouts did not demonstrate a reproductive phenotype, suggesting that compensatory genetic mechanisms may be responsible for maintaining adequate reproductive function

[198-200]. Removal of just a few neuropeptides can have minimal impact, as co-expressed gonadotropin stimulators can remain functionally intact. Therefore, there is still a need to discover other key players in the neuropeptide system with a plausible role in reproduction [197].

4.7.3 Process of oocyte maturation and ovulation

Zebrafish are considered income breeders, meaning they allocate energy to oocyte maturation throughout the breeding season, which in the lab can occur year-round and in the wild lasts for 4-5 months [201, 202]. Depending on feeding and culture conditions, female zebrafish have a daily reproductive potential of around 20-40 fertilizable eggs per day. When females are prevented from mating daily, their reproductive capacity increases with the number of days of separation until it reaches a plateau after about a week [203, 204]. Reproduction requires more energy expenditure in females than in males, as they have to nourish and rear their offspring, making the female reproductive system more sensitive to energy reserves [205].

Oocyte development involves 5 stages in zebrafish. In the early stages of oogenesis, primordial germ cells (PGCs) transform into oogonia and then into primary oocytes, which are arrested in their first meiotic prophase. Subsequently, the primary oocytes increase in size and accumulate nutrient-rich reserves for the development of the future embryo during the vitellogenesis phase. In addition, oocyte maturation continues from prophase I to metaphase II, where it undergoes a second arrest (Figure 4). During this resting period, various messenger RNAs (mRNAs) are translated into proteins to ensure good oocyte quality, and the connections between the oocytes and the granulosa cells begin to break. During the first and second meiotic arrest, the oocytes sense the signals from the gonadotropins, after which they release their arrest [206]. Reports from several studies suggest that the release of first arrest is triggered by Fsh, which supports oocyte growth and development, and the second release by Lh, which is the main regulator of oocyte maturation and ovulation [207-209].

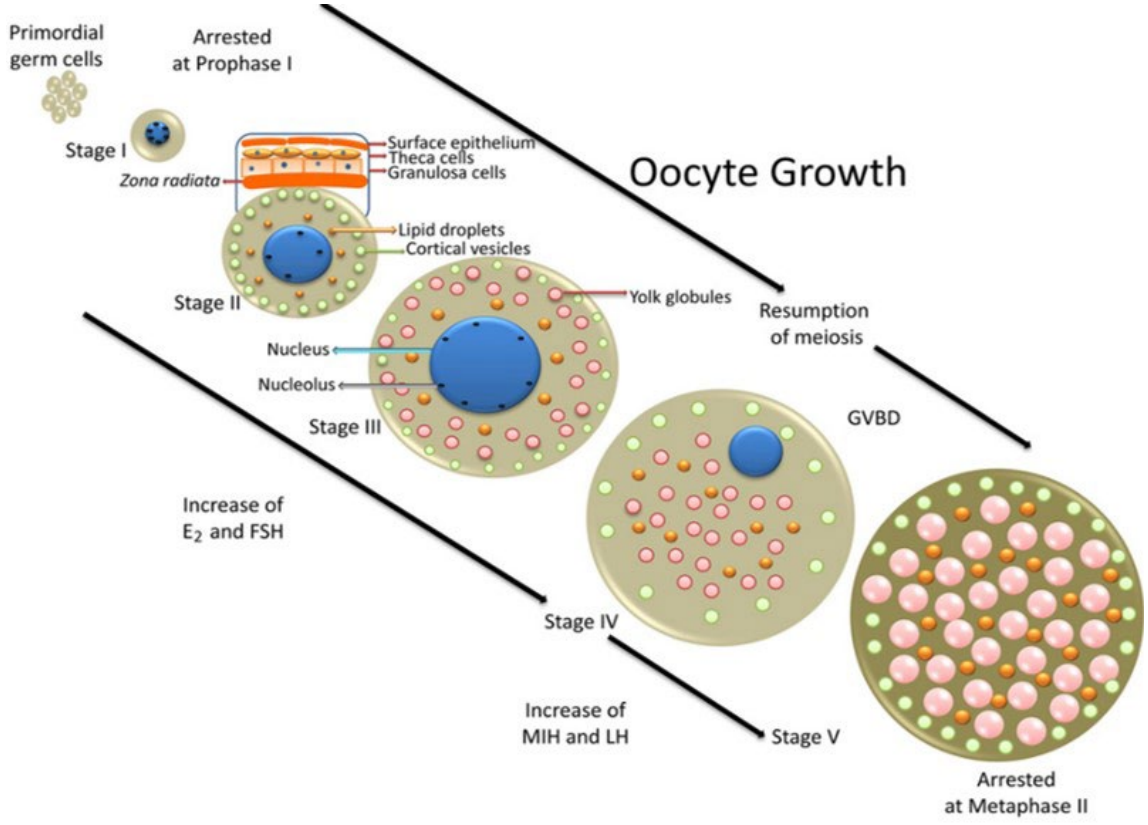

Figure 4: Scheme recapitulating the stages of oogenesis in zebrafish.

Primordial germ cells (PGCs) transform into primary oogonia, which further transform into primary oocytes. In stage I (primary growth stage), oocytes are first in nests with other oocytes (stage IA) and then in a final follicle (stage IB). Towards the end of stage I, they are arrested in meiotic prophase I, where they greatly increase in size. In stage II (cortical alveolar stage), oocytes are characterized by the appearance of cortical alveoli of various sizes and a protruding yolk sheath. In response to signals from E2 and Fsh, the oocytes are released from a standstill and continue to grow. In stage III (Vitellogenesis), yolk proteins appear in oocytes and yolks with crystalline yolk accumulate, resulting in the growth of an oocyte. At this stage, oocytes develop the ability to respond to the steroid 17a,20P-dihydroxy-4-pregnen-3-one (DHP) in vitro by undergoing oocyte maturation. At stage IV (oocyte maturation), the oocytes increase slightly in size, become translucent, and their yolks become non-crystalline as they undergo final meiotic maturation in vivo (and in response to DHP in vitro). In stage V (mature egg), oocytes (about 0.75 mm) are ovulated into the ovarian lumen and are capable of fertilization [206]. Adapted from **[210]**.

After LH binds to its cognate receptor (Lhr) on the granulosa cells of mature follicles, 17a,20b-dihydroxy-4-pregnen-3-one (17,20bP or DHP), a maturation-inducing hormone (MIH), is produced. DHP then binds to its cognate receptors on the oocyte membranes and stimulates the activation of the maturation-promoting factor (MPF), which releases the oocytes from their first meiotic prophase arrest and resumes maturation [189, 211]. Simultaneously, LH and DHP stimulate key downstream factors and regulators of oocyte maturation and ovulation, such as progestin membrane and nuclear receptors, prostaglandins, and matrix metalloproteinases [212]. When ovulation occurs, proteolytic enzymes digest the follicular cell layers that surround the oocytes, causing the rupture of the follicle and the release of mature oocytes [208, 213, 214]. Advanced targeted gene knockout studies have also confirmed the specificity of LH's actions during these two phases of oogenesis. Lh deficient fish appear to have normal gonadal development, but they are sterile and cannot reproduce [215-217].

According to studies, female zebrafish have an ovarian cycle that lasts between 5 and 7 days, after which fecundity and oocyte quality begin to decline while oocyte mass remains constant. This decline in oocyte quality is believed to be caused by follicular atresia, the resorption of mature oocytes (Fig 5). It has been discovered that this serves as a quality control mechanism to determine the proportion of oocytes that will mature, a mechanism that maintains the constant development of new oocytes [218, 219].

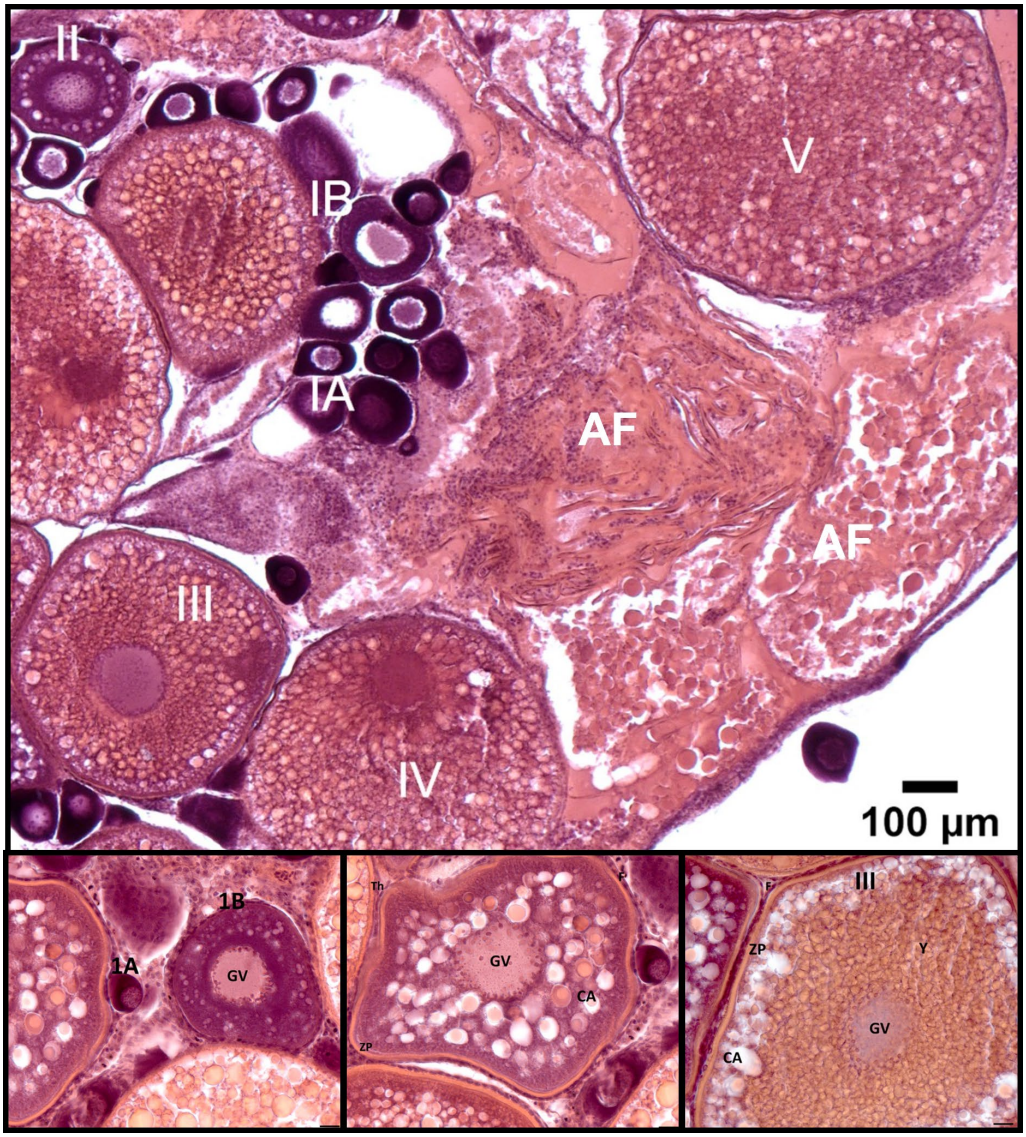

Figure 5: Ovarian section showing different stages of oocytes in zebrafish.

In the top image (A) shows different stages during oocyte development. Stages 1a, 1b and II are immature stages, III is vitellogenic stage, stage IV and V are mature stages and AF refers to atretic follicles. The bottom B, C and D figures are higher magnification images of each stage. GV refers to Germinal vesicle; CA is Cortical alveoli; ZP is Zona pellucida and Y refers to Yolk material. The scale of the top image bottom images is 100 µm and that of bottom images is 20 µm.

4.7.4 Regulation of Melanocortin and somatotropic systems in zebrafish

In zebrafish, the somatotropic and melanocortin system are highly conserved. Similar to mammals, zebrafish melanocortin system is comprised of first order and second-order neurons. First order neurons (*pomc* and *agrp*) are located in the Nucleus lateralis tuberis (NLT) in the hypothalamic domains homologous to mammalian arcuate nucleus. Two genes orthologues to the mammalian POMC gene, namely *pomca* and *pomcb* have been identified as a result of additional whole genome duplication as mentioned before [188]. Zebrafish *pomca* can give rise to same mature peptides as in mammals, however, posttranslational modification of *pomcb* is said to result only in α-MSH and β-endorphin (Heiko Löhr, unpublished data from Hammerschmidt Lab). The processed peptides *agrp* and *alpha-MSH* released from Agrp and Pomca neurons respectively, bind onto second-order target neurons expressing melanocortin receptors (*mc3r* and *mc4r*) [220]. These neurons also express orthologues to the mammalian peripheral hormones and their receptors, which signal to the first order neurons to detect the body's energy status. For instance, the zebrafish orthologue of the *leptin* receptor (*lepr*) has been cloned and observed to be expressed in the adult zebrafish's arcuate nucleus. Projections of first-order neurons innervate similar target regions in the hypothalamus, as identified in mammals, which suggests that the neuroanatomical circuits of the melanocortin system are conserved between mammals and zebrafish [151].

In addition to the role of regulating energy homeostasis, the melanocortin system in zebrafish is further involved in the regulation of somatic growth. It is interesting to note that zebrafish, despite their capacity to store energy as neutral lipids in adipose tissue, they do not become heavily obese in response to increased energy supply but display linear growth instead. Studies in zebrafish have shown that transgenic overexpression of *agrp* increased *gh1* expression and linear body growth [221] and morpholino-based *agrp* knockdown resulted in an opposite effect [222]. Further, zebrafish Pomc and Agrp neurons indirectly regulate the hypophysiotropic system via the Sst1.1 second order neurons of the melanocortin system, which project into the *gh1*-expressing cells of the adenohypophysis [151]. This suggests that the release of growth hormone, via the action of Gnrh, might be under the direct control of the hypothalamic melanocortin system.

4.7.5 Leptin system in zebrafish

Zebrafish express two leptin paralogs, *lepa* and *lepb*, which are found to play a pleiotropic role and are expressed in various tissues. Lepa and lepb, like all vertebrate leptin genes, consist of four alpha helices and two cysteine residues that form a disulphide bond. Three receptor interaction sites have been identified in these genes, and primary amino acid sequence conservation has been observed in each of these regions. [159, 163, 223]. Although *lepa* and *lepb* genes share only 24% amino acid identity with each other and 18% with their human orthologous gene, the gene synteny and predicted protein structures are well conserved. Both the leptin paralogs mediate their signal through a single leptin receptor *(lepr)* gene [224, 225]. This gene is expressed during all stages of embryonic and larval development, with the strongest expression in the brain, kidney, and testis of adults. The available evidence for the conservation of leptin function at a physiological level in fish is inconclusive, and it is unclear whether in fish leptin signals the status of peripheral adipose stores in the same way as in mammals [160, 162].

Some studies using genome editing technologies on one of the two *leptin* paralogs suggest that it plays an essential role in zebrafish development and energy homeostasis. For example, *lepa* knockdown embryos and larvae had body malformations and larger yolk sacs, compared to wild-type embryos or larvae at the same developmental stages [226]. Additionally, inhibition of *lepa* led to lower metabolic rates in larvae , while treatment with heterologous human leptin increased their energy expenditure [227]. Leptin has also been linked to obesity, as *lepa* knockdown males and females displayed an obese phenotype with increased body weight and linear growth [225]. Similarly, *lepb* deficient adult zebrafish showed obese phenotypic characteristics, including significantly higher blood glucose levels compared to their wildtype counterparts [228]. However, conflicting results have been reported in other studies using mutations of *lepr* gene. The major body composition changes observed in mammalian loss-of-function models (morbid obesity) are either mild or absent in fish models [229]. In adult zebrafish, a point mutation in the *lepr* gene resulting in truncated polypeptide did not exhibit hyperphagia or increased adiposity but did have higher insulin mRNA levels and changes in glucose homeostasis [230]. During larval development, a 16bp insertion

in the *lepr* gene caused transcriptional differences in several genes related to food intake and digestion, while no differences were observed in metabolism, energy allocation, or growth in adults [231]. Another study conducted showed no significant phenotypic differences in *lepr* mutant zebrafish up to 2.5 months post-fertilization. However, adult *lepr* mutants exhibited the traits of obesity, such as increased food intake, weight, and body fat percentage. Furthermore, overfeeding caused significantly impaired glucose tolerance in the adult mutants compared to wild type siblings [232, 233]. While some evidence suggests conservation of leptin function at a physiological level between fish and mammals, the available data are inconclusive, and further research is needed to clarify the mechanisms and which extent leptin regulates energy homeostasis in zebrafish [189]. Additionally, the divergent results within the same species raise the question of whether there is a biological reason for this.

While research on leptin in teleost species has focused primarily on its effects on appetite, food intake and energy metabolism, very few studies have looked at its role in reproduction [189]. Some studies suggest that leptin may be important in the later stages of fish maturation, but research in this area remains limited. In vitro studies with recombinant mammalian leptin have shown that it could potentially regulate the reproductive system of fish. For example, in pituitary cell cultures from pubescent and adult male European sea bass, recombinant mouse leptin was found to have a direct, beneficial effect on Lh release [234]. Furthermore, human recombinant leptin was observed to stimulate Lh and Fsh secretion in pituitary cells during early gametogenesis in male and female rainbow trout [235]. Recently, a study in zebrafish with loss of *lepr* function showed centrally mediated subfertility, in which the authors found decreased expression of the luteinizing hormone beta subunit (*lhb*) in the pituitary gland [189]. However, subfertility was partially restored by administration of human chorionic gonadotropin [236].

5 AIMS OF THE THESIS

Maintaining energy balance is vital to the survival of species, as energy plays a crucial role in all physiological processes, including animal growth and reproduction. In recent decades there has been increasing interest in using fish for comparative neuroendocrinological research. The overall aim of the thesis was to systemically elucidate aspects of energy homeostasis control using zebrafish as a model organism.

At the onset, we investigated the effects of diet-induced obesity (DIO) and its effect on metabolic endpoints in adulthood using zebrafish as a model organism. We observed the signs of compensatory or catch-up growth after being subjected to long-term obesogenic diet across different time points. Additionally, we examined whether early food restriction exacerbated the effects of long-term DIO or predisposed the fish to metabolic syndromes (Publication 1).

Considering the controversial data about role of leptin in obesity, we examined whether mutations in *lepr* and/or *lepa* leads to obesity related growth phenotypes in zebrafish (Publication 2). Subsequently, we explored the central and peripheral effects of leptin on the reproductive system along the hypothalamic-pituitary-gonadal (HPG) axis in female zebrafish using *in vitro* and *in vivo* methods (Publication 3).

Further, in an attempt to explore if body growth and reproduction are regulated by the central melanocortin system, we used Agrp1 LOF model in zebrafish (Publication 4). In sum, these studies were aimed to provide a systematic and functional analysis of the regulation of growth and reproduction across neuroendocrine axis in zebrafish.

6 PUBLICATIONS OF THE THESIS

6.1 Publication 1

"Long-term obesogenic diet leads to metabolic phenotypes which are not exacerbated by catch-up growth in zebrafish"

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Supplementary Material is provided as SM1

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Author Contributions

Sandra Leibold - Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing – original draft.

Amrutha Bagivalu Lakshminarasimha - Data curation (Figure 4 and 5), Investigation and validation.

Felix Gremse - Resources, Software

Matthias Hammerschmidt - Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Maximilian Michel - Conceptualization, Formal analysis, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

PLOS ONE

RESEARCH ARTICLE

Long-term obesogenic diet leads to metabolic phenotypes which are not exacerbated by catch-up growth in zebrafish

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Abstract

Obesity and metabolic syndrome are of increasing global concern. In order to understand the basic biology and etiology of obesity, research has turned to animals across the vertebrate spectrum including zebrafish. Here, we carefully characterize zebrafish in a long-term obesogenic environment as well as zebrafish that went through early lifetime caloric restriction. We found that long-term obesity in zebrafish leads to metabolic endpoints comparable to mammals including increased adiposity, weight, hepatic steatosis and hepatic lesions but not signs of glucose dysregulation or differences in metabolic rate or mitochondrial function. Malnutrition in early life has been linked to an increased likelihood to develop and an exacerbation of metabolic syndrome, however fish that were calorically restricted from five days after fertilization until three to nine months of age did not show signs of an exacerbated phenotype. In contrast, the groups that were shifted later in life from caloric restriction to the obesogenic environment did not completely catch up to the long-term obesity group by the end of our experiment. This dataset provides insight into a slowly exacerbating time-course of obesity phenotypes.

Introduction

Metabolic syndrome is becoming an increasingly prevalent global health concern. Metabolic syndrome is an umbrella term used for various pathologies surrounding long-term obesity such as hypertension or type 2 diabetes [1] and whether an individual develops obesity and metabolic syndrome is a complex interplay of genetic and environmental contributions. While studies suggest that heritable factors account for 40% to 85% of the variation in adiposity [2] the environmental contribution is still immense, even at birth [3–5]. Maternal body weight and overnutrition during the gestational period have been implicated in increasing the risk of metabolic syndrome for offspring [5, 6]. Further, high birth weight and/or rapid infant growth [7, 8] as well as low birth weight / pre-term birth and a period of subsequent catch-up growth

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[9–11] are associated with an increased risk of metabolic syndrome later in life. Evidence in rodents shows that a period of early food restriction or malnutrition—especially in the perinatal period–followed by compensatory growth is correlated with metabolic risk in later life [9, 12]. Caloric restriction leads to growth stagnation or reduced growth and when conditions are favourable of growth again, animal growth increases again. This phenomenon has been most frequently referred to as "compensatory growth" [13] and can be observed across vertebrates including people [1, 14], livestock [15, 16], amphibians [17], birds [18], reptiles [19, 20] or fish [21, 22]. However, an important distinction is that differences can be seen in the growth rate of the organism after the growth restricting conditions are alleviated. The increased growth is called catch-up growth if it occurs at the same growth rate as matched (size or age) animals that were never restricted; or compensatory growth if growth rates are higher than in matched animals [13]. Importantly, in mammals, compensatory responses with above normal growth rates are associated with altered body composition later in life [23], particularly after prenatal [24, 25] or early postnatal food restriction [26, 27], while catch-up growth has not been associated with long-term detrimental outcomes. However, the effect of compensatory of catch-up growth (CG) on long term metabolic outcomes is controversial in children, and it has been argued that when measured as a matter of gestational age, the effect of birthweight on long-term metabolic outcomes disappears and therefore the long-term detrimental outcomes have potentially more to do with premature birth (and therefore low birthweight) than with low birthweight in babies carried to term [1].

Zebrafish have emerged as an important vertebrate model system for several reasons. Not only are genes largely conserved but there is also a high degree of physiological and anatomical similarity. Genetic models of obesity have been established in zebrafish such as overexpression of agouti related peptide [28] or semaphorin 3 [29, 30]. Metabolically, zebrafish show evidence for metabolic syndrome endophenotypes such as diet induced obesity, diabetes (reviewed in [31], dyslipidemia (reviewed in [32], non-alcoholic or alcoholic fatty liver disease (reviewed in [33, 34]. Further, key obesity hallmarks have been identified in zebrafish downstream of overfeeding. Obesity is associated with increased body growth due to overfeeding [35–38] as well as increased lipid deposition in subcutaneous and visceral adipose depots [37, 38]. However, to long-term detrimental effects of long-term obesity are not entirely clear in people [39, 40] let alone in fish. However, recent studies in fish support that obesity also leads to long-term effect in fish such as cognitive decline and inflammation of the central nervous system [41, 42]. Peripherally, it has been shown histologically that adipocytes not only undergo hypertrophy but also hyperplasia in zebrafish exposed to an obesogenic diet [36]. Hyperplasia in mammals is considered to be an adaptive response allowing the safe storage of fat while subcutaneous adipocyte hypertrophy is correlated with metabolic complications [43]. Additionally, fish store triglycerides not only in fat but also in muscle and liver under normal conditions [44], so increased hepatic lipid metabolism [37] or deposition [38] could be normal. Abnormalities consistent with hepatic steatosis have also been seen in DIO zebrafish [35, 45, 46]. A reduced metabolic rate has been linked to metabolic syndrome in people [47, 48], however this association has been questioned in mouse model studies [49–51]. In zebrafish, a recent study showed an increased metabolic rate as well as mass for overfed fish [52], suggesting that the effect may be driven by mass rather than a decreased energy expenditure in the obese animals. Similarly, obesity and in particular insulin resistance have been linked to functional changes in mitochondria in people [53] as well as mice [54, 55]. In zebrafish, mitochondrial function has to our knowledge only been looked at in the context of a CG response, where hepatic oxidative phosphorylation was shown to be enhanced in animals undergoing CG [56] but not in the context of obesity. Lastly abnormalities in glucose metabolism particularly hyperglycemia, impaired glucose tolerance and insulin resistance are hallmarks of type 2 diabetes, a condition

intricately linked to metabolic syndrome and have been described in mammals [1], as well as fish [38, 57, 58].

Consequently, a variety of metabolic complications due to an obesogenic environment have been described in zebrafish. Further, a recent study in zebrafish has found that maternal overfeeding has a negative effect on early obesity markers in larvae of the next generation, comparable to evidence in mammals [42]. Therefore, zebrafish provide an elegant system to study the basic biology of energy homeostasis. However, it is unclear whether early undernutrition followed by CG could exacerbate markers of obesity—even though CG is a well-studied phenomenon in fish (reviewed in [22].

Consequently, we carried out a systematic characterization of DIO as well as CG. We compared CR with DIO and tested whether CG of fish which were shifted from CR to DIO at different timepoints showed an exacerbated long-term DIO phenotype. Zebrafish are externally fertilized and feed for the first 5 days-post fertilization (dpf) on maternally provided egg yolk. We therefore restricted food from 5dpf onwards and induced CG by overfeeding comparable to the DIO group. We induced CG at three timepoints associated with different growth rates in wildtype animals: 1) a juvenile phase (one month) which exhibits naturally high growth rates (CG1), 2) three months old fish, a stage when growth begins to slow (CG3) and 3) nine months of age when growth mostly stagnates in wildtype fish (CG9). We investigate growth parameters, fat distribution and metabolic genes in order to establish whether zebrafish a) show signs of compensatory or catch-up growth, b) show signs of metabolic syndrome after a long-term obesogenic diet in support of and beyond previous studies and c) whether early food restriction exacerbates the effects of long term DIO and pre-disposes fish for or exacerbates metabolic syndrome.

Materials and methods

Zebrafish maintenance

Zebrafish of the Ekkwill strain were raised and maintained at 28˚C under a 14 h light / 10 h dark cycle and in tanks with continuous water exchange and recycling (AquaSchwarz, Göttingen, Germany) [59]. Embryos were obtained by natural mating while siblings derived from the same mating of a single parental pair were used for comparative analyses of aged-matched fish.

Fish were raised as previously reported in groups of 5 (DIO, CG after induction) or 50 fishes (CR, CG before induction) in 3 L tanks [36]. In our previous publication, the DIO condition was labelled as "high amounts of food, low density" (HF-LD) and the CR condition was called "low amounts of food, high density" (LF-HD). Compensatory growth was induced by splitting one tank with 50 fish in 10 tanks containing 5 fish each. Fish were sorted for sex as soon as visible and maintained at a female:male ratio of 3:2. Body length of 10 fish of each group was measured at least every other week and density was checked weekly. Number and sex ratio of each tank were checked before each measurement but at least once per month.

We carried out a control experiment for CG1 where we compared changing the amount of food at a constant density versus changing density at constant food levels (S2 Fig). For this, we fed as follows.

Changing food levels, constant density: 5 fish per 2.5 liter (L) fed either

1. DIO: 100 ml paramecia daily (5–13 days post fertilization (dpf)) and 2x 10 drops of artemia (14 dpf onwards).
2. CR: daily feeding of 10 ml paramecia (5–13 dpf) and 2x 1 drop of artemia (14 dpf onwards).

3. CG1: CR conditions from 5-30dpf, DIO conditions 30dpf and older

Constant food levels, changing density: feeding under DIO conditions as above, density either

1. DIO: 5 fish per 2.5L tank for the extent of the experiment
2. CR: 50 fish per 2.5L tank for the extent of the experiment
3. CG1: 50 fish per 2.5L tank from 5dpf until 30dpf which were split into 10 times 5 fish per 2.5L tank at 30dpf onwards

All zebrafish experiments were approved by the national animal care committee (LANUV Nordrhein-Westfalen; 8.87–50.10.31.08.130; 84–02.04.2012.A253) and the University of Cologne. Animals were sacrifices with an overdose of Tricaine MS-222 according to [60].

Determination of body length and body weight

For determination of body length and body weight, fish were anesthetized in 0.13% Tricaine (w/v). Body length was measured from the anterior tip of the mouth to the base of the caudal fin (standard length, SL) using millimeter paper (to 0.5 mm). For determination of body weight, anesthetized fish were dried on Whatman paper and measured to one decimal point in milligrams. Per measurement 10 fish (as soon as visible 5 males and 5 females) were measured individually per condition.

Calculation of specific growth rate

We calculated specific growth rate (SGR) in %weight per day according to [61] calculation 9 which states SGR = (Ln(weight 2)-Ln(weight 1))/(Day 2—Day 1) x 100 where day 1 and weight 1 is the first measurement and day 2 and weight 2 the subsequent measurement.

RNA extraction and quantitative real-time PCR

Total RNA was purified from pool of 3 brains or individual bodies with the PureLink RNA Mini Kit (life technologies, Carlsbad, USA) after homogenization of the frozen material with mortar and pestle on liquid nitrogen and RNA extraction with Trizol (Invitrogen, CA, USA). cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen, CA, USA) according to the manufactor‘s instructions.

Quantitative real-time PCR (qRT-PCR) was performed in quadruplicates with an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, USA), SYBR Select Master Mix (Applied Biosystems, Foster City, USA) and primers for *agrp* [62] and *insa* [63]. Relative mRNA expression levels were determined with Biosystems Prism SDS and Excel software, using the expression level of *rps23* [64] as an internal standard.

Quantification of food intake

After determination of body weight, fish were put in single tanks (1 L) to acclimate and were starved for at least 24 hours. Feeding experiments took place at room temperature in a separated room without any disturbance to avoid stressing the fish. At least 5 fish were tested per condition.

Because there is no standard method available for quantification of food intake in adult zebrafish, a new method for quantification of food intake in adult zebrafish was established. Instead of commercial fish food, living third instar larvae of wild-type *Drosophila melanogaster* (*D. melanogaster*) were used for quantification of food intake because they can survive several hours in water without changing weight and keep moving making them an attractive prey for zebrafish. *D. melanogaster* provided by the Roth laboratory at the University of Cologne were

used to raise larvae for the feeding experiment. Third instar larvae of *D. melanogaster* were washed out of the feeding medium, counted, dried using Whatman paper and weighted. Larvae with a weight of at least 10% of the fish's body weight were given to the fish. During the following 8 hours, every 1–2 hours a counted number of fresh larvae were given to the fish. After one and after eight hours, fish were put in single tanks with fresh water and remaining larvae were counted and weighted. Weight was measured to one decimal points in mg using a precision scale. Food intake during the first hour was calculated in fed larvae in % bodyweight, while food intake during eight hours (whole day) was calculated in fed larvae/100 mg body weight.

μCT analysis

For μCT imaging, adult zebrafish were fixed and decalcified in Bouin's solution at room temperature for 7 days, stored in PBS and imaged using a micro-computed tomography (μCT) device (SkyScan1272, Bruker BioSpin GmbH, Ettlingen, Germany). Zebrafish were placed individually in 1.5ml Eppendorf tubes using and an ultra-focus scan over the whole body was performed in a full-rotation in step-and-shoot mode. 322 projections (1008x672 pixels, 4x4 binning) were acquired per subscan with an x-ray tube voltage of 60 kV, power 0.166 mA, aluminum filter 0.25 mm,exposure time of 363 ms, 6 averages and a object-source distance of 86 mm. All CT images were reconstructed at an isotropic voxel size of 18 μm using a Feldkamp type algorithm (filtered back-projection).

Fat-containing regions were appear hypo intense in μCT data and were segmented using Imalytics Preclinical (Gremse-IT GmbH, Aachen, Germany [65]. The volumetric fat percentage was calculated as the ratio of subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT) fat volume compared to the entire volume of the body cavity anterior of the anal fin and expressed per skeletal segment.

Histology

For histological analyses, adult zebrafish were fixed and decalcified in Bouin's solution at room temperature for 7 days and stored. After μCT imaging, male fish were fixed in 4% PFA overnight at 4˚C followed by PBS washes the next day. Viscera were removed, livers dissected from the fixed samples and oriented in 3% agarose in 30% sucrose/PBS and mounted in tissue freezing medium (Leica). The head and tail regions were removed by cutting out the tissue posterior to the operculum and anterior to the dorsal fin. The rest of the trunk (without the viscera) was subsequently bisected, decalcified in 0.5 M EDTA, embedded in the same way as mentioned for the livers. 8–10 μm transverse sections of the trunk and livers were obtained using a Leica CM1850 cryostat. The slides containing sections were air dried for 60 minutes at room temperature and fixed in ice cold 4% PFA for 10 minutes. After fixation, the samples were washed thrice in PBS for 5 minutes each and treated with 100% propylene glycol for 5 minutes. Meanwhile, the Oil Red O (ORO) (Sigma-Aldrich) stock solution (0.5g Oil Red O in 100 ml propylene glycol) was filtered and prewarmed at 60˚C. The samples were stained with ORO for 8–10 minutes at 60˚C. The slides were differentiated in 85% propylene glycol for 5 minutes, rinsed twice in distilled water and counterstained with Gill's haematoxylin for 30 seconds. Excess stain was removed by washing with running tap water for a minute, rinsed in distilled water and mounted with Aqua-Mount® (Avantor).

For the quantification of subcutaneous adipocytes, ORO and haematoxylin-stained transverse sections of three levels were selected, total numbers and areas per side around the pleural ribs (PR) region was measured using ImageJ. Average adipocyte cell size along both sides of the pleural rib in each section was calculated using area divided by cell number. The single

adipocyte cell sizes were further validated using ImageJ. For each condition, two fish and three consecutive sections were analysed, and the mean was recorded.

Analysis of oxygen consumption. After determination of body weight, fish were put in single tanks (1 L) to acclimate and were starved for at least 24 hours. Determination of oxygen consumption was performed at room temperature (19–20˚C) in a separated dark room without any disturbance to avoid stressing the fish. Individual fish were placed in respiratory chambers and allowed to acclimate for at least 30 min with continuous water flow before measurements started.

The experimental set-up consisted of 4 respiratory chambers (Loligo Systems, Tjele, Denmark; for adult fish: diameter = 40 mm, volume = 38 ml) with ministirrers inside which are placed in a water bath. Fresh air-bubbled water is provided using a Minipuls 3 flushing pump (Gilson, Limburg-Offheim, Germany) which is controlled by a computer. Dissolved oxygen levels were measured using Oxy 4 mini (Presens GmbH, Regensburg, Germany), a multichannel oxygen measuring system which connected via optical cables with oxygen sensors (Presens GmbH, Regensburg, Germany) which are glued inside the chambers. Oxygen levels were recorded every second during a 5 min phase in each interval (20 min flushing, 15 min waiting, 5 min measuring). Flushing is sufficient to exchange more than 99% of the water [66]. Oxygen sensors and optic fibres were calibrated using air (100% air saturation) and nitrogen bubbled water (0% air saturation) while a temperature probe was calibrated using ice (0˚C) and boiling water (100˚C) as described in the manual. Calibrations were done prior to each experiment. Oxygen consumption was calculated using the programme AutoResp2 (Loligo Systems, Tjele, Denmark) and determined during at least three days (72 h). Measured values with the coefficient of determination (R^2) > 0.9 were used to calculate routine and standard metabolism. Mean of oxygen consumption rate during 24 hours was defined as routine metabolism [67], while mean of the 5 lowest oxygen consumption rates were assumed to reflect SMR [68, 69]. Both rates were expressed as mg O_2 / kg/ h.

Analysis of mitochondrial function using respirometry

After determination of body length and body weight, fish were sacrificed, cut in small pieces and digested with trypsine on ice for 40 min. Intact mitochondria were isolated according to [70]. The mitochondrial protein concentration was determined using a Bradford assay.

Oxygen consumption rates were measured with Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) at 28˚C using 150–200 μg crude mitochondria as described before [71]. In short, mitochondrial complex I activity was measured with the addition of 1 mM ADP, 5 mM pyruvate, 2 mM malate, and 10 mM glutamate (maximal rate of oxidative phosphorylation of complex I, OXPHOS CoI). Then, mitochondrial coupling was evaluated by the inhibition of ATP synthase by adding 1.5 mg/ml oligomycin and uncoupling by a multiple-step carbonyl-cyanide p-trifluoromethoxyphenylhydrazone titration (membrane leakage, LEAK). Afterwards the uncoupler FCCP is added till a maximal plateau is reached to determinate maximal oxygen consumption flux due to electron transfer system (ETS). By adding 0.5 mM rotenone, complex I is inhibited to assess oxygen consumption flux due to reactive oxygen species (ROX). Of note, oxygen concentration below 100 nmol/ml were avoided.

Analysis of blood glucose levels in adult zebrafish

Blood sugar levels were determined using the glucose meter Accu-Chek Aviva (Roche Diagnostics, Risch, Switzerland) as described [72]. In short, fish were anesthetized in ice for 60 seconds and sacrificed afterwards by decapitation. The cut was immediately anterior to the articulation of the pectoral fin with the girdle, and severed the heart. Whole blood was analysed immediately by applying a test strip directly to the cardiac blood.

Statistical analysis

Statistics were carried out in GraphPad Prism version 8.4.3 for Windows, GraphPad Software, San Diego, California USA. The particular test used is indicated in each section. P-values less than 0.05 were considered significant.

Primary data

We have made the primary data available through GFBio [73] and the primary data is available under https://doi.org/10.1594/PANGAEA.941313 while the CT scans are available under https://doi.org/10.1594/PANGAEA.940201.

Results

CG in zebrafish—characterization

We compare calorically restricted (CR) fish with overfed / diet induced obese (DIO) fish and fish that were shifted from CR to an obesogenic environment after 1, 6 or 9 months of CR (compensatory or catch-up growth; CG1, CG3 and CG9 respectively, see also Fig 1A and 1B).

Consistent with previous work, overfeeding leads to increased somatic growth [35, 36]. We carried out multiple 2-Way ANOVA which in all cases showed a significant interaction of

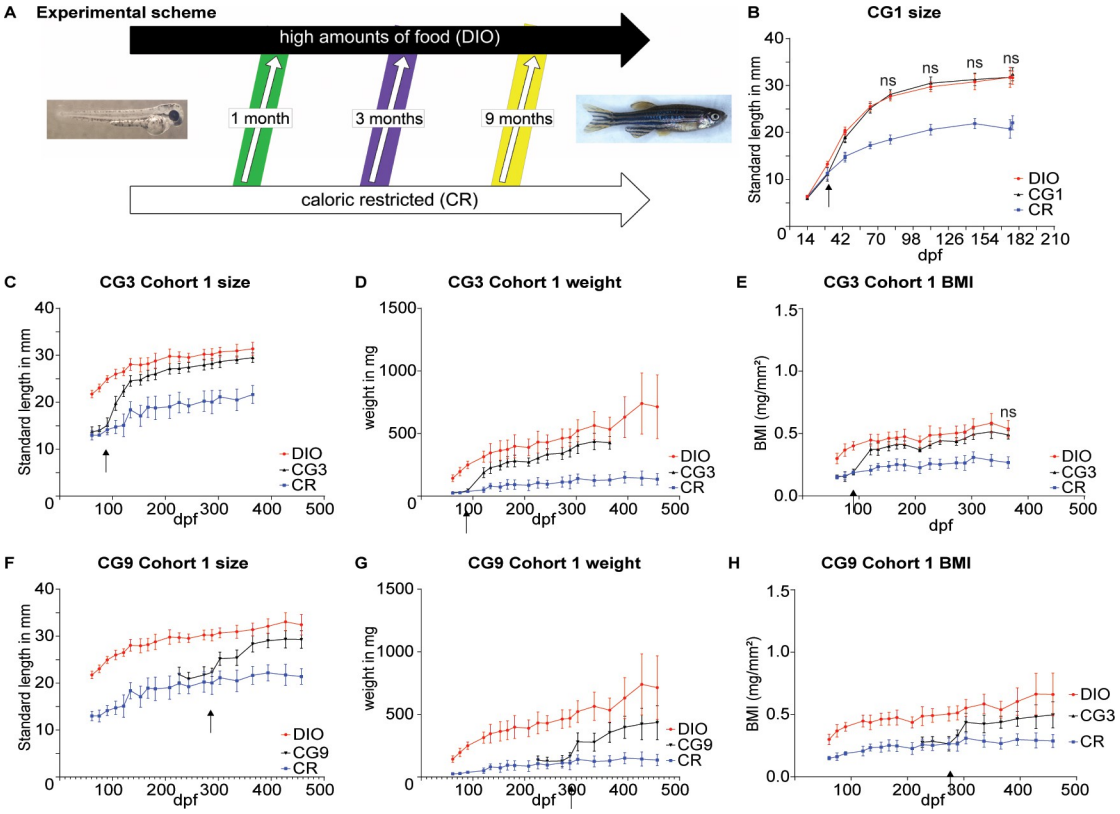

Fig 1. Compensatory growth can be induced in juvenile and adult zebrafish by excessive feeding following caloric restriction and results in compensation for differences in body length and BMI. (A) Scheme showing changes in feeding regime to induce compensatory growth (CG) in zebrafish with an age of one, three or nine months; (B) Standard length of fish undergoing CG at 1 month of age (arrow); (C, F): Standard Length; (D, G) body weight; (E, H) and body mass index of a cohort of fish (n = 10) undergoing CG at 3 months of age (C-E) and at 9 months of age (F, H); error bars indicate STDEV, ns indicates a lack of significant difference between the CG and the DIO group at that timepoint as indicated by a 2-Way ANOVA followed by Tukey's multiple comparison test.

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time (fish grow longer and heavier over time) and food (DIO fish grow faster than CR fish). For all datasets, CG fish started growing significantly when transferred to the obesogenic environment. We were interested in whether growth approaches the DIO curve and therefore only indicate on the graphs where the difference between the DIO and the CG group is not significantly different anymore according to Tukey's multiple comparison test.

For the early CG1 group we measured only the standard length. DIO fish grow significantly faster than CR fish. After transfer of the CG1 fish from CR to DIO conditions, fish compensate quickly. While we still saw a significant difference between DIO and CG1 at 44 and 64dpf, the two groups are not significantly different from 80 days onwards (Fig 1B; indicated as not significantly different between CG1 and DIO as indicated by a 2 Way ANOVA MCT; effect of time $F(9, 252) = 1301$, $p<0.0001$; effect of food $F(2, 252) = 1095$, $p<0.0001$; interaction $F(18, 252) = 40.18$, $p<0.0001$). When we induced CG at 3 months of age (CG3), we similarly saw that DIO fish grow faster than CR fish in terms of standard length (Fig 1C; 2 Way ANOVA effect of time $F(16, 451) = 175.6$, $p<0.0001$; effect of food $F(2, 451) = 2014$, $p<0.0001$; interaction $F(32, 451) = 10.96$, $p<0.0001$); body weight (Fig 1D; 2 Way ANOVA effect of time $F(15, 424) = 93.75$, $p<0.0001$; effect of food $F(2, 424) = 1291$, $p<0.0001$; interaction $F(30, 424) = 10.26$, $p<0.0001$) as well as body mass index (BMI, Fig 1E; 2 Way ANOVA effect of time $F(15, 424) = 79.29$, $p<0.0001$; effect of food $F(2, 424) = 1032$, $p<0.0001$; interaction $F(30, 424) = 7.508$, $p<0.0001$). During the compensatory response, CG3 fish showed an initial fast growth followed by a plateau that slowly approached the DIO group but in the time we measured remained significantly different to the DIO group in the two way ANOVA MCT.

When we induced CG at 9 months of age we found a comparable result in that the CG group never quite reached the DIO groups growth, either for standard length (Fig 1F; 2 Way ANOVA effect of time $F(9, 265) = 35.09$, $p<0.0001$; effect of food $F(2, 265) = 900.4$, $p<0.0001$; interaction $F(18, 265) = 7.444$, $p<0.0001$), fish weight (Fig 1G; 2 Way ANOVA effect of time $F(9, 265) = 23.10$, $p<0.0001$; effect of food $F(2, 265) = 511.2$, $p<0.0001$; interaction $F(18, 265) = 4.511$, $p<0.0001$) or BMI (Fig 1H; 2 Way ANOVA effect of time $F(9, 265) = 17.26$, $p<0.0001$; effect of food $F(2, 265) = 363.1$, $p<0.0001$; interaction $F(18, 265) = 3.285$, $p<0.0001$).

We repeated the experiment in a separate cohort with a comparable result–the CG group responded with a period of fast growth and never quite approached growth in the DIO group except in one timepoint (S1 Fig). We again induced CG at 3 months of age and measured standard length (S1A Fig; 2 Way ANOVA effect of time $F(11, 324) = 206.9$, $p<0.0001$; effect of food $F(2, 324) = 1050$, $p<0.0001$; interaction $F(22, 324) = 14.44$, $p<0.0001$), body weight (S1B Fig; 2 Way ANOVA effect of time $F(11, 324) = 57.69$, $p<0.0001$; effect of food $F(2, 324) = 351.3$, $p<0.0001$; interaction $F(22, 324) = 7.463$, $p<0.0001$) and BMI (S1C Fig; 2 Way ANOVA effect of time $F(11, 324) = 51.61$, $p<0.0001$; effect of food $F(2, 324) = 357.8$, $p<0.0001$; interaction $F(22, 324) = 5.066$, $p<0.0001$). For CG9 we also measured standard length (S1D Fig; 2 Way ANOVA effect of time $F(10, 297) = 37.99$, $p<0.0001$; effect of food $F(2, 297) = 1259$, $p<0.0001$; interaction $F(20, 297) = 9.571$, $p<0.0001$), fish weight (S1E Fig; 2 Way ANOVA effect of time $F(10, 297) = 15.28$, $p<0.0001$; effect of food $F(2, 297) = 464.4$, $p<0.0001$; interaction $F(20, 297) = 2.251$, $p = 0.0019$) and BMI (S1F Fig; 2 Way ANOVA effect of time $F(10, 297) = 14.61$, $p<0.0001$; effect of food $F(2, 297) = 358.1$, $p<0.0001$; interaction $F(20, 297) = 1.661$, $p = 0.0389$).

We previously showed that growth at different densities is equivalent to overfeeding at the same density [36]. However, it is conceivable that CG would be impacted in a different way by density changes. Therefore, we also tested the 1month CG condition keeping the number of fish per tank consistent between the CR, CG and DIO groups but varying the food given. We saw no difference in CG between changes in feeding regime with a constant density (S2A Fig; 2 Way ANOVA effect of time $F(6, 179) = 198.5$, $p<0.0001$; effect of food $F(2, 179) = 339.3$,

$p<0.0001$; interaction $F(12, 179) = 16.82$, $p<0.0001$) and constant feeding regime with changes in density S2B Fig; 2 Way ANOVA effect of time $F(6, 186) = 180.6$, $p<0.0001$; effect of food $F(2, 186) = 463.8$, $p<0.0001$; interaction $F(12, 186) = 16.13$, $p<0.0001$). If anything, the change in density lead to a faster catch-up period than the change in feeding regime.

CG in zebrafish–catch up growth in the absence of compensatory growth

In order to establish the mode of growth in zebrafish, we compared growth trajectories. Zebrafish show a typical sigmoidal growth curve. We started measuring too late to see the exponential phase of growth, but in every group, we saw a clear segment of linear growth. Puberty onset occurs at an SL of around 18mm [74] which in the DIO group occurs around 45 dpf and in the CR group occurs around 3 months of age. A decline in growth rate is seen in many fish after sexual maturity [13], and particularly in the DIO curve, fish growth is linear until shortly after maturity and starts becoming asymptotic around 20-25mm SL (Fig 1C, 1D, 1F, 1H and 1J)). Looking at the growth curves, we noticed that CG fish grow in a similar manner to DIO fish of a comparable SL when they are transferred to *ad libitum* conditions, suggesting that fish resume a normal growth trajectory after the period of growth stagnation, which suggests catch-up growth and not compensatory growth.

We calculated growth rates according to [61]. The results did not differ between the cohorts so we only show the results for the second cohort of fish we raised. The growth rate of the DIO fish is the highest during the period of linear growth and rapidly declines during the period of asymptotic growth around 45–90 days followed by a very low residual growth rate (Fig 2A–2C). The growth rate in the CG groups peaks briefly after release from the growth restricting conditions and indeed during this time is significantly higher than the DIO or CR group. Importantly however, the growth rate of the CG group does not rise above the highest growth

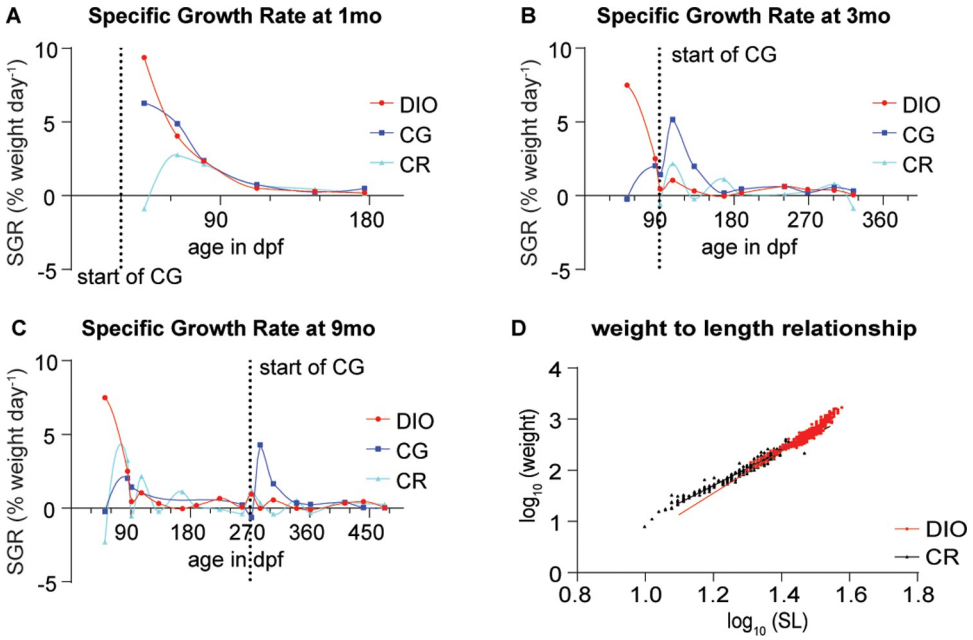

Fig 2. Growth rate and weight-length relationship in fish growing under different conditions. (A-C) Specific Growth rates (SGR) of fish fed under DIO, CG and CR conditions and undergoing CG at (A) one month of age, (B) 3 months of age (C) or 9 months of age. Data fit with an Akima spline curve; (D) The weight-length relationship of CR and DIO fish. The slope is significantly larger in the case of the DIO group compared to the CR group $F(1, 970) = 133$, $p<0.0001$.

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rate seen in the DIO group at an earlier timepoint, providing evidence that the observed growth is catch-up growth [13].

We also looked at the weight-length relationship for CR and DIO fish with a linear regression analysis of the log weight to log standard length relationship. For DIO fish we found that log(DIO weight) = 4.134*log(SL) -3.404, R^2 = 0.9037 and for CR fish that log(CR weight) = 3.363*log(SL) - 2.329, R^2 = 0.9575 (Fig 2D). Therefore, we measure b at 4.134 for DIO fish and 3.363 for CR fish which provides evidence for positive allometric growth in zebrafish (b>3 according to [75]).

CG in zebrafish—CR and early CG fish are hyperphagic

During the early CG period, we expect animals to be hyperphagic when transferred to *ad libitum* food access. We first looked at the expression levels of *agouti related protein (agrp)* which is known to be an indicator of hunger in animals including zebrafish [62]. As expected we saw an increase in *agrp* expression in fish brains in the CR group compared to the DIO group, suggesting increased hunger in the calorically restricted group. When we tested CG9 fish one to two weeks after the transfer from CR to DIO conditions we found that *agrp* levels were significantly reduced compared to CR but had not yet returned to baseline compared to DIO (Fig 3A, ANOVA F(2, 6) = 114.4, p<0.0001). We measured food intake by counting living third instar larvae of *D. melanogaster*. Larvae can survive several hours in water without changing weight and continue to move which makes them an attractive prey for zebrafish. We first tested feeding in DIO and CR fish and found that food intake in DIO fish rises in proportion with body weight and therefore remains relatively constant if expressed in relation to body weight. At both timepoints tested, we found that CR fish when exposed to *ad libitum* feeding conditions are severely hyperphagic and eat nearly twice the amount of food as DIO fish (Fig 3B). The hyperphagia was significant at CG3 (Fig 3C, t(17) = 3.498, p = .0028) as well as CG9 (Fig 3D, t(18) = 3.622, p = 0.0020). This hyperphagia persisted for at least one week after the transition of CR fish into the obesogenic environment in both the CG3 (Fig 3C, t(16) = 6.150, p<0.0001) as well as the CG9 (Fig 3D, t(18) = 5.380, p<0.0001) groups.

Adipose distribution in an obesogenic environment–the DIO phenotype

We first investigated adipose distribution in DIO fish. Evidence for the development of diet induced obesity has been shown in zebrafish for 8 weeks of overfeeding. However, the authors did not look at adipose distribution [35]. We carried out histology of DIO fish at 8, 9.5 and 12 months of age, a period where growth has ceased but which is associated with an increase in body circumference as zebrafish show positive allometric growth (Fig 2D). We saw that SAT adipocyte number increased between 8 and 9.5 months but not between 9.5 and 12 months of DIO (Fig 4A, ANOVA F(2,3) = 19.83, P = 0.0186). Adipocyte size increased between 8 and 9.5 months as well as between 9.5 and 12 months (Fig 4B, ANOVA F(2,3) = 144.9, P = 0.0010). This data shows that fish show hypertrophy across all three timepoints but gradually loose the capacity to store fat through adipocyte hyperplasia.

In order to get a better idea of the quantitative distribution of adipose tissue in zebrafish, we carried out μCT on adult DIO fish after increasing amount of time on the obesogenic diet (representative transverse section and 3D rendering see Fig 4C and 4D respectively). We quantified fat volume per skeletal segment across the body cavity and found that visceral adipose tissue (VAT) showed a significant increase in VAT at 12 months of DIO (Fig 4E, ANOVA F (2,10) = 17.76, P = 0.0005). We found the same increase in SAT in the 12 month DIO group (SAT Fig 4F, ANOVA F(2,10) = 18.56, P = 0.0004).

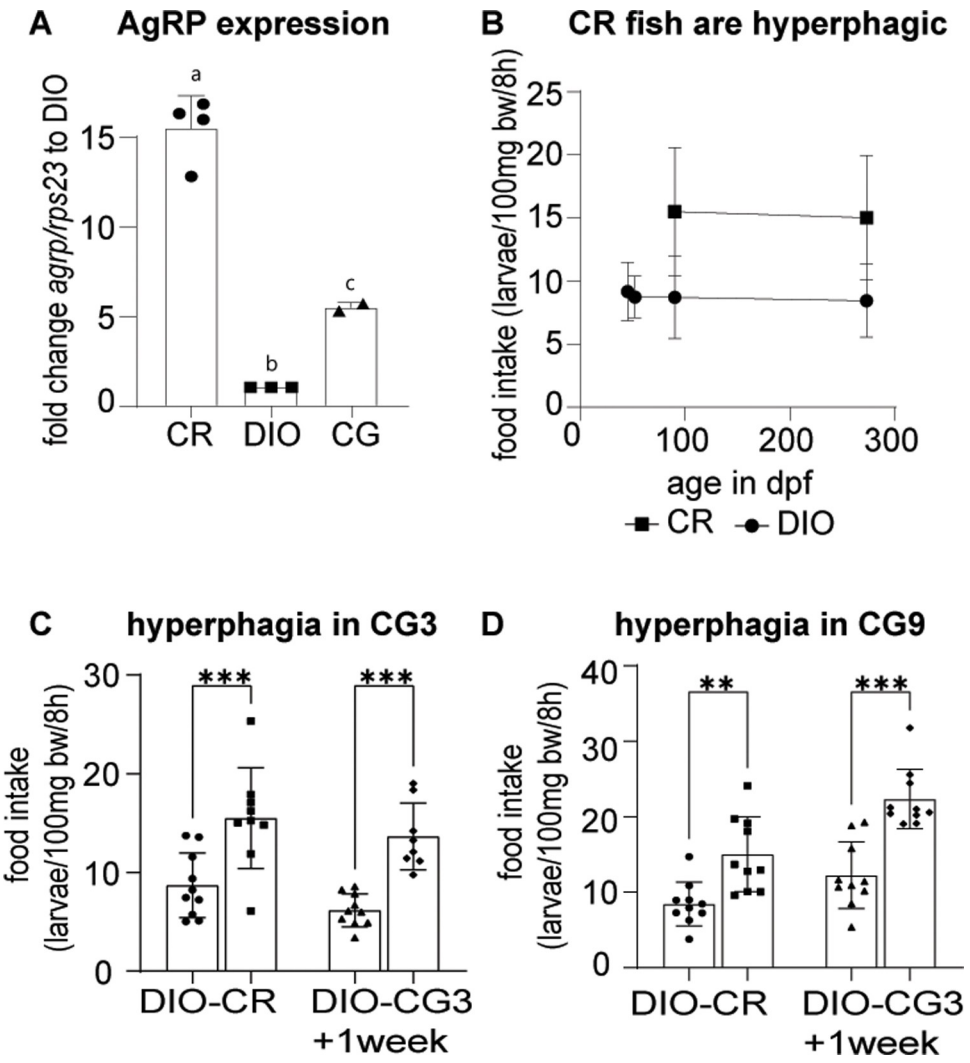

Fig 3. Fish undergoing CR and CG are hyperphagic. (A) *agrp* expression analysis of CR and DIO fish brains as well as CG9 fish brains 1–2 weeks after the shift to the obesogenic environment. Expression is standardized to *rps23*; (B) calorically restricted fish are severely hyperphagic when exposed to *ad libitum* feeding conditions compared to the DIO group that has been raised in an obesogenic environment; (C) CG3 fish are hyperphagic before (CR) transfer to DIO conditions as well as at least one week after transfer (CG3); (D) The CG9 group is similarly hyperphagic before as well as at least one week after the transfer to DIO conditions. Pooled data of male and female fish, n of 4–10; error bars indicate STDEV, groups were compared with ANOVA followed by Tukey's multiple comparisons test (A) or an unpaired t-test (C, D), significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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We tested liver sections for signs of fatty liver by staining cryo-sections with Oil red O and found evidence for increased hepatic adiposity between 8 months and 12 months of DIO. Further we saw early signs of steatohepatitis in the 9.5 and 12 months old hepatic tissue which we did not see at 8 months of age (Fig 4F). In our hands, the lipid droplets stained with oil red O (ORO) partly floated off and consequently not all lesions are positive for ORO.

Adipose distribution–CG does not exacerbate the DIO phenotype

We next looked at whether CG exacerbates the DIO phenotype by both histology as well as μCT. Based on our histological examination of SAT, we found that the CG1 group catches-up in both adipocyte number as well as size while the CG3 group catches up in size but not

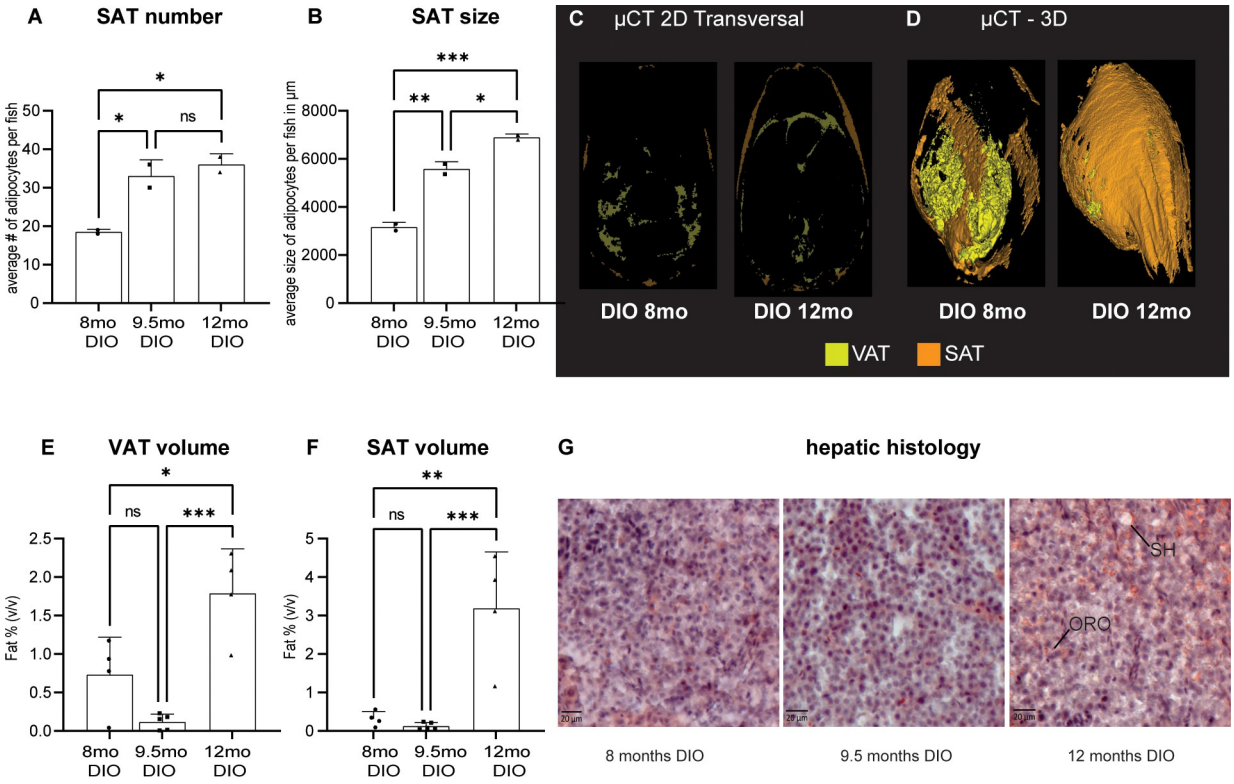

Fig 4. Adipose distribution under overfeeding conditions. (A) Subcutaneous adipose (SAT) cell number after 8 to 12 months of DIO; (B) average SAT adipocyte size after long-term DIO, Data from two representative male fish of the cohort; (C-F) Adult DIO fish of different ages and length on an obesogenic diet were compared for whole body adiposity using µCT imaging; (C) representative transverse section through DIO fish at 8 months and 12 months of age (D) representative three-dimensional volume rendering of visceral adipose tissue (VAT) and SAT; (E) quantification of VAT and (F) SAT, Data from 4–5 mixed gender fish and (G) representative hepatic histology of the 8, 9.5 and 12 month endpoints in DIO feeding conditions. Cryosections stained with hematoxylin for nuclei and oil red O (ORO) for lipids. Sections showed progressive signs of lesions indicative of steatohepatitis (SH). Error bars indicate STDEV, groups were compared with an ANOVA followed by Tukey's multiple comparisons test, significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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number (Fig 5A adipocyte number ANOVA $F(5,6) = 33.55$, $P = 0.0003$ and Fig 5B adipocyte size ANOVA $F(5,6) = 198.3$, $P<0.0001$). Therefore, at an earlier timepoint, adipocyte hypertrophy is the predominant mode of compensation. At the later CG9 timepoint, the CG9 group catches up to the DIO group in number but not (quite) size of adipocytes (Fig 5A and 5B).

We also scanned CG animals with µCT (Fig 5C, compare to 4C) and found that VAT and SAT volume is caught up completely at the CG1 and CG3 timepoints but not the CG9 timepoint (VAT Fig 5C ANOVA $F(5,20) = 6.564$, $P = 0.0009$; SAT Fig 5D ANOVA $F(5,20) = 15.57$, $P<0.0001$). Staining hepatic tissue with ORO showed an increase in adipose deposition as well as steatic hepatosis in the CG9 group compared to the CG3 group but less adipose staining or lesions in the CG9 group compared to the DIO liver of the same age (Fig 5F, compare to 4G).

CG in zebrafish—metabolic rates support a role for lean body mass in the late CG delay

We set-up intermittent respirometry to measure oxygen consumption in zebrafish and calculated metabolic rates. Oxygen consumption can be used to calculate routine and standard metabolic rate (RMR and SMR). We validated the procedure with fed and fasted animals since there should be a difference between these two groups due to the thermic effect of food. Consistent with previous publications [76–79], we found a significant increase in metabolic rate in

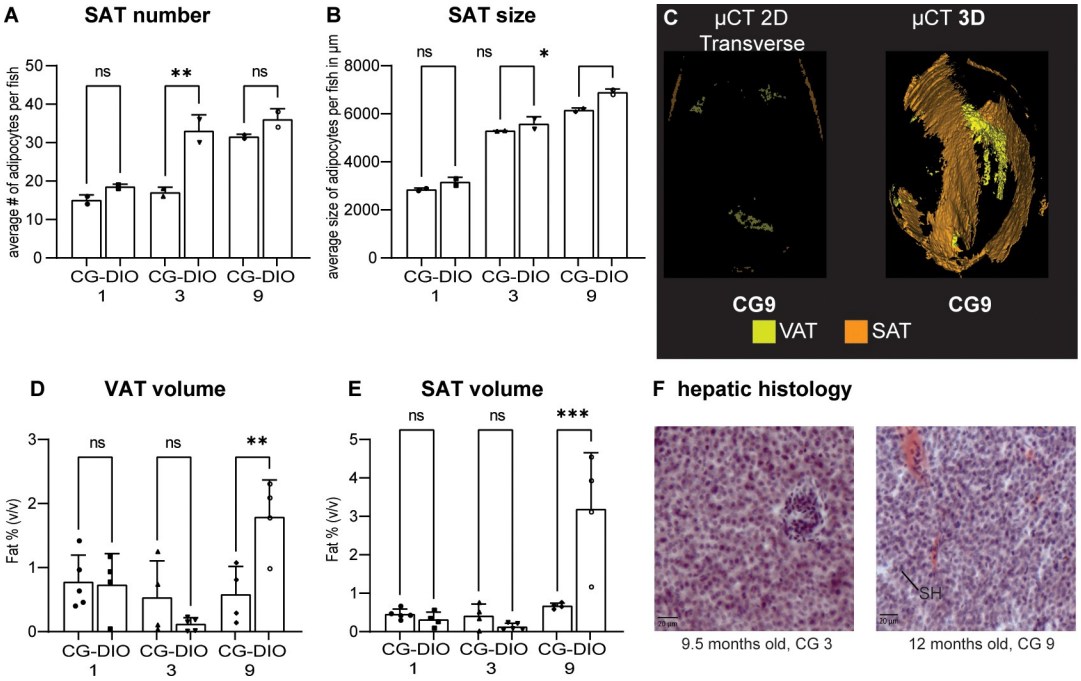

Fig 5. Early CG does not exacerbate DIO. Endpoint comparison of CG1, CG3 and CG9 animals with their respective DIO control groups at 8, 9.5 and 12 months respectively. (A) Subcutaneous adipocyte (SAT) number and (B) size. Data is from two representative male fish of the cohort; (C) CG1-9 fish were compared for whole body adiposity with DIO fish using µCT imaging; (D-E) quantification of visceral (VAT) and subcutaneous (SAT) adipose tissue by µCT, data from 4–5 mixed gender fish. (F) representative hepatic histology of the CG group stained with hematoxylin and oil red O (ORO). Sections show little ORO staining and few lesions (compare with Fig 4F). Error bars indicate STDEV, groups were compared with an ANOVA followed by Šídák's multiple comparisons test, significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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fed fish compared to fasted fish. Respiration from bacteria (oxygen chambers without fish) was negligible (Fig 6A; ANOVA $F(2,7) = 181.1$, $p<0.0001$). When we looked at the CG groups, we found no difference in either RMR (Fig 6A; ANOVA $F(5, 28) = 2.243$, $p = 0.0778$) or SMR (Fig 6B; ANOVA $F(5, 28) = 2.961$, $p = 0.0287$) compared to the DIO groups.

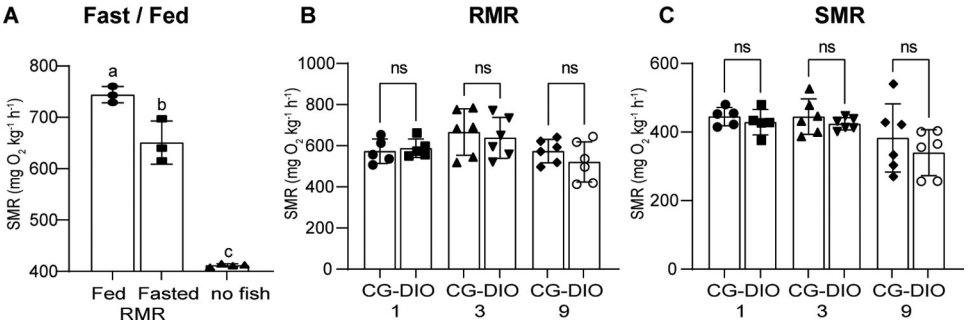

Fig 6. CG does not lead to changes in metabolic rate. (A) Shows the effect of fasting on resting metabolic rate in adult fish. RMR is lower during fasting conditions, n = 3 for fed and fasted, n of 4 for chambers without fish; (B) We compared CG1, CG3 and CG9 fish with their respective DIO control fish and found no difference in RMR; (C) We similarly found no difference in standard metabolic rate between the CG and the DIO control groups. n = 6 each, error bars indicate STDEV, groups were compared with an unpaired t-test (A) or an ANOVA followed by Šídák's multiple comparisons test (B-C), significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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CG in zebrafish–role of mitochondria

We tested for mitochondrial function in CR and DIO fish. As there were significant size difference between CR and DIO fish we also included a size-matched DIO group which reached this size at a significantly younger age (1.5 months versus 9 months). Therefore, the CR and DIO groups are matched in age and the CR and size-matched DIO groups in size. We found that the maximal rate of oxidative phosphorylation did not differ between age matched or size matched calorically restricted or overfed fish (Fig 7A, ANOVA F(3,8) = 1.129, p = 0.3936). We next tested the leakage of the mitochondrial membrane which also did not differ between age matched or size matched CR and DIO fish (Fig 7B, ANOVA F(3,8) = 1.172, p = 0.3793).

When we compared the CG groups with the respective CR and DIO control groups, we found no evidence for changes in mitochondrial function at either CG3 (Fig 7C, ANOVA F (3,8) = 2.197, p = 0.1662) or CG9 (Fig 7D, ANOVA F(3,8) = 3.609, p = 0.0651). We saw a trend specifically in the CR to CG3 comparison with a t-test (t(4) = 2.766, p = 0.0505) which is

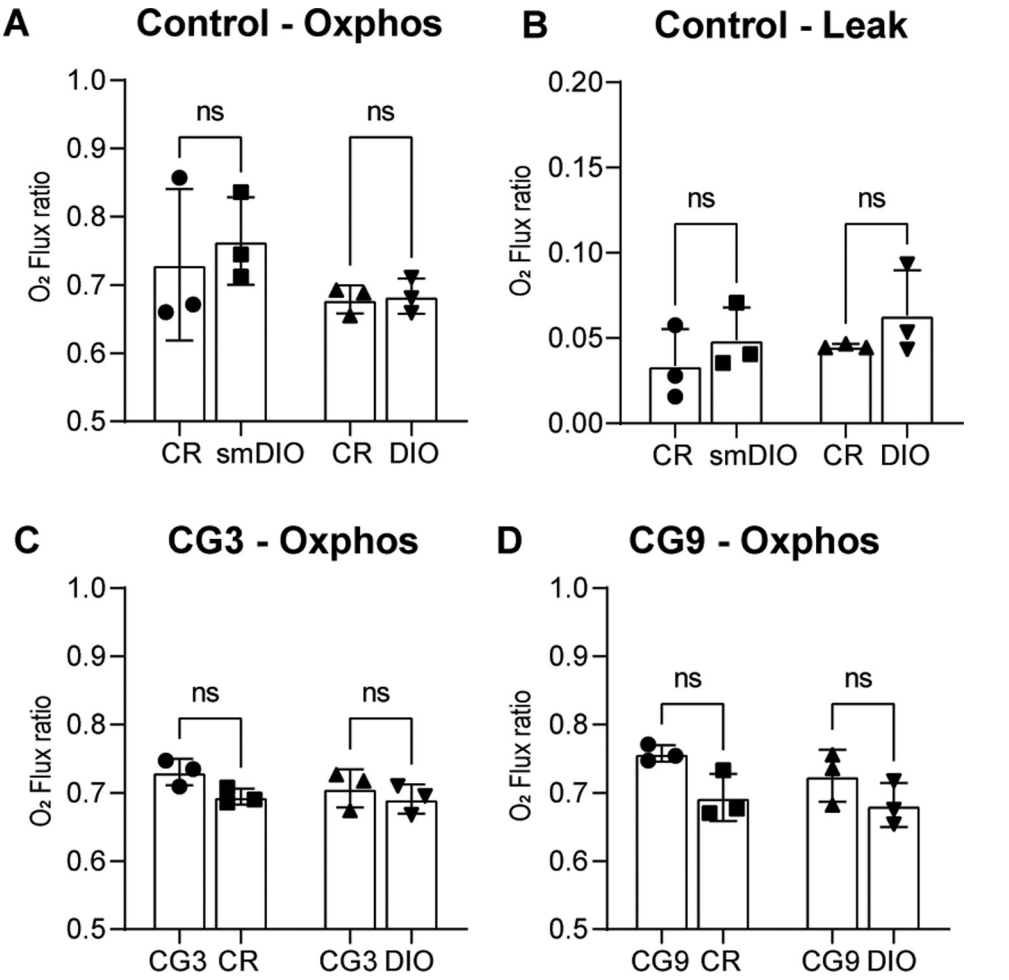

Fig 7. The shift to CG leads to an increase in maximal oxidative phosphorylation. (A) Due to the size difference between CR and DIO fish we tested age matched (CR and DIO) as well as size matched (CR and size matched (sm) DIO) maximal rate of oxidative phosphorylation and (B) the leakage of the mitochondrial membrane; (C-D) the maximal rate of oxidative phosphorylation in CG compared to control CR and compared to control DIO fish for (C) CG3 and (D) CG9; n = 3 for each group, error bars indicate STDEV, groups were compared with an ANOVA followed by Šídák's multiple comparisons test.

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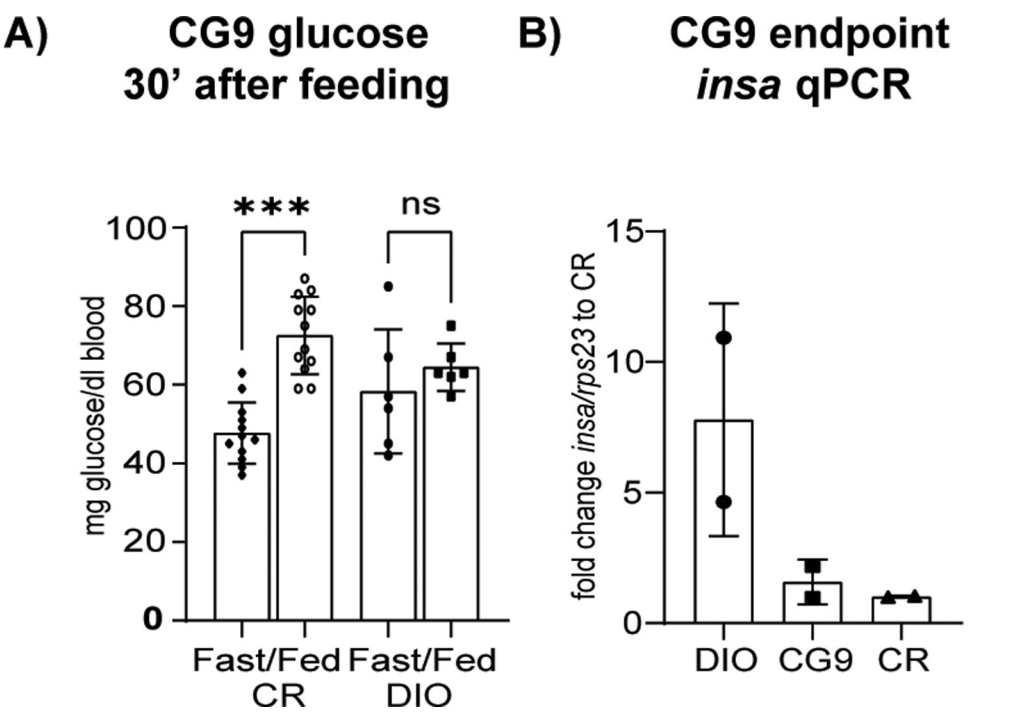

Fig 8. Glucoregulation at 9 months. (A) Blood glucose levels in fish fasted for 90 hours and tested 30 minutes after refeeding, CR n = 12 and DIO n = 6; (B) *insa* qPCR of whole fish body, n = 2; Error bars indicate STDEV, groups were compared with an ANOVA followed by Tukey's multiple comparisons test (A) or Šídák's multiple comparisons test (B-C), significance is indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

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significant in the CR vs CG9 group ($t(4) = 3.034$, $p = 0.0386$). Since we have multiple comparisons we used an analysis of variance followed by Šídák's multiple comparison which is more stringent than an unpaired t-test. We found no differences between any of the groups in the mitochondrial membrane leak current (not shown).

Traces of metabolic disease—glucoregulation

Elevated blood glucose levels at rest are an early sign of metabolic disturbance in obesity. We tested 9 months old fish fasted for 90 hours. We fed granulated food and tested blood glucose thirty minutes after feeding and found that CR fish respond with an elevated blood glucose level while DIO fish showed a reduced excursion (Fig 8A, ANOVA $F(3, 32) = 2.075$, $p<0.0001$). In order to support this data, we tested for insulin expression. In adult zebrafish, the islets are not in a distinct location but spread along the intestines. We therefore tested expression in whole bodies of fish. We only had two fish of the cohort for this analysis so the data is only indicative but we saw a trend towards an increased *insa* expression in DIO fish but not in CG9 fish compared to CR fish (Fig 8B, ANOVA $F(2, 3) = 4.102$, $p = 0.1385$).

Discussion

Here we carried out a systematic characterization of growth responses in zebrafish. On one hand we aimed to establish whether we could observe obesity like endophenotypes in long-term overfed zebrafish, on the other hand we wanted to establish whether CG leads to an exacerbated DIO phenotype. Multiple authors have reported an increase in adiposity after overfeeding [35, 36], however long-term detrimental effects and markers are largely unknown.

Further, there is controversy regarding genetic obesity models in the field such as the effect of leptin receptor mutations [30, 80]. Consequently, it is possible that fish (or given the wide range of life histories amongst fish at least zebrafish) store energy differently or at least not in form of adipose stores such as in mammals. In our previous study we showed that large adult females shunt energy primarily towards reproduction, followed by somatic growth and only then storage of fat [36]. Zebrafish are income breeders and as such given excess food could potentially increase daily reproductive capacity instead of storing energy for a later point [81]. This would circumvent increased adiposity and instead lead to increased body growth but no increase in body to mass index or conditioning index and we would expect no downstream deleterious effect of increased body adiposity such as lipotoxicity or hyperglycemia.

We therefore aimed to establish a more in-depth characterization of diet induced obesity in zebrafish. Linear regression of our growth curves showed that zebrafish undergo positive allometric growth. Positive allometric growth means that large specimens in the cohort show an increased circumference rather than an increased length. Therefore, at a higher SL, weight gain plays a predominant role while linear growth stagnates and/or plays a lesser role. From a mammalian and metabolic syndrome perspective, this is an interesting observation as obesity in mammals similarly is a lack of linear growth and instead a deposition of excess energy in adipose tissue. This data shows that while zebrafish grow linearly well into adulthood, this linear growth eventually stagnates and instead fish start depositing energy into circumference and likely adipose deposition. Looking at adipose depots in more detail, we found that SAT adipocyte number and size increased significantly over time, particularly at the late timepoint of 12 months. Further, SAT and VAT volume significantly increased in at 12 months of DIO but not before. Fish growth is sigmoidal with an early exponential phase, a linear phases and late growth stagnation. This suggests that energy allocation slowly shifts from linear growth to energy storage with a visible impact upon internal adipose tissue by one year of age. Further, previous studies have shown hepatic steatosis as a consequence of long-term overfeeding [35, 45, 46] which we were able to confirm in our long-term DIO fish which showed increased hepatic adipose deposition as well as increased number of hepatic lesions, which are reminiscent of the scarring seen in long-term obesity in mammals. When we looked at metabolic rate and mitochondrial function we did not see an overt phenotype in DIO animals. We saw a statistically not significant trend in the data comparing mitochondrial function between the CR and CG groups. A similar effect has been observed in mice during the first weeks after initiating a high fat diet where animals show changes in oxidative phosphorylation which normalize once animals adapt to the obesogenic diet [82]. For metabolic rate we looked at routine and standard metabolic rate (RMR and SMR). RMR is the energy required by an unfed animal which exhibits normal, spontaneous activity and is expected to be higher than SMR which is the energy needed to maintain proper body function [78, 83]. We found metabolic rate to be significantly reduced in DIO mammals due to the relative increase in adipose and decrease in muscle tissue. The lack of a difference between CR and DIO fish may be due to the fact that these animals are poikilothermic. The role of core temperature in obesity is controversial, but as a whole, obesity seems to lead to a reduced core temperature [84] similar to genetic obesity models [85]. In fish conversely, this variable is tightly controlled by the experimenter as core temperature is according to water temperature and the same across conditions. Further, a reduced temperature is known to reduce the metabolic rate. Lastly, we investigated glucose metabolism, specifically how fish responded to a food stimulus. Interestingly, DIO fish showed less of a glucose excursion 30 and 120 minutes after feeding compared to control animals suggesting an increased resorption of postprandial glucose from the bloodstream. In support of this, DIO fish showed an increased level of *insa* transcripts. This data suggests that fish undergo an adaptive response to overfeeding by increasing insulin production and thereby

glucose resorption as opposed to an impaired glucose handling such as in evident in type 2 diabetes supporting previous evidence in zebrafish that oral glucose tolerance is impaired in long-term overfed fish [58]. It would be interesting to see whether specific strains of fish are more or less predisposed to DIO induced changes in glucose handling–adaptive as well as maladaptive as it is still largely unclear why some people with obesity respond with adaptive changes such as we saw in these fish and others develop type 2 diabetes. In conclusion, DIO zebrafish share hallmarks of long-term obesity with mammals. While the data presented here confirms that a long-term obesogenic environment leads to excess lipid deposition [35–38], hepatic lipid deposition and hepatic steatosis [35, 45, 46] as well as hyperglycemia and impaired glucose handling in zebrafish [38, 58], we did not find evidence for significant alterations in metabolic rate of mitochondrial function.

The other main direction of this study aimed to characterize CG in zebrafish. We wondered whether we could utilize the fish to model aspects of metabolic disease, particularly a predisposition to develop metabolic disease later in life after caloric restriction or undernourishment in early life / childhood. We found that body weight and length catch-up and do not compensate. Historically the two terms are often interchangeable, but Malcolm Jobling has pointed out key differences, specifically that compensation involves a period of increased growth rate above normal growth rates while catch-up growth involves animal growth at normal growth rates after a period of depressed growth [13]. Importantly, above-normal growth rates are associated with detrimental effects on animal physiology that could contribute to the aetiology of metabolic syndrome. Fast growth is correlated with increased oxidative stress [86], reduced protein turnover, increased telomere shortening, and leads to an accumulation of cellular damage and indeed reduced longevity [23, 87]. We did not observe increased growth rates after food restriction from 5dpf onwards. This was surprising to us as we initiated CG very early and had hypothesized that we would see compensatory growth. However, while growth rates in fish that were catching up increased upon transfer to *ad libitum* food conditions, they never exceeded those we saw in DIO conspecifics at a similar (earlier) period and standard length in life. This result suggests that either zebrafish do not respond with compensatory growth upon food restriction or that there is a critical period where growth stunting conditions lead to compensatory growth with an increased growth rate and that this period is already over 5 days post fertilization. Indeed, there is controversy in the mammalian literature about the effect of early growth restriction on compensatory growth and the most solid evidence involves food restriction during the peri-natal period, particularly by food restricting dams during pregnancy or pre-weaning [12]. In the medical literature, it appears that increased growth rates and downstream metabolic syndrome are mostly associated with pre-term birth and it has been suggested that the variable literature on catch-up growth in low weight infants is due to an unclear definition of low gestational age and compensatory growth is rather associated with pre-term birth when compared to term infants [1, 88]. Our study supports this notion as larvae feed off of maternally provided egg yolk until 5dpf, after which they need to provide sustenance themselves. Therefore, our experimental setup is analogous to caloric restriction immediately after a term birth / after weaning. Given the wealth of knowledge on zebrafish embryo development, it would be interesting to expand this study to look at the long-term effects of yolk depletion or maternal CR. Indeed, a recent study looked at partial yolk depletion in embryos and found changes in gene expression associated with metabolic syndrome at 2dpf [89]. However, the authors did not measure growth rates or characterized long-term responses between yolk depleted and sham treated siblings. This implies that malnutrition during these 5 days of yolk supplied energy could be comparable with pre-term birth and subsequent predisposition for metabolic syndrome. Therefore, it would be interesting to study which neural circuits are particularly predisposed to alteration by yolk depletion.

Based on the literature, one would predict that in the case of compensatory growth, the shift from CR to DIO would lead a long-term exacerbation of metabolic effects while in the case of catch-up growth one would expect effects similar to DIO or slightly behind the DIO group still catching up. Our growth curves support catch-up and not compensatory growth, and we indeed observed that none of the CG groups showed metabolic endpoints with an exacerbated phenotype compared to the DIO group. Indeed, some of the CG groups did not show a complete catch up process to the DIO group yet. While the early shift (CG1) caught up completely with the DIO group endpoints, the CG3 group mostly did and the CG9 group never caught up completely. The growth curves approximated each other, but especially in the metabolic endpoints there were still significant differences which are suggestive of a sequential process in the development of metabolic endpoints. For example, the CG3 group did catch up to the DIO3 group in adipocyte size but not number suggesting that SAT adipocytes undergo hypertrophy before the undergo a renewed period of hyperplasia. In mammals, hypertrophy during obesity is considered to be indicative of metabolic complications while hyperplasia is considered to be protective [43]. Fish appear to undergo both but sequentially. As far as overall levels of SAT and VAT around the body cavity are concerned, our μCT analysis showed that CG1 and 3 fish catch up to DIO fish but CG9 fish do not catch up to DIO9 fish in volume of adipose depots. This shows that adipose storage is a long-term process which specifically happens at a later timepoint of growth and that CG9 fish have not been in an obesogenic environment for long enough. Therefore, this data does not support that early lifetime caloric restriction (after 5dpf) is a risk factor for a later development and exacerbation of metabolic complications. Similarly, the hepatic steatosis we observed in the DIO9 groups could not be seen to the same extent in the CG9 group and the increased glucose resorption after feeding we saw in the DIO group but not the CG9 group suggesting that the islets had not undergone as significant an expansion as in the DIO9 group in order to compensate for the increased food intake. Therefore, the period of caloric restriction we utilized in these experiments was if anything protective of long term overfeeding induced effects and the CG9 fish never caught up to the DIO controls which were never privy to any food restriction. More studies would be needed as to whether maternal food restriction or overfeeding and/or food restriction before 5dpf would lead to long-term effects. This study lays the groundwork to explore this topic further.

In summary, it is notable that fish exhibit similar trends in the storage of lipids in an obesogenic environment compared to mammals and that fish of the Ekwill strain show several adaptive responses to DIO. However, significant variation exists between people in terms of response to obesity. Similarly, a recent paper showed differences between two strains of fish in fasting induced central gene expression changes [90]. It would be interesting to see whether similar strain differences exist in fish between metabolic endpoints such as a predisposition for adipose storage or a predisposition to type 2 diabetes. Further, while this study is used two large cohorts to establish the growth phenotype, we looked at metabolic markers (Figs 3–8) with a relatively moderate number of biological replicates. Obesity in mammals is known to induce relatively strong effects. We therefore decided to look for large biological effects in multiple metabolic markers. We saw such effects in the DIO groups compared to the CR groups. However, we did not see evidence that CG significantly exacerbates DIO (or that there is an observable trend in the data). Consequently, we cannot rule out that there are small biological effects in this data that would require significantly larger cohorts to establish. Interestingly, while there is controversy about the role of the leptin receptor in obesity in zebrafish [80] in studies that scored growth results before 6 months of age, a recent study showed that loss of the leptin b paralog in zebrafish leads to increased adiposity and a phenotype reminiscent of type 2 diabetes specifically in older fish between one and two years of age [91]. That is

consistent with the data presented in this paper in that some metabolic endophenotypes only develop at a more advanced age. It would further be interesting to see whether infiltration of lipid into the liver, kidney, muscle or indeed the heart led to functional consequences for the zebrafish such as a reduced filtration rate or a reduced swim capacity.

Supporting information

S1 Fig. Compensatory growth can be induced by excessive feeding following caloric restriction: Cohort 2. (A, D) standard length; (B, E) fish weight and (C, F) body mass index of fish undergoing CG3 (A, B, C) of CG 9 (D, E, F); error bars indicate STDEV, ns indicates a lack of significant difference between the CG and the DIO group at that timepoint as indicated by a 2-Way ANOVA followed by Tukey's multiple comparison test.
(TIF)

S2 Fig. Compensatory growth (CG) can be induced by changes in feeding conditions and densities as well as with changes in feeding conditions only. (A,B) Growth curves of fish kept at different feeding conditions and densities; (A) Body lengths of fish raised in low density (5 fish per tank) but different feeding regimes; fish raised with ad libidum feeding conditions which is able to induce diet-induced obesity (DIO) show significantly increased linear growth compared to fish raised under caloric restriction (CR); fish raised under caloric restriction before 1 month of age but with ad libitum conditions afterwards (CG) show briefly increased growth rates and compensate for differences in body length suggesting that these fish exhibit compensatory growth (CG); (B) Body lengths of fish raised with different densities while every tank received the same amount of food resulting in different feeding conditions; fish raised in low density (5 fish per tank) with ad libidum feeding conditions (DIO) show significantly increased linear growth compared to fish raised in high density (50 fish per tank) and therefore under caloric restriction (CR); fish raised in high density (50 fish/tank, caloric restriction) before 1 month of age but in low density (5 fish/tank, ad libitum conditions) afterwards (CG) show briefly increased growth rates suggesting that these fish exhibit CG; n = 10 for each condition;; error bars indicate STDEV, ns indicates a lack of significant difference between the CG and the DIO group at that timepoint as indicated by a 2-Way ANOVA followed by a multiple comparison test.
(TIF)

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6.2 Publication 2

“Leptin system loss of function in the absence of obesity in zebrafish”

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RESEARCH

Leptin system loss of function in the absence of obesity in zebrafish

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Abstract

The leptin system plays a crucial role in the regulation of appetite and energy homeostasis in vertebrates. While the phenotype of morbid obesity due to leptin (LEP) or leptin receptor (LEPR) loss of function is well established in mammals, evidence in fish is controversial, questioning the role of leptin as the vertebrate adipostat. Here we report on three (Lepr) loss of function (LOF) and one leptin loss of function alleles in zebrafish. In order to demonstrate that the Lepr LOF alleles cannot transduce a leptin signal, we measured *socs3a* transcription after i.p. leptin which is abolished by Lepr LOF. None of the Lepr/Lepa LOF alleles leads to obesity/a body growth phenotype. We explore possible reasons leading to the difference in published results and find that even slight changes in background genetics such as inbreeding siblings and cousins can lead to significant variance in growth.

Key Words
- leptin
- leptin receptor
- zebrafish

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Introduction

Research into the genetic basis of obesity was initiated when breeding experiments at Jackson labs gave birth to a pair of plump mice in the 1960s. Tracing the genes responsible for the aptly named *obese (ob/ob)* and *diabetes (db/db)* alleles led to the milestone discovery of the leptin system. The hormone leptin (*ob* gene) is released by adipose tissue in proportion to fat stores. Leptin signals the status of peripheral adipose stores to the brain, where it is received by the melanocortin system which expresses the leptin receptor (*db* gene, reviewed by Coleman 2010). Leptin deficiency in humans as well as mice leads to morbid obesity, which can be cured by leptin supplementation (Farooqi & O'Rahilly 2014). The genes involved in energy homeostasis are conserved across all vertebrates (Volkoff 2016) including leptin (Lep), the hormone receptor (Lepr), and the leptin pathway which involves Janus kinase 2 (Jak2), signal transducer and activator of transcription 3 (Stat3) and suppressor of cytokine signaling 3 (Socs3) (Liongue *et al.* 2016). While fish Lep and Lepr share minimal amino acid similarity with their vertebrate counterparts, gene synteny and predicted protein structure are well conserved (Denver *et al.* 2011, Prokop *et al.* 2012). Recently, Jak2 and Stat3 were shown to be phosphorylated by trout leptin in trout cell culture, providing evidence for a functional leptin pathway (Gong & Bjornsson 2014). However, evidence for conservation of leptin function at a physiological level has been mixed and it is unclear whether fish leptin signals the status of peripheral adipose stores in the same manner as in mammals (Londraville *et al.* 2014, Deck *et al.* 2017).

To better understand the role of leptin in teleost physiology, several groups have turned to knockout models which have targeted mutations in either *lep* or *lepr*.

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There are currently six studies investigating a global loss of function (LOF) in fish: Lepr in medaka (Chisada *et al.* 2014), Lepr in zebrafish (Michel *et al.* 2016, Fei *et al.* 2017, Ahi *et al.* 2019), Lepa in zebrafish (Michel *et al.* 2016, Audira *et al.* 2018), and Lepb in zebrafish (He *et al.* 2021). The reported results, however, are not consistent amongst studies: two alleles for Lepr LOF in zebrafish have no significant obesity phenotype (Michel *et al.* 2016, Ahi *et al.* 2019) while a third allele was found to display several characteristics of obesity (Fei *et al.* 2017). Loss of Lepa was found to have an obesity phenotype in zebrafish (Audira *et al.* 2018) and LOF of Lepb led to obesity at 1.5 years of age (He *et al.* 2021). The obesity phenotype in medaka was only seen at an early age, after which WT and LOF animals do not differ in weight or length (Chisada *et al.* 2014). It is worth noting at an equivalent developmental stage in mice, body weight doubles as a result of LEP or LEPR LOF and nearly all the additional weight is in adipose tissue (Ingalls *et al.* 1950, Hummel *et al.* 1966). Similarly, in children, leptin deficiency leads to morbid obesity early in childhood (Montague *et al.* 1997). While results differ among fish studies, the reported phenotype is comparatively mild.

We wondered whether there was a biological reason for the divergent results among the fish studies, as the differences were not only between species where life history could explain the divergence (medaka and zebrafish) but also among the different LOF alleles in zebrafish. We hypothesized that the *lepr*^{sa1508} mutation we studied could be a hypomorph. Importantly, none of the studies into fish leptin system dysfunction provide functional evidence indicating whether *lepr* or *lepa* gene function is ablated. Here, we show evidence that Lepr signaling in three different Lepr LOF alleles is ablated by measuring *socs3a* transcription after leptin injection, including the previously studied *lepr*^{sa1508} allele.

Another possible reason for the different results among the fish studies could be the experimental design. The studies which report an obesity phenotype raised WT or LOF fish from genotype incrosses separated from each other (Chisada *et al.* 2014, Fei *et al.* 2017, Audira *et al.* 2018). In comparison, the studies that found no growth difference raised sibling WT and LOF animals together in a tank (Michel *et al.* 2016, Ahi *et al.* 2019). There are two factors in this difference – genetic distance and behavior in the same tank. On the genetic level, fish from heterozygous incrossing are siblings, and the genetic background varies only very little. In contrast, keeping WT and LOF lines separated from each other increases the genetic distance and could segregate background mutations. This may not be a critical factor in a highly inbred species such as the laboratory mouse, but it could lead to significant differences in a highly outbred model such as the zebrafish (LaFave *et al.* 2014, Doran *et al.* 2016). On the behavioral level, raising WT and heterozygous fish together with homozygous LOF fish in the same tank could lead to food competition among genotypes, and homozygous Lepr LOF fish might not be able to develop an obese phenotype in this environment. Factors such as appetite, stress, and social dominance could be relevant. This is circumvented when genotypes are raised in separate tanks.

Here, we test body growth in three Lepr LOF lines and one Lepa LOF line. We test WT and LOF siblings raised together in tanks and cousin WT and LOF raised in separate tanks. A body growth phenotype indicative of obesity was not found in any of the Lepr LOF lines, nor in an independent Lepa LOF line when siblings were raised together in a tank. When cousins were raised separated from each other, significant growth differences between genotypes were observed, however, in some cases, the LOF animals were smaller and in others bigger. A consistent obesity phenotype was not found, providing independent evidence that Lepr or Lepa LOF do not lead to obesity in zebrafish.

Materials and methods

Zebrafish strains and maintenance

Animals were raised in an AquaSchwarz system on a 14 h light:10 h darkness cycle according to Brand *et al.* (2002). Groups of 30 larvae were raised and were fed with 100 mL paramecia as well as a pinch of red breeze food supplement (Preis Aquaristik) each day. After 14 days until the end of the experiment, fish were fed with 2 × 3 mL of 2-days old dense Artemia per day plus either a pinch of red breeze food supplement or a pinch of spirulina powder on alternating days. We reduced density to 10 fish per 2.5 L tank at 30 dpf.

Zebrafish strains used

The Lepr mutant strain *lepr*^{sa1508} was obtained from the Zebrafish International Resource Center (Oregon, USA). The other lines were established using CRISPR/Cas9 gene-editing according to Jao *et al.* (2013), with genotyping performed using a heteroduplex mobility assay according to Ota *et al.* (2013). Fish were outcrossed at least five generations before this study.

Specifically, the Lepr lines *lepr*^{vu624} and *lepr*^{vu625} were established using a gRNA against the target ATGATGAAGACAGACCTAGG in exon 3, from which

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two F1 lines were generated. Compared to GenBank BN000731.1 or RefSeq NM_001113376.1, the mutations are as follows: *lepr*^{vu624}: deletion of A34, insertion of GTC; *lepr*^{vu625}: deletion C29-G35, insertion of TGTCTTGATCATGCAGATGTCCTCTCTT. These mutations in exon 3 delete 1 bp and insert 3 bp in the *lepr*^{vu624} allele and delete 7 bp and insert 28 bp in the *lepr*^{vu625} allele resulting either in a frameshift and early truncation of the open reading frame or a disruption of the splice site depending on the predicted isoform analyzed. The Lepa mutant line *lepa*^{vu622} was established using a gRNA to the target AATCTCTGGATAATGTCCTGG as described (Michel *et al.* 2016) which compared to RefSeq NM_001128576 has a deletion of C184-A188.

For genotyping, a heteroduplex mobility assay was employed using the following primers: *lepr*^{vu624} and *lepr*^{vu625} 5'ACCCACACTAATGCGTCTCTG3' × 5'AAGCTTAAAGATCGGACCATTCCA3'; *lepa*^{vu622} 5'CAAAGACCATCATCGTCAGA3' × 5'ACCCAGAAG TGTGGATAGAT3'. For *lepr*^{sa1508}, we designed cleaved amplified polymorphic sequence primers according to Hodgens *et al.* (2017) and amplified with 5'ATGTGGAAGGATGTTCCAAATCCCAACAAGaG3' × 5'ACAGGGGGTAAAATGACTTCAGAAAGATAATA3'; the product WT allele is cleaved by SacI, the loss of function is not.

All procedures were approved by the national animal care committee (LANUV Nordrhein-Westfalen) and the University of Cologne and/or the University of Michigan Animal Care and Use Committee.

Body growth

All animals from one experimental cohort were assessed on the same day by an experimenter blinded to the genotype. At indicated times, fish were anesthetized with tricaine, blotted dry, photographed on millimeter paper, weighed, fin-clipped, and placed in a recovery tank. Standard length was measured according to Parichy *et al.* (2009) from pictures with Fiji (ImageJ). Fulton's condition factor (K) was calculated according to Stevenson & Woods (2006) and is a parameter analogous to BMI.

IP injection

Recombinant mouse leptin (SIGMA) was made up to a stock concentration of 1 mg/mL in DPBS (Gibco, Thermo Fisher) and injected into female zebrafish of 3–4 months of age at 2.5 mg/kg with a phenol red concentration of 1 µL/100 mg according to Kinkel *et al.* (2010) with the modification that we used a 10 µL Hamilton syringe with a 28 gauge needle.

RNA extraction and qPCR

Animals were sacrificed 50 min after injection and whole livers were isolated, immediately placed on dry ice, and stored at −80°C until RNA extraction was performed. Total RNA was extracted using Trizol (Invitrogen) and purified using a PureLink™ RNA mini kit (Thermo Fischer) following the manufacturer's protocols. Residual genomic DNA was removed using the PureLink DNase mixture included in the kit. Final RNA concentration was determined by optical density reading at 260 nm using NanoDrop 2000c and 1 µg of RNA with a 260/280 ratio > 1.8 was used for RT using a high-capacity cDNA kit (Applied Biosystems). Primers were designed using Primer-BLAST (NCBI) with similar melting temperatures. Additionally, we attempted to span an exon–exon junction or an intron where possible. The sequences are listed in Table 1. Oligonucleotide primers were ordered from IDT.

Q-RT-PCR was performed with Sybr Select Master Mix (Life Technologies, Thermo Fisher Scientific) on an ABI-Prism 7500 Fast Detect system under the following conditions: initial PCR activation 50°C for 20 s, 95°C for 2 min; PCR cycles, 95°C for 15 s denaturation, 60°C for 1 min annealing and 72°C for 30 s extension, repeated for 40 cycles. Each assay was performed in triplicate including a no-RT and no-template control. Relative expression levels were calculated following a modified ΔΔCt method to include primer efficiency (Pfaffl 2001) with *eef1a1l1* as

Table 1 Oligonucleotides used for qPCR experiments.

Gene name	Refseq No.	Primer sequence (5′–3′)	Amplicon length (bp)
eef1a1l1	NM_200009	Forward AACATGCTTGAGGCCAGTCC Reverse ACGGTTCCGATGCCTCCA	188
socs3a	NM_199950	Forward AAGCAGGGAAGACAAGAGCC Reverse AGAGCTGGTCAAAAGAGCCTAT	95
lepr	NM_001309403	Forward GGGGAACCGGCTCGATACAC Reverse GCTCTCCATGGTGTCAATCTGCC	210
lepa	NM_001128576	Forward GGAACACATTGACGGGCAAA Reverse ATGGGTTTGTCAGCGGGAAT	87

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the reference gene. Efficiencies and R^2 were assessed using a five-point cDNA serial dilution. All the primer pairs had PCR efficiencies between 96% and 102%, and the amplicon for each primer pair was sequenced. Data are presented as fold change relative to WT, calculated based on primer efficiency using a pooled sample of the relative dataset's cDNA, and then standardized to *eef1a1l1* expression. The reference gene has previously been shown to be a stable housekeeping gene (McCurley & Callard 2008) and was tested here to verify that it did not vary among genotypes.

µCT imaging and analysis

Zebrafish were anesthetized, fixed in 4% PFA, and imaged using a micro-CT (µCT) device (U-CT OI, MILabs B.V., Utrecht, the Netherlands). Zebrafish were placed on an acrylic glass plate and an ultra-focus scan over the whole body was performed in a full-rotation in step-and-shoot mode. One thousand four hundred and forty projections (1944 × 1536 pixels) were acquired per sub scan with an x-ray tube voltage of 60 kV, power 0.16 mA and exposure time of 75 ms and 4 averages. All CT images were reconstructed at an isotropic voxel size of 20 µm using a Feldkamp type algorithm (filtered back-projection). The fat-containing tissue regions, which appear hypo-intense in the µCT data, were segmented, and the volumetric fat percentage was computed as the ratio of (s.c. and visceral) fat volume to the entire body volume using Imalytics Preclinical (Gremse-IT GmbH, Aachen, Germany (Gremse *et al.* 2016).

Statistical analysis

Statistics were carried out in GraphPad Prism version 9.1.1 for Windows, GraphPad Software. Two-tailed, unpaired *t*-tests were carried out to compare LOF and WT data. One-way ANOVA was followed by Dunnett's multiple comparisons test. *P*-values less than 0.05 were considered significant. Violin plots were chosen to represent the data because in addition to the frequency distribution of the data, these also highlight the median and quartiles.

Results

All *lepr* alleles abolish Lepr signal transduction

To determine whether we had functional Lepr ablation, we tested whether the mRNA undergoes non-sense-mediated decay (NMD) in any of the Lepr LOF alleles. Interestingly, none of the alleles underwent NMD (Fig. 1). No significant difference was found in *lepr* mRNA in WT compared to LOF siblings in the $lepr^{sa1508}$ allele ($t(16) = 0.09094$, $P = 0.9287$, Fig. 1A), the $lepr^{vu624}$ allele ($t(16) = 0.6938$, $P = 0.4978$, Fig. 1B) or the $lepr^{vu625}$ allele ($t(16) = 0.03766$, $P = 0.9704$, Fig. 1C). Consequently, the *lepr* mRNA is stable in each of the Lepr LOF alleles compared to their respective WT siblings.

To determine whether leptin can induce *socs3a* expression in WT control animals, heterologous mouse leptin was injected intra-peritoneally into adult fish. We found that 2.5 mg/kg was the minimal effective dose to induce hepatic *socs3a* expression 50 min after injection compared to vehicle-injected animals (Fig. 2A). When leptin was injected into control and $lepr^{sa1508}$ LOF animals, we saw a significant increase in *socs3a* expression in WT but not LOF siblings (ANOVA $F(3,40) = 145.6$, $P < 0.0001$, Fig. 2B). The loss of leptin-induced *socs3a* expression replicated in the $lepr^{vu624}$ line (ANOVA $F(3,32) = 107.5$, $P < 0.0001$, Fig. 2C) as well as the $lepr^{vu625}$ line (ANOVA $F(3,32) = 8.644$, $P < 0.0002$, Fig. 2D). These data provide evidence that all three Lepr LOF alleles abolish leptin-induced *socs3a* transcription.

Growth parameters of $lepr^{vu624}$, $lepr^{vu625}$ and $lepa^{vu622}$ – evidence from a heterozygous incross

We tested whether the $lepr^{vu624}$ or $lepr^{vu625}$ lines develop an obesity phenotype. The study of Fei *et al.* suggests that there is a feeding-induced component of the obesity

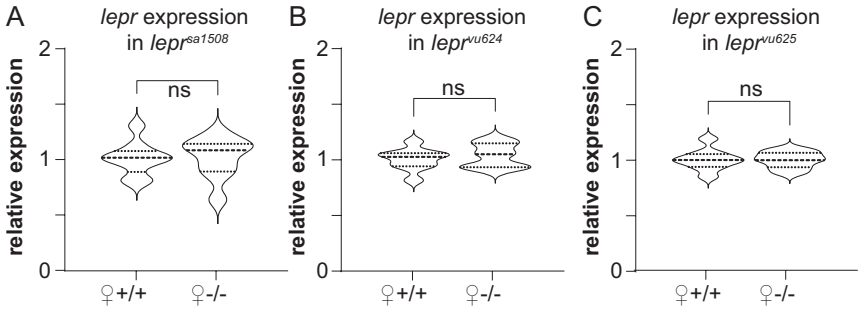

Figure 1
No evidence of *lepr* mRNA decay in any of the Lepr LOF alleles (A) the $lepr^{sa1508}$ allele; (B) the $lepr^{vu624}$ allele; (C) the $lepr^{vu625}$ allele. (ns, no significant difference; *t*-test). Expression data are shown as fold change relative to controls. Data represented as violin plots ($n = 9$ fish/group).

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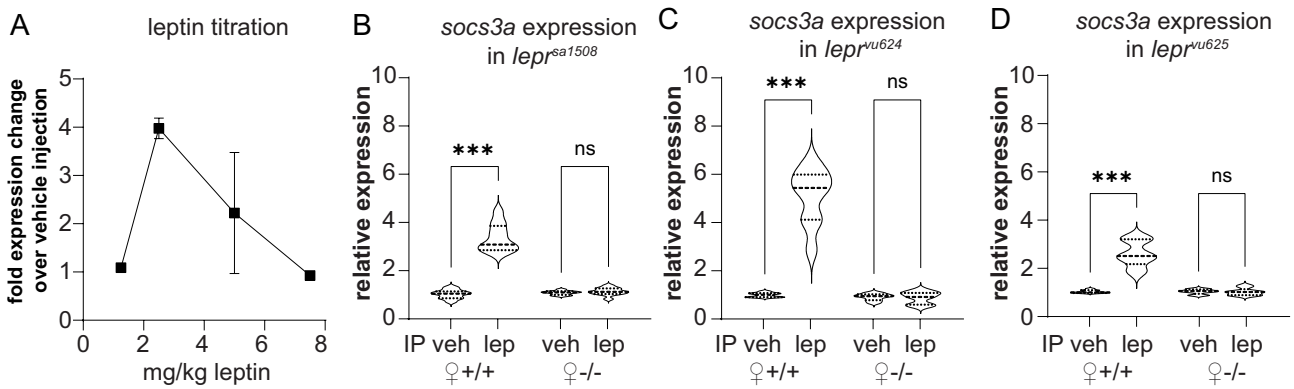

Figure 2
Intraperitoneal leptin injection can induce hepatic *socs3a* expression and Lepr LOF can block this effect. (A) titration of IP leptin on hepatic *socs3a* expression; (B) The effect of IP leptin in the *lepr*^{sa1508} allele; (C) the *lepr*^{vu624} allele and (D) the *lepr*^{vu625} allele. One-way ANOVA was followed by Dunnett's multiple comparisons test. Asterisks indicate significant effects against vehicle-injected control (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; ANOVA). Expression data are shown as fold change relative to controls. Data represented as violin plots ($n = 9$–11 fish/group).

phenotype (Fei *et al.* 2017), and therefore, animals raised at low density were fed excess food according to Leibold & Hammerschmidt (2015). Offspring of a heterozygous *lepr*$^{+/-}$ x *lepr*$^{+/-}$ cross were raised, and these experiments were performed in animals derived from the same cross to control for cryptic background alleles in the genome. The *lepr*^{sa1508} allele was previously tested under these conditions with no observed differences in weight (Michel *et al.* 2016).

At 3 months of age (89 dpf), animals of the *lepr*^{vu624} allele displayed no difference in standard length (SL) in either gender (Fig. 3A; SL female: $t(41) = 1.583$, $P = 0.1212$; male: $t(20) = 0.7716$, $P = 0.4494$). We scored the mass of the different genotypes and again saw no significant difference in either gender (Fig. 3B; weight, female: $t(41) = 1.574$, $P = 0.1233$; male: $t(20) = 1.337$, $P = 0.1963$). The condition factor of these fish was also calculated and again no significant difference was found between genotypes in either gender of fish (Fig. 3C; CF female: $t(41) = 0.1413$, $P = 0.8883$; male: $t(20) = 0.9979$, $P = 0.3303$).

We also tested growth in the *lepr*^{vu625} allele. Comparing to the other two alleles under these raising conditions, no obesity phenotype at 3 months (92 dpf) of age was noted in standard length (Fig. 3D; SL female: $t(47) = 1.464$, $P = 0.15$; male: $t(23) = 0.9099$, $P = 0.3723$), animal weight (Fig. 3E; weight, female: $t(47) = 0.1956$, $P = 0.8458$; male: $t(23) = 0.9468$, $P = 0.3536$) or in animal condition factor (Fig. 3F; CF female: $t(47) = 1.326$, $P = 0.1912$; male: $t(23) = 0.6883$, $P = 0.4981$).

In the mouse, mutations in the leptin receptor and its ligand, leptin, result in nearly identical phenotypes. We previously reported that a trans-heterozygote for *lepa*$^{vu621/vu622}$ alters β-cell number in a similar way to the *lepr*^{sa1508} allele in overfeeding conditions (Michel *et al.* 2016), but the effect of mutations in leptin on growth was not examined. *lepa*^{vu622} fish raised in the same conditions as described above did not show or size phenotype: fish from a heterozygous × heterozygous cross of the *lepa*^{vu622} allele were unaltered in standard length (Fig. 3G; SL, female $t(27) = 0.5626$, $P = 0.5784$; male: $t(22) = 1.657$, $P = 0.1116$), body weight (Fig. 3H; female: $t(27) = 0.2222$, $P = 0.8258$; male: $t(22) = 2.045$, $P = 0.0530$) and condition factor (Fig. 3I; female: $t(27) = 0.8852$, $P = 0.3839$; male: $t(22) = 0.3593$, $P = 0.7228$).

These results confirm that loss of leptin signaling function, either through loss of the receptor or the ligand, does not result in a change in mass, size, or condition factor in zebrafish.

Does competition among genotypes influence weight gain in LOF animals?

In zebrafish work, WT x WT (*lepr*$^{+/+}$ × *lepr*$^{+/+}$) and LOF × LOF (*lepr*$^{-/-}$ × *lepr*$^{-/-}$) crosses are sometimes used instead of het × het (*lepr*$^{+/-}$ × *lepr*$^{+/-}$) as this can simplify the experimental setup. Fish are subsequently raised in separate tanks. For body growth, that means that the genetic background is further removed than in siblings of a het × het crossing (i.e. cousins or further removed) and that genotypes do not compete in the same environment (tank) for resources. Importantly, the studies in zebrafish that do not report a growth phenotype raise animals as a mixed genotype (Michel *et al.* 2016, Ahi *et al.* 2019), while the studies that do report a growth phenotype do not (Fei *et al.* 2017, Audira *et al.* 2018). To investigate whether this methodological difference could underlie the different outcomes among the studies, we raised offspring of a

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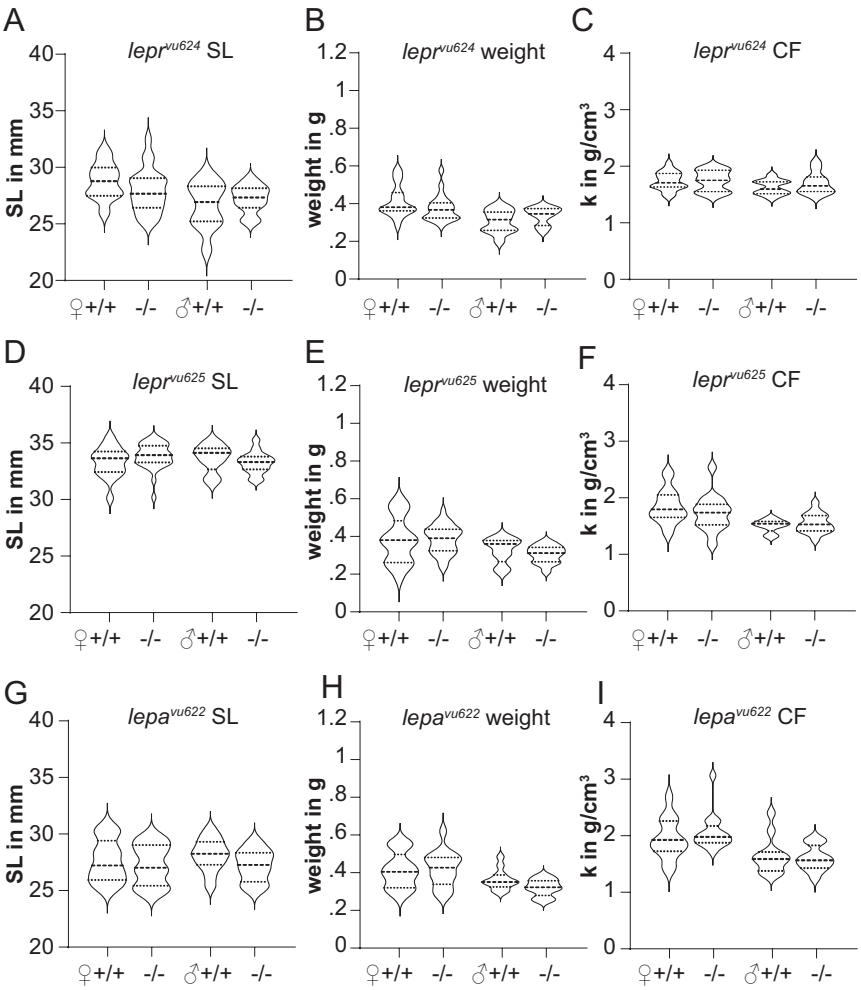

Figure 3
Offspring of a heterozygous × heterozygous cross raised in tanks of mixed genotypes do not show a growth phenotype. Animals of either line were raised in tanks of ten sibling animals under the same feeding conditions until (A, B and C) *lepr*^{vu624} – 89dpf; $n = 21/22/11/11$ (as seen on the graph female +/+ & –/–; male +/+ & –/–); (D, E and F) *lepr*^{vu625} – 92dpf; female $n = 17/32/9/16$; (G, H and I) *lepa*^{vu622} – 110dpf; female $n = 13/16/12/12$; Indicated is the standard length (A, D and G), weight (B, E and H) and condition factor (C, F and I). Unpaired *t*-tests were used to compare between genotypes. Asterisks indicate significant effects against the control (*$P < 0.05$; *t*-test). Data represented as violin plots (+/+ wildtype; –/– loss of function).

WT × WT cross and offspring of a Lepr LOF × Lepr LOF cross in separate tanks. Interestingly, significant growth differences were observed at sporadically 146 dpf.

In the *lepr*^{vu624} allele, no difference in standard length between genotypes was found (Fig. 4A; SL *lepr*^{vu624}; female: $t(40) = 1.877$, $P = 0.0678$; male: $t(24) = 0.3463$, $P = 0.7322$), however standard length in female animals trended toward shorter LOF animals. Moreover, a significant reduction in *lepr*^{vu624} weight was observed in female but not male animals, while there was a trend for reduced weight in male animals as well (Fig. 4B, weight female: $t(40) = 2.079$, $P = 0.0441$; male: $t(24) = 1.769$, $P = 0.0896$). In the conditioning factor, a trend for a reduced K in male animals was seen (Fig. 4C; female: $t(40) = 0.9529$, $P = 0.3464$; male: $t(24) = 2.055$, $P = 0.0509$).

In the *lepr*^{vu625} allele, we found that standard length was significantly higher in male but not female LOF animals (Fig. 4D; female: $t(30) = 0.5241$, $P = 0.6040$; male: $t(35) = 2.2$, $P = 0.0345$). No differences in animal weight were observed (Fig. 4E; female: $t(29) = 0.6418$, $P = 0.5261$; male: $t(36) = 1.086$, $P = 0.2846$), but there was a significant reduction in condition factor in male LOF animals (Fig. 4F; female: $t(29) = 0.04389$, $P = 0.9653$; male: $t(36) = 2.449$, $P = 0.0193$). In this case, the significant increase in length without an equal increase in weight resulted in a slightly reduced condition factor in Lepr LOF animals.

However, as these results are not completely congruent with the results from the other Lepr LOF allele (Fig. 4A, B and C), we also tested the previously published *lepr*^{sa1508} allele which has no altered growth phenotype in a heterozygous incross breeding setup (Michel *et al.* 2016). When raised in separate tanks as offspring of a WT × WT and LOF × LOF crossing scheme, no difference was found between *lepr*^{sa1508} LOF and WT animals in standard length (Fig. 4G; female: $t(26) = 0.5140$, $P = 0.6116$; male: $t(30) = 0.2472$, $P = 0.8064$) or weight (Fig. 4H; female: $t(26) = 0.2504$, $P = 0.8043$; male: $t(30) = 0.9736$, $P = 0.3381$). Surprisingly, the condition factor of *lepr*^{sa1508} LOF male fish but not female fish was significantly higher (Fig. 4I; female $t(26) = 0.1948$, $P = 0.8471$; male: $t(30) = 2.858$, $P = 0.0077$).

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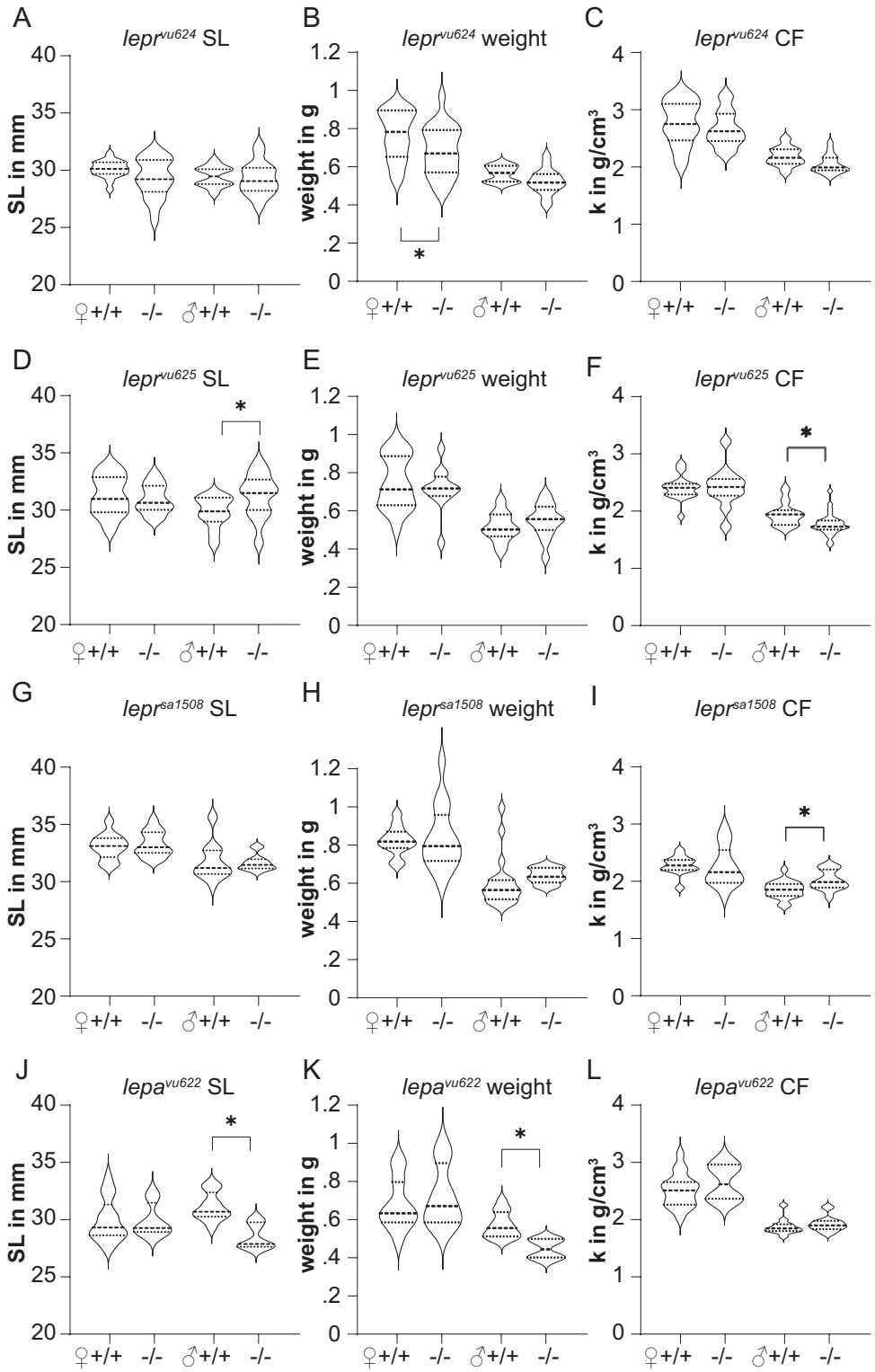

Figure 4

Offspring of a WT × WT and LOF × LOF crosses raised separately in tanks of ten animals with the same access to food until 146 dpf. (A, B and C) the *lepr*^{vu624} allele, $n = 18/24/10/16$ (as seen on the graph female +/+ & –/–; male +/+ & –/–); (D, E and F) the *lepr*^{vu625} allele, $n = 20/12/10/27$; (G, H and I) the *lepr*^{sa1508} allele $n = 12/16/19/13$ and (J, K and L) the *lepa*^{vu622} allele $n = 17/4/12/8$. Indicated are the standard length (A, D, G, and J), weight (B, E, H and K) and condition factor (C, F, I and L). Unpaired *t*-tests were used to compare between genotypes. Asterisks indicate significant effects against the control (*$P < 0.05$; *t*-test). Data represented as violin plots (+/+ wildtype; –/– loss of function).

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Table 2 Summary of growth experiments performed for the different alleles and crossing conditions.

	% Change LOF compared to WT					
	SL, female	SL, male	Weight, female	Weight, male	CF, female	CF, male
Het inx						
lepr^{vu624}	–3.1	+2.0	–9.8	+9.2	+0.4	+4.1
lepr^{vu625}	+3.8	–3.0	+1.5	–7.1	–7.1	+2.8
lepa^{vu622}	–1.4	–3.6	+1.9	–13.2	+5.5	–2.5
WT and LOF inx						
lepr^{sa1508}	+0.7	–0.4	+1.6	+5.8	–1.0	**+8.2**
lepr^{vu624}	–2.8	–0.6	**–12.4**	–8.2	–3.9	–6.6
lepr^{vu625}	–1.0	**+4.7**	–4.3	+6.2	+0.2	**–10.0**
lepa^{vu622}	–0.1	**–9.4**	+5.2	**–27.7**	+5.1	+2.4
Mouse		**Weight**				
ob/ob		**+210.3**	Male/female mixed			
db/db		**+58.8**	Male/female mixed			
Human						
ob/ob		**+100**	9-year-old boy			

Data are expressed as % increase or % decrease from control animals. Significant differences for increases and decreases are shown as bold. The mouse and human data are adapted from: *ob/ob* (Ingalls *et al.* 1950), *db/db* (Hummel *et al.* 1966) and a human patient from Montague *et al.* (1997).

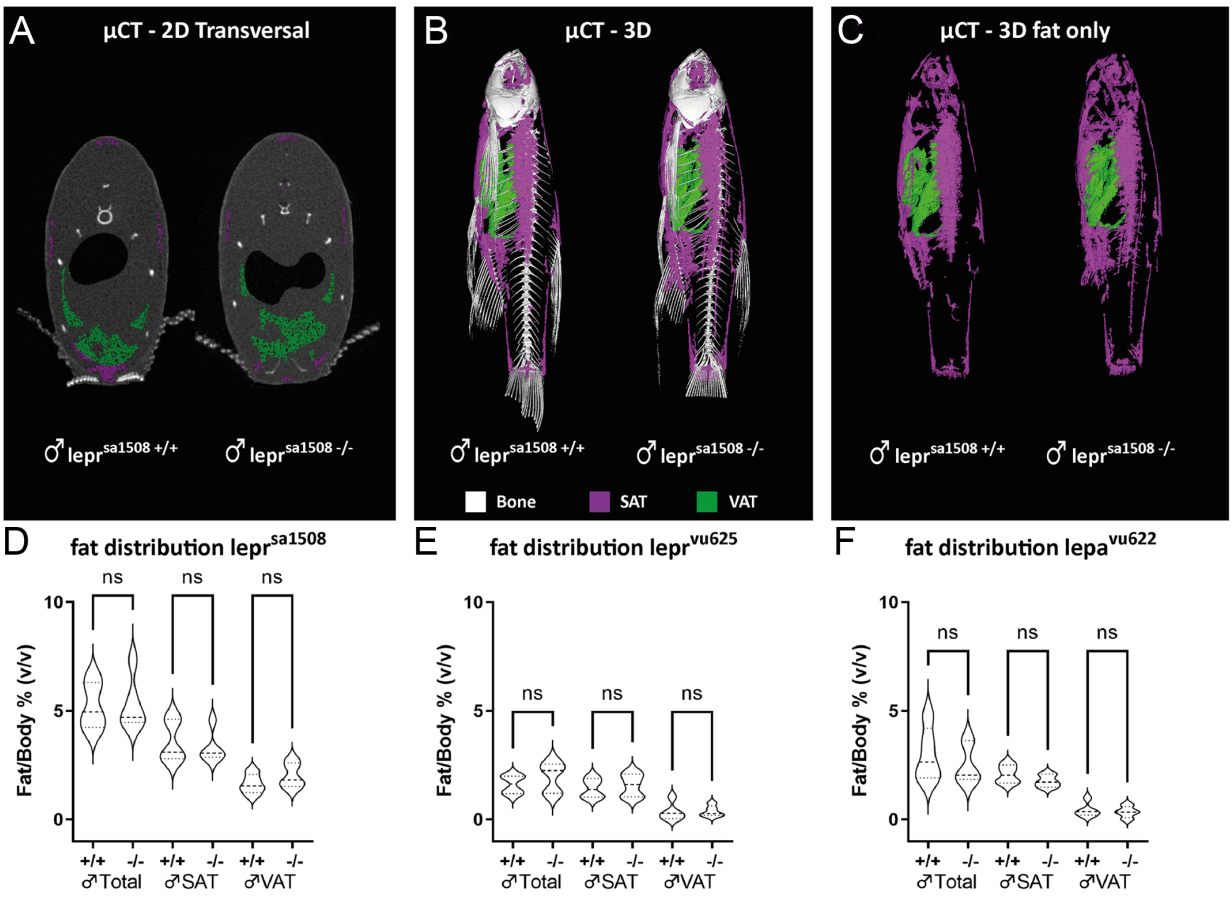

Figure 5
Size-matched adult fish were compared for whole-body adiposity using µCT imaging. (A) representative transverse section through *lepr*^{sa1508} WT and LOF zebrafish including the abdominal cavity. Fat-containing tissue regions appear hypo-intense and are colored. (B) Representative three-dimensional volume rendering of segmented bones and fat (left panel) as well as (C) fat alone (right panel). (D, E and F) quantification of fat subdivided into total body fat, s.c. adipose tissue (SAT) and visceral adipose tissue (VAT) for the (D) *lepr*^{sa1508} allele, the (E) *lepr*^{vu625} allele, and the (F) *lepa*^{vu622} allele. Unpaired *t*-tests were used to compare between genotypes. Asterisks indicate significant effects against the control (*$P < 0.05$; *t*-test). Data represented as violin plots (+/+ wildtype; $^{-}/^{-}$ loss of function).

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This result is in contrast to the overall trend seen with the other two alleles.

Lastly, we also tested the Lepa LOF allele *lepa*^{vu622}. Here, a significant reduction was seen in male standard length but not female standard length (Fig. 4J; female: $t(19) = 0.04163$, $P = 0.9672$; male: $t(18) = 4.908$, $P = 0.0001$), as well as in male but not female weight (Fig. 4K; female: $t(19) = 0.4742$, $P = 0.6408$; male: $t(18) = 4.349$, $P = 0.0004$). As the significant growth reduction is represented in both LOF length and weight, this did not reflect as a difference in condition factor in either male or female fish between the genotypes (Fig. 4L; female: $t(19) = 0.8227$, $P = 0.4209$; male: $t(18) = 0.7406$, $P = 0.4685$).

Since we saw differences in size that were inconsistent among alleles, we re-calculated the average change in LOF over WT animals and expressed the data in a table to gain a better overview (Table 2). For comparison, we included examples from published vertebrate studies.

Is there a lipodystrophy phenotype underlying the growth parameters?

One possibility is that LOF affects body fat accumulation and/or distribution, even without leading to a change in overall weight or length. Indeed, a recent paper reported that Lepb LOF zebrafish show an increased body fat percentage at 1.5 years of age (He *et al.* 2021). µCT analysis was carried out in 1-year-old *lepr*^{sa1508} WT and LOF fish of comparable weight and standard length, but no differences were found in total body fat (Fig. 5A, $t(8) = 0.1315$, $P = 0.8986$), s.c. adipose tissue (SAT, $t(8) = 0.5358$, $P = 0.6067$) or visceral adipose tissue (VAT, $t(8) = 1.179$, $P = 0.2722$). In order to ascertain that this is not a very late developing phenotype, we followed animals with the *lepr*^{vu625} and *lepa*^{vu622} LOF alleles to 1.5 years of age. Again, no differences were observed among genotypes for either *lepr*^{vu625} (Fig. 5B, total fat $t(7) = 0.8490$, $P = 0.4240$; SAT $t(7) = 0.4168$, $P = 0.6893$ or VAT $t(8) = 0.1573$, $P = 0.8789$) or *lepa*^{vu622} (Fig. 5C, total fat $t(6) = 0.5592$, $P = 0.5962$; SAT $t(5) = 1.055$, $P = 0.3396$ or VAT $t(7) = 0.4059$, $P = 0.6944$).

Discussion

Can the study of different alleles explain the difference in growth phenotypes?

Several groups working with engineered mutations in the zebrafish leptin signaling system have reported different phenotypes, either finding a role for leptin in body weight regulation (Chisada *et al.* 2014, Fei *et al.* 2017, Audira *et al.* 2018, He *et al.* 2021) or not (Michel *et al.* 2016, Ahi *et al.* 2019). Several scenarios could explain these divergent results. It could be that some alleles are hypomorphs and do not, in fact, ablate function. The *lepr*^{sa1508} mutation, for example, leads to a stop codon in the 17th exon. This truncation allele is predicted to abolish JAK/STAT signaling, however, the mutation is far downstream, raising the possibility that the intact N terminal domain may retain some cryptic signaling ability. Indeed, none of the above-published articles presents functional evidence regarding the ablation of leptin signaling. Therefore, we tested whether the canonical downstream Lepr pathway was intact in our alleles by measuring *socs3a* expression induced by leptin injection.

The canonical signaling pathway of the Lepr is through Jak/Stat activation (Gong & Bjornsson 2014, Peelman *et al.* 2014) and downstream *socs3a* transcription (Kwon *et al.* 2016). Recombinant mouse leptin was chosen because the predicted structure of leptin is well conserved between vertebrates (Denver *et al.* 2011, Prokop *et al.* 2012). Indeed, a previous study in tilapia showed comparable effects of recombinant mammalian leptin with recombinant tilapia leptin (Baltzegar *et al.* 2014). The major concern against the use of heterologous leptin is that the heterologous hormone will have a different dissociation constant and higher off-target activity than the species-specific source and will possibly activate a different receptor system altogether. Previous studies have reported different kinetics between species-specific and heterologous leptin in fish (Baltzegar *et al.* 2014, Shpilman *et al.* 2014). That is possible in this system as well; however, we picked a timepoint where *socs3a* is significantly elevated after heterologous leptin injection and a minimal effective dose to achieve this. Therefore, the induction of the canonical Lepr signaling pathway through *socs3a* expression is specifically blocked by the presented mutations in *lepr*. It remains possible that aspects of the Lepr signaling system which do not act through *socs3a* remain intact especially since the mRNA of *lepr* does not decay in any of these lines.

As sometimes an allele can have unintended effects such as being an unexpected hypomorph rather than the predicted amorph, we generated two new alleles of *lepr* using CRISPR-mediated mutagenesis. These two alleles were tested with an injection of recombinant mouse leptin, and again leptin-induced *socs3* expression was abolished in these Lepr LOF fish. The presented evidence suggests that a false-negative result is unlikely.

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Can the experimental setup explain the difference in growth phenotypes?

We noticed two key differences in the experimental setup among the published Lep/Lepr LOF studies: the breeding scheme and the way animals were raised. One set of studies reported crossing heterozygous parents and raising the offspring in tanks of mixed genotype and no growth phenotype (Michel *et al.* 2016, Ahi *et al.* 2019). The other reported breeding from a WT line and a LOF line and raising in separate tanks and found a phenotype (Chisada *et al.* 2014; X Wang, personal communication) or an unreported cross raised separately (Audira *et al.* 2018). As far as we can see, each study represents one cohort of animals.

The breeding scheme can have a large effect on body growth. We found significant growth differences in WT animals not only among individuals within the same clutch but also among clutches of different parental pairs (personal observation). This is likely due to the significant variation found in zebrafish laboratory stocks which are highly outbred (Parichy *et al.* 2009, LaFave *et al.* 2014). This stands in contrast to reasonably uniform growth among individuals in mouse research, where mouse lines have been bred to be free of significant variation (LaFave *et al.* 2014). Background variation would lead to differences in growth among clutches of WT and LOF fish that are not consistent among cohorts.

Raising genotypes separated from each other lead to a loss of competition between WT and LOF animals which may uncover a growth phenotype. One could hypothesize a different stress sensitivity between genotypes for example (Deck *et al.* 2017) or competition for a scarce resource such as food.

When we raised Lepa and Lepr LOF alleles as siblings (from a heterozygous incross) in mixed genotype tanks no difference in body growth among genotypes was observed. To test whether background genetics or competition could have affected the different results across labs we also raised WT and LOF crosses separately while very carefully ascertaining that density and access to food were the same across genotypes and tanks. To our surprise, we found significant growth differences between genotypes in this setup. However, there was no consistent obesity phenotype in LOF animals and instead, we found that in some cases, LOF animals showed decreased and in others increased growth (Table 2). This is particularly evident in male fish, where we expect weight fluctuations could be more evident as energy is primarily used for muscle or storage, while female fish carry up to 25% of their body weight in egg mass which undergoes constant flux (Leibold & Hammerschmidt 2015). This observation is consistent with the hypothesis that inbreeding of the WT and the LOF allele separately can exacerbate cryptic variation which in turn can lead to differences in growth, particularly in male fish.

These results stand in contrast to the role of leptin in mammalian biology, where leptin has a high penetrance in obesity. The original studies in mice report an increase of body adiposity from 9% body fat to up to 42.3% body fat in LEPR and LEP LOF mice on the background of a >40% weight increase without effect on length (Coleman 1978). The reported body growth phenotype of Lepr or Lep LOF in fish in contrast is relatively small (Chisada *et al.* 2014, Fei *et al.* 2017, Audira *et al.* 2018).

Toward the biological role of leptin in fish

If the reported body growth phenotype for Lepr/Lep LOF fish can be partially or fully attributed to experimental differences, what then is the biological role of leptin in fish, and does leptin still play a role in metabolic processes?

The observation that different Lepr or Lep LOF alleles do not lead to a reproducible obesity phenotype in fish argues against the role of leptin as the fish adipostat. Leptin is not even expressed in adipose tissue of fish (Londraville *et al.* 2014, Deck *et al.* 2017). In mammals, loss of leptin signaling leads to the inability of the brain to perceive peripheral fat stores resulting in severe hyperphagia and morbid obesity (Barsh & Schwartz 2002). This suggests that the role of leptin as the main adipostat in mammals could be a neofunctionalization of the leptin gene.

It is, however, unclear whether results can be generalized across fish or whether fish families have adapted differently due to the large diversity in the fish orders (Ronnestad *et al.* 2017). A recent study in trout adipocytes, for example, found that leptin can be secreted in cell culture (Salmeron *et al.* 2015). In mammalian LOF models, dysfunction can be seen in blood sugar control, reproduction, osteogenesis, thermogenesis, angiogenesis, immune function, hematopoiesis, and arterial pressure control, and it is not easy to tease specific and local effects apart from morbid obesity and lipotoxicity (Friedman 2014). Indeed, in LEPR LOF *db/db* mice, severe hyperglycemia precedes the obesity phenotype (Hummel *et al.* 1966). In fish, a role for leptin in glucose mobilization was identified in tilapia (Baltzegar *et al.* 2014, Mankiewicz *et al.* 2021) and zebrafish (Michel *et al.* 2016, Fei *et al.* 2017, Audira *et al.* 2018, He *et al.* 2021). Dysregulated glucose levels could in turn lead to a later

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adiposity phenotype and authors report an elevation of visceral fat in Lepr LOF medaka (Chisada *et al.* 2014) and a trend toward an increased whole-body triglyceride level in Lepr LOF animals at 1 year of age (Michel *et al.* 2016) as well as increased adiposity at an advanced age in Lepb LOF animals (He *et al.* 2021). We did not observe an increase in body adiposity in this study even at 1.5 years of age, but it would be interesting to elucidate the exact interplay between Lepa, Lepb and Lepr in the etiology of glucose dysregulation and possible late adiposity changes.

This study provides evidence that growth conditions need to be very carefully monitored throughout fish life in order to study metabolism and obesity, and that fish do not show signs of complete adipose dysregulation after the loss of leptin signaling that is comparable to the role of leptin in mice. The lack of a gross bodyweight phenotype up to at least a year of age is a strength of fish LOF models in the study of the biological role of leptin as the level of adiposity in mouse LOF models significantly confounds mammalian studies. It is, therefore, feasible in fish to study the role of leptin on single organs and paracrine/endocrine systems.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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6.3 Publication 3

“Leptin modulates oocyte maturation by a central and a direct pathway in zebrafish”

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Madhuri Puvvada- Investigation, Data curation and validation.

Matthias Hammerschmidt – Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Maximilian Michel - Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

RESEARCH

Leptin modulates oocyte maturation by a central and a direct pathway in zebrafish

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Abstract

Loss of LEPR function (LOF) in mammals leads to diverse phenotypes including morbid obesity and infertility while zebrafish show relatively minor phenotypes. This however allows the study of LEPR LOF in the absence of the detrimental effects of hyperglycemia or obesity. Here, we show evidence that leptin plays a role in the central as well as peripheral regulation of the hypothalamic–pituitary–gonadal (HPG) axis in zebrafish. Animals with a Lepr LOF show dysregulated pituitary HPG genes as well as evidence that oocytes mature slower and/or exhibit an increased rate of atresia. In culture, Lepr LOF attenuates the effect of 17α-20β-dihydroxy-4 pregnen-3-one in promoting germinal vesicle breakdown (GVBD) and increases the rate of GVBD as well as attenuates the rate of oocyte atresia.

Key Words

- LepR
- leptin
- oocyte
- GVBD
- zebrafish

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Introduction

Both low BMI, with low leptin levels, and high BMI, with high leptin levels, are associated with infertility (Chou & Mantzoros 2014). The underlying mechanisms are unclear. Leptin is a hormone signaling peripheral energy stores and mammals with leptin (LEP) or leptin receptor (LEPR) loss of function (LOF) are infertile due to leptin's permissive role in maintenance of the hypothalamic–pituitary–gonadal (HPG) axis upstream of melanocortin signaling. Briefly, leptin acts via melanocortin neurons on gonadotropin-releasing hormone (GNRH) neurons which cause luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release (Chou & Mantzoros 2014). Critically, infertility in LEPR LOF mice can be reversed centrally by an additional LOF in the agouti-related neuropeptide (AGRP) locus, while significant peripheral defects persist (Sheffer-Babila *et al.* 2013). Peripherally, significant expression of *LEPR* can be seen in the mammalian ovary, specifically the granulosa cells (Karlsson *et al.* 1997, Spicer & Francisco 1997, Ruiz-Cortes *et al.* 2003, Ryan *et al.* 2003). In the pre-ovulatory follicle, the granulosa cells surround the oocytes and provide important trophic support as well as produce critical steroids to guide the oocyte through its development and differentiation. *In vitro* studies of mammalian granulosa cells have shown that leptin modulates estradiol and progesterone production (Dupuis *et al.* 2014). Further, leptin was shown to be secreted into the follicular fluid by human chorionic gonadotrophin, providing evidence for a local, paracrine effect of leptin (Karlsson *et al.* 1997). Additionally, leptin also appears to affect follicular atresia. Atresia is a degenerative process that eliminates 99.9% of non-ovulating oocytes in the mammalian ovary through apoptosis, a phenomenon critically involving the granulosa cells. Interestingly, *db/db* LEPR LOF mice (Hamm *et al.* 2004) and *ob/ob* LEP LOF animals (Serke *et al.* 2012) show an increase in granulosa cell apoptosis while leptin injection *in vivo* in rats attenuates follicular atresia (Almog *et al.* 2001). Together, these data suggest that leptin plays a supportive role in oocyte maturation not only via a central but also a peripheral pathway and may additionally be involved in the modulation of follicular atresia.

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A significant confounding factor in mammalian leptin research is the morbid obesity of leptin LOF models. Excessively high lipid levels have negative consequences on organ function in and of themselves (Engin 2017). In contrast, the major body composition changes of LOF models seen in mammals (morbid obesity) are either mild or not present in normally fed fish (*lepr* in Medaka (Chisada *et al.* 2014), zebrafish (Michel *et al.* 2016, Fei *et al.* 2017, Ahi *et al.* 2019, Bagivalu Lakshminarasimha *et al.* 2021, Kamstra *et al.* 2022), *lepa* in zebrafish (Audira *et al.* 2018, Bagivalu Lakshminarasimha *et al.* 2021, Kamstra *et al.* 2022) and *lepb* in zebrafish (Kamstra *et al.* 2022)). Importantly, leptin and its receptor are well conserved between mammals and non-mammalian vertebrates on a structural but not amino acid level (Denver *et al.* 2011, Prokop *et al.* 2012). Evidence for a functional leptin pathway via Janus kinase 2 and signal transducer and activator of transcription 3 phosphorylation was shown in trout (Gong & Bjornsson 2014) and leptin-induced activation of suppressor of cytokine signaling 3 (*socs3*) expression was shown in zebrafish. We therefore investigated the reproductive function in Lepr LOF zebrafish in the *lepr*^{sa1508} line in which *socs3* signaling is impaired (Bagivalu Lakshminarasimha *et al.* 2021).

Lep as well as *lepr* expression is found in gonadal tissue in zebrafish (Liu *et al.* 2010, 2012), Jian Carp (Tang *et al.* 2013), yellow catfish (Gong *et al.* 2013), blunt snout bream (Zhao *et al.* 2015), Japanese pufferfish (Kurokawa & Murashita 2009) and chub mackerel (Ohga *et al.* 2015). Evidence is starting to be published on the role of peripheral leptin action in fish. Recent *in vitro* work in the yellow catfish showed that leptin can modulate β-oxidation in ovarian follicle cells (Song *et al.* 2018). A trout study using hepatic cells showed that leptin can block estrogen-induced vitellogenin release (Paolucci *et al.* 2020). Oocyte maturation in fish occurs via pituitary FSH acting on follicle cells to secrete estrogen which acts on the hepatic estrogen receptor to induce vitellogenin synthesis, the main egg yolk lipoprotein (Levavi-Sivan *et al.* 2010). It is unclear at this stage whether these effects exist *in vivo* and how they integrate. Centrally, leptin was shown to have a positive effect on Lh production in sea bass pituitary cell culture (Peyon *et al.* 2001) and on Lh as well as Fsh production in rainbow trout (Weil *et al.* 2003). However, the knockout of several genes critical for the central regulation of reproduction results in relatively subtle phenotypes in fish, compared to those observed in mammals (Trudeau 2018). Particularly baffling was the result that neither Gnrh3 LOF (Spicer *et al.* 2016) nor Gnrh2 LOF (Marvel *et al.* 2019) or the double LOF (Marvel *et al.* 2018) showed a reproductive phenotype, and the Zohar group recently showed that the Gnrh LOF is compensated for by a central mechanism (Marvel *et al.* 2021). Our previous results suggest that the *lepr*^{1508} is dispensable for reproductive function in the nutrient-rich laboratory environment (Michel *et al.* 2016). Since centrally, leptin acts upstream of Gnrh, we wondered whether similar to the Gnrh LOF zebrafish we would see central compensation that results in the observed lack of a reproductive phenotype.

Here, we show centrally that *lepr*^{sa1508} LOF leads to reduced pituitary *fshb* and *lhb* expression. Since we saw no overt subfertility, we investigated Lepr LOF oocytes and found a delay in maturation and/or an increase in oocyte degradation. Peripherally, we show that cultured oocytes respond to 17α-20β-dihydroxy-4 pregnen-3-one (DHP) as well as leptin and this depends on Lepr function in the cultured oocytes. This data provide evidence for a central as well as the peripheral role of leptin in oocyte modulation.

Materials and methods

Zebrafish maintenance

The zebrafish used in the study were raised and maintained at 28°C in an Aqua Schwarz system (AQUA SCHWARZ GmbH, Göttingen, Germany) on a 14 h light:10 h darkness cycle (Brand *et al.* 2002). The embryos were obtained by natural breeding. Groups of 30 larvae were each raised in embryo medium and were fed 3 × 100 mL paramecia from 5 to 13 days post fertilization (dpf) each day. From 14 to 60 dpf, fish were fed twice daily with 3 mL artemia and on alternating days a pinch of dry red breeze food supplement or a pinch of spirulina powder. From 60 dpf, they were fed with artemia, fish flakes (Tetra GmbH, Melle, Germany) and frozen food (Fimö Aquaristik, Bünde, Germany). From 3 months of age, fish were sorted for gender and maintained in 12 L tanks with appropriate female: male ratios until the time of the experiment.

Zebrafish strains

The Lepr mutant strain *lepr*^{sa1508} was obtained from the Zebrafish International Resource Centre (Eugene, Oregon, USA). The fish were outcrossed at least five generations before this study. For comparative analyses, the siblings derived from the mating of a single heterozygous *lepr*$^{sa1508+/-}$ pair were used. Offspring was genotyped as described before (Bagivalu Lakshminarasimha *et al.* 2021). All experimental procedures were approved by the national

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animal care committee (LANUV Nordrhein-Westfalen) and the University of Cologne.

Zebrafish immunohistochemistry

For Lepr immunohistochemistry, ovaries were dissected from *lepr:lepr–mCherry* BAC reporter adult females (Kang *et al.* 2016) or brains from *pomca:eGFPras;lepr:mcherry*, (SL 2.5 cm) (Kang *et al.* 2016, Löhr *et al.* 2018) and fixed in 4% PFA (Sigma) overnight at 4°C, cryoprotected with 3% agarose in 30% sucrose, embedded in O.C.T cryomedium (Leica Biosystems) and frozen at −30°C. Coronal sections of 10 µm (ovaries) or 30 µm (brain) thickness were cut with a Leica cryostat. Sections were blocked in 10% fetal calf serum in PBST (0.3% Tween-20) for 2–3 hours at room temperature, incubated with primary chicken anti-GFP (ThermoFisher Scientific) (cat no. A10262) and rabbit anti-RFP antibody (MBL) (cat no. PM005) overnight at 4°C. The next day, the samples were visualized with species-specific secondary antibodies coupled to Alexa Fluor 488 or 555 (Thermo Fischer Scientific) after incubation for 2 h at room temperature. Chicken anti-GFP antibody labels the *Pomc* neurons and rabbit anti-RFP antibody labels the *Lepr* neurons. Nuclear counterstaining was performed with DAPI.

RNA extraction and quantitative real-time PCR

Animals were euthanized, whole ovaries were isolated and stored at −80°C until RNA extraction. Total RNA was extracted using Trizol (Invitrogen) and purified using a PureLink™ RNA mini kit (Thermo Fischer) according to the manufacturer's protocols. Residual genomic DNA was removed using the PureLink DNase mixture provided in the kit. cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Primers were designed using Primer-BLAST (NCBI) as described (Bagivalu Lakshminarasimha *et al.* 2021). Sequences are listed in Table 1. Oligonucleotide primers were ordered from IDT.

Quantitative real-time PCR (qRT-PCR) was performed according to (Bagivalu Lakshminarasimha *et al.* 2021). The reference gene *eef1a1l1* was chosen as the internal standard as it has previously been shown to be a stable housekeeping gene (McCurley & Callard 2008) and was tested here to verify that it did not vary among control conditions.

Purging of oocytes

To stage the oocytes in a synchronous way, the existing oocytes were purged from adult females (9 months old) of both $lepr^{sa1508+/+}$ as well as $lepr^{sa1508-/-}$ animals by natural

Table 1 Oligos used for q-PCR experiments.

Gene name	Refseq no.	Primer sequence (5′–3′)	Amplicon length (bp)
eef1a1	NM_200009	Forward AACATGCTTGAGGCCAGTCC Reverse ACGGTTCCGATGCCTCCA	188
hsd3b1	NM_001386297	Forward TGGAGTTAATGTCAAAGCAAC Reverse GTTTTCTTGGATGCATGTCT	55
star	NM_131663	Forward TGTCAAGCAAGTCAAGATTC Reverse TTACAAATCCCTTCTGCTCA	200
pgrmc1	NM_001007392	Forward ACACTCATGATGATCTCTCC Reverse AATCGTACTTCTGTGTGAAC	87
pgrmc2	NM_213104	Forward GATGCAGTTCATGGAGAAAT Reverse AGATTCTGAGGGGTTTTCTT	203
lhb	NM_205622	Forward AACGCCTGTCAAGATGTTAT Reverse AGAAGAGAAAGAAGACACCA	50
fshb	NM_205624	Forward TAATGAGCGCAGAATCAGAA Reverse GGTAAACTCGATCCATTGTC	144
ctsba	NM_213336	Forward GACTGGGGTGATAATGGATA Reverse TCAGGGGCTTCTTTTAGATT	122
casp3a	NM_131877	Forward TGTGGATACAACAGATGCTA Reverse GTTGTTGATGATGATGCAGT	136
bcl2a	NM_001030253	Forward GAAAATGGAGGTTGGGATG Reverse CTGTCACTTCTGAGCAAAAA	153
baxa	NM_131562	Forward CCTTGTCATCAAGGCTATTT Reverse TGTTCCCTGATCCAGTTAAT	108
becn1	NM_200872	Forward TTGCCATTGTATTGTTCAGG Reverse TTGTCCACATCCATTCTGTA	155
fsta	NM_131037	Forward CAAAAAGTGCAAGCTGGTAA Reverse TTGAAGATCATCCACCTGAA	173

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mating. The couples were placed in mating cages separated by a baffle. Couples were allowed to breed from 8:00 h to 16:00 h (8 hours). Females that laid a good and comparable clutch of eggs were sorted by eye, separated from males in a 12 L tank and fed.

Histological analysis

Six adult WT and LOF females were sampled at each time point of 0-, 2-, 4-, 8-, 12- and 16-days post purging (DPP). They were anesthetized in 0.13% tricaine (w/v) and euthanized in an ice bath. The standard length (SL in cm) of each fish was measured from a photo using Fiji (ImageJ) and the body weight (W in g) of each fish was recorded. Fish were decapitated after being euthanized, fixed in Bouin's solution at room temperature for 48 h, decalcified in 0.5 M EDTA for 7 days, dehydrated in a graded series of increasing ethanol concentrations, cleared in Roti-Histol (Carl Roth, Karlsruhe, Germany) and embedded in paraffin (Carl Roth). Coronal sections of 8–10 µm thickness were cut from the ventral side and stained with hematoxylin and eosin (H&E), using standard protocols. The slides were mounted and imaged using a Zeiss Axiovision 4.8 light microscope. The different stages of oocytes were identified and staged as described before (Selman *et al.* 1993). In addition to the oocyte stages, atresia and post-ovulatory complexes were examined.

In vitro GVBD assay

Adult zebrafish ovarian follicles were isolated and incubated 8 DPP according to (Seki *et al.* 2008, Pang & Thomas 2009, Welch *et al.* 2017). Briefly, gravid females from WT and LOF animals were anesthetized in 0.13% tricaine (w/v) (Sigma) for 2 min and euthanized in an ice bath 3 h prior to the end of the daylight period. Ovaries were dissected and transferred to a 35 × 10 mm culture dish containing 4 mL Leibovitz's L-15 medium. Immature (stages I and II) and translucent mature (stage V) oocytes were separated with fine forceps and discarded. Oocytes characterized by dark, opaque cytoplasm and readily apparent germinal vesicle were sorted and pooled, randomly distributed in culture dishes containing; 60% Leibovitz's L-15 medium (vehicle), 60% Leibovitz's L-15 medium + DHP or 60% Leibovitz's L-15 medium + 10 nM of recombinant mouse leptin (Sigma). Follicles were incubated for 4–5 h and % germinal vesicle breakdown (GVBD) was scored. The viability of the oocytes was tested based on trypan blue staining. Viable oocytes which did not undergo GVBD were counted as immature and non-viable oocytes were counted as dead at the end of 5 h.

Quantification and statistical analysis

For the quantification of total ovary area, total numbers and area occupied by different stages or atretic oocytes (mm^2) in WT and LOF females, three consecutive sections from at least three individual fish (for histology) were analyzed from two independent experiments using Fiji (ImageJ) software. Groups of 3 × 30 oocytes were analyzed for each GVBD assay with at least six independent experiments and GVBD was scored under a Zeiss Axiovision 4.8 light microscope. Data collection and analysis were performed using Microsoft Excel and GraphPad Prism version 9.1.1 for Windows, GraphPad software. Two-tailed, unpaired *t*-tests were carried out to compare LOF and WT data. One-way ANOVA or two-way ANOVA was followed by Dunnett's multiple comparisons test (MCT) or Šídák's MCT as indicated. *P*-values less than 0.05 were considered significant.

Results

lepr is centrally expressed and gonadotrope expression is reduced in Lepr LOF fish

We tested whether the *lepr* is centrally expressed in zebrafish using a *lepr:lepr–mCherry* BAC reporter line from the Kang and Poss labs (Kang *et al.* 2016) and found a *lepr* signal throughout the brain. Since in mammals, *LEPR* is expressed on proopiomelanocortin (*POMC*) and on *AgRP* neurons (Cone 2006), we tested for *lepr* expression in a pomca:EGFPras line (Löhr *et al.* 2018) and found neurons co-expressing *lepr* and *pomc* in the hypothalamus (Fig. 1A). We also tested whether the gonadotropins lh (*lhb*) and fsh (*fshb*) were dysregulated in the pituitary and found that both genes showed significantly reduced expression in the $lepr^{sa1508}$ line in a mixed sample of male and female pituitaries (*fshb* Fig. 1B, $t(16)=6.232$, $P < 0.0001$ and *lhb* Fig. 1C, $t(15)=7.947$, $P < 0.0001$). We replicated this result in a separate cohort of female fish pituitaries only and similarly found reduced expression of both genes (*fshb* $t(6)=7.450$, $P=0.0003$ and *lhb* $t(6)=8.013$, $P=0.0002$).

The $lepr^{sa1508}$ line shows a delay in oocyte maturation only after synchronization of oocytes

This result was surprising since we previously did not observe subfertility in these animals (Michel *et al.* 2016) which we would expect downstream of reduced *lhb* and *fshb* expression. We first took animals from our holding tanks (of mixed gender) and looked at ovarian histology

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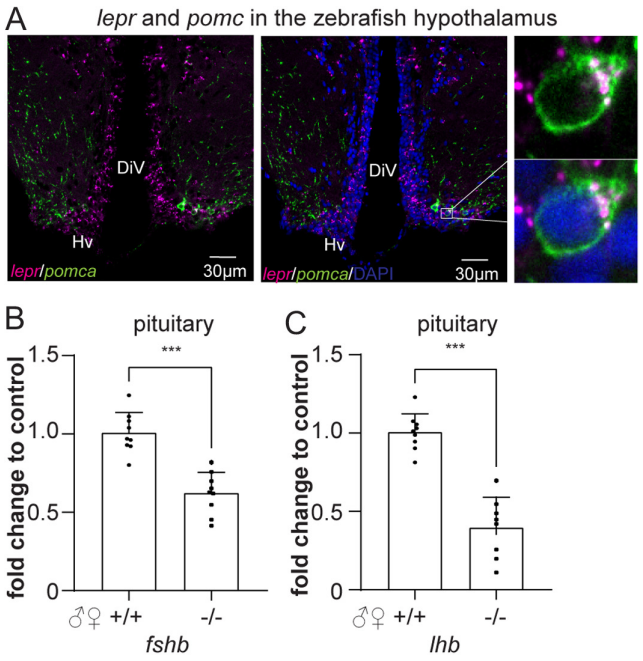

Figure 1
Evidence for Lepr in the central regulation of gonadotropins. (A) Confocal images showing Pomca cells expressing Lepr in the hypothalamus of the adult zebrafish brain. *pomca:eGFPras;lepr:mcherry* zebrafish were labeled with chicken anti-GFP to label Pomc neurons and rabbit anti-RFP to label Lepr neurons. Scale bar 30µm; (B) pituitary expression of *fshb* and (C) *lhb* in from lepr^{sa1508} WT and LOF animals, each datapoint represents a pool of two male (♂) and/or female (♀) adult fish pituitaries. Expression data are shown as fold change relative to controls. Asterisks indicate significant effects against WT (***$P < 0.001$) on a two-tailed, unpaired *t*-test. DiV, diencephalic ventricle; Hv, ventral zone of periventricular hypothalamus. A full color version of this figure is available at https://doi.org/10.1530/JOE-22-0026.

with H&E staining at comparable planes between individuals and genotypes. We counted oocytes based on stage and found that there was a borderline significant difference between genotypes (Fig. 2A, two-way ANOVA effect of oocyte stage $F(4,40)=201.7$, $P < 0.0001$ and effect of genotype $F(1,40)=4.781$, $p=0.0347$, however no effect of the Šídák MCT). However, when we looked at the source of the difference with an MCT *post hoc* test, we found that staged oocytes did not significantly differ between genotypes, suggesting that there was no difference between genotypes based on the oocyte stage, from immature stage 1 until mature stage 5 oocytes. We therefore purged existing oocytes from female fish in order to synchronize oocyte development and allowed them to rest for a defined number of days separated from males (Fig. 2B for a representative example). We found that there was no difference in mature (stages 4 and 5) oocytes between genotypes on the day of purging, however oocytes matured at a significantly more rapid rate in WT compared to *lepr*^{sa1508} LOF animals (Fig. 2C, two-way ANOVA, effect of DPP $F(5,24)=95.70$, $P < 0.0001$ and effect of genotype $F(1,24)=166.4$, $P < 0.0001$). Interestingly, the difference is strongest at 8 DPP, after which the number of mature oocytes reduces in WT animals to reach an equilibrium with *lepr*^{sa1508} LOF animals by 16 DPP (Fig. 2C). The reduction in the number of mature oocytes in the LOF animals could be driven by delayed maturation or by atresia. Consequently, we also scored atresia between genotypes. Atretic oocytes are difficult to count as only the initial stages of atresia lead to discernible oocytes but during later stages of atresia, there are areas that carry hallmarks of atresia but are not countable as distinct atretic oocytes. Therefore, we scored

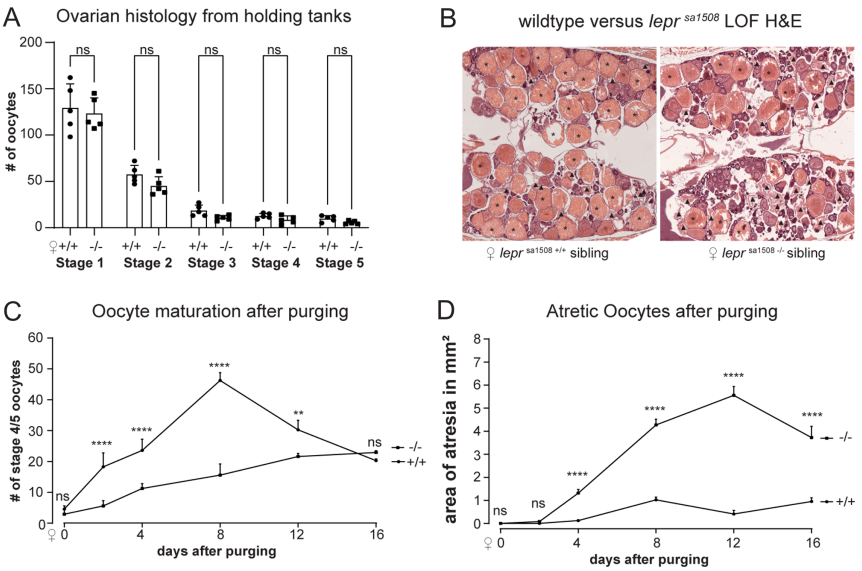

Figure 2
Ovarian histology from lepr^{sa1508} WT and LOF animals (A) staged oocytes of ovaries dissected from zebrafish taken directly out of mixed gender holding tanks ($n = 5$ each); (B) a representative example of H&E histology at 8 days post purging for WT and Lepr LOF, asterisks (*) label mature oocytes and triagles (▲) areas with atretic oocytes. Scale bar 1000µm; (C) Development of mature oocytes (stages 4 and 5) post purging with females kept in isolation from males after breeding ($n = 6$ each) and (D) time-course of atretic area as a function of days post purging between genotypes ($n = 6$ each); Two-way ANOVA was followed by Šídák's multiple comparisons test. Asterisks indicate significant effects against vehicle-injected control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). ns, no significant difference. A full color version of this figure is available at https://doi.org/10.1530/JOE-22-0026.

atresia as the area of atretic oocytes between genotypes. We found in WT siblings that oocytes start to degrade at 8 DPP which increases toward 16 days. In contrast, the *lepr*^{sa1508} LOF animals show a significant increase in atresia from 4 DPP onwards (Fig. 2D, two-way ANOVA effect of time $F(5,24)=247.0$, $P < 0.0001$ and effect of genotype $F(1,24)=866.6$, $P < 0.0001$).

Oocyte gene expression is altered in *lepr*^{sa1508} LOF animals after purging

In order to validate the histological results, we tested oocytes for a set of genes involved with maturation and atresia respectively. For maturation, we tested the following genes according to (Levavi-Sivan *et al.* 2010, Shang *et al.* 2019, Li & Ge 2020): 3β-Hydroxysteroid dehydrogenase (*hsd3b*); StAR protein (*star*), progesterone receptor membrane component 1 (*pgrmc1*); progesterone receptor membrane component 2 (*pgrmc2*), *lhb* and *fshb*. For atresia, we tested the following genes according to Gonzalez-Kother *et al.* (2020): cathepsin Ba (*ctsba*), caspase 3, apoptosis-related cysteine peptidase a (*casp3*), BCL2 apoptosis regulator (*bcl2a*), BCL2 associated X, apoptosis regulator a (*baxa*), beclin 1, autophagy related (*becn*) and follistatin (*fsta*).

Overall, we see an upregulation of maturation genes in WT animals at 8 DPP compared to 4 DPP which goes down again by 16 DPP (Fig. 3A, two-way ANOVA effect of day post purging $F(7,48)=32.96$, $P < 0.0001$, Dunnett's MCT see Table 2). In the *lepr*^{sa1508} LOF ovaries, we see an overall reduction in maturation-induced genes (Fig. 3A). In contrast, atresia involved genes rise in expression at 12- and 16-DPP in WT animals while they are significantly upregulated at 4, 8 and 12 days in *lepr*^{sa1508} LOF ovaries (Fig. 3B, two-way ANOVA effect of day post purging $F(7,48)=848.7$, $P < 0.0001$, Dunnett's MCT see Table 2).

Lepr is present on follicle cells of the ovary

Having seen an effect of the *lepr*^{sa1508} LOF in oocyte maturation and follicular atresia, we looked at oocytes histologically in the *lepr–mCherry* line in order to establish whether the Lepr is present on follicle cells of the ovary. In the *lepr–mCherry* line, we found that indeed the Lepr is present on follicle cells in the zebrafish ovary (Fig. 4).

Leptin promotes Lepr-dependent oocyte maturation and survival in an *in vitro* GVBD assay

The maturation and atresia phenotype could be centrally regulated via the HPG axis. Alternatively, or additionally,

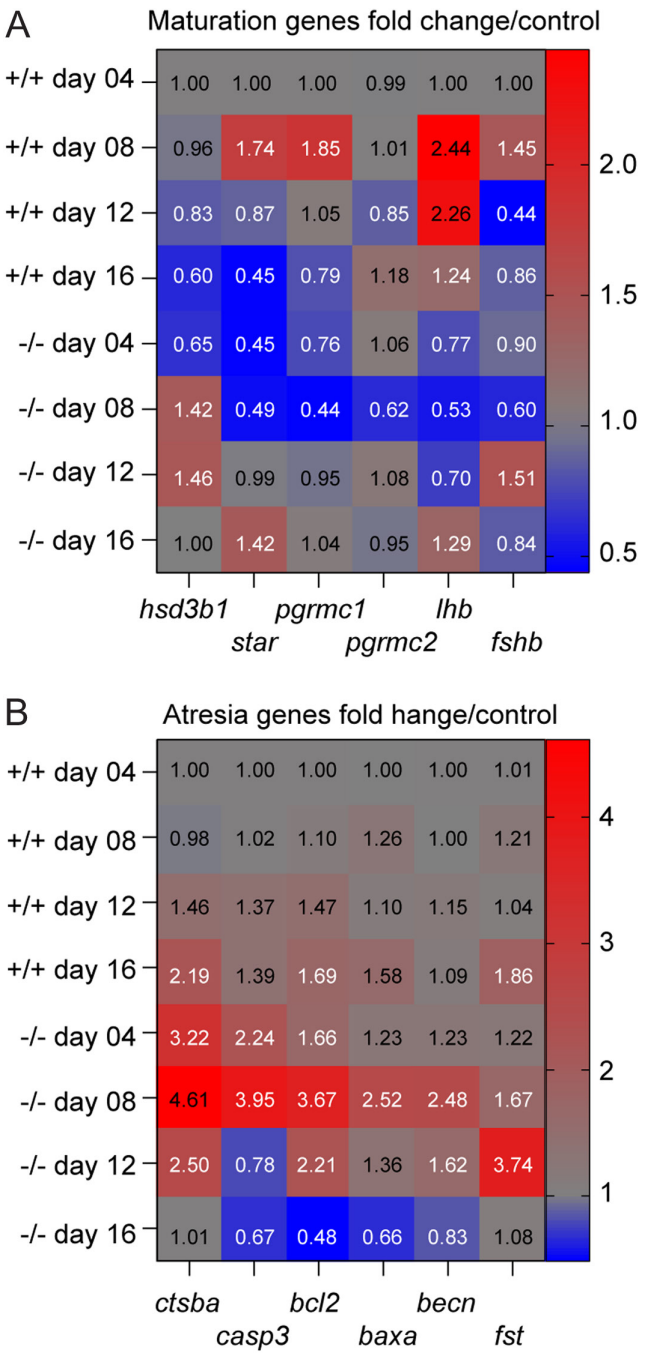

Figure 3
Gene Expression changes in Lepr LOF ovaries compared to WT ovaries from 4 to 16 days post purging;, data expressed as a heatmap with the color coding scale indicated on the right and the fold difference to the respective control indicated in each field; (A) genes involved in maturation and (B) genes involved in atresia, $n = 3$ each timepoint; Two-way ANOVA was followed by Šídák's multiple comparisons test; for results of the MCT as well as significant changes please see Table 2. Expression data are shown as fold change relative to controls. A full colour color version of this figure is available at https://doi.org/10.1530/JOE-22-0026.

Table 2 Statistical significance on a two-way ANOVA for the heat maps in Fig. 3A and B using Dunnett's MCT, comparing each cell mean with the control cell mean of the relative gene expression for WT day 4. Represented is the adjusted *P* value for each test.

Maturation	***hsd3b1***	***star***	***pgrmc1***	***pgrmc2***	***lhb***	***fshb***
wt_d4 vs wt_d8	0.9996	**0.0003**	**<0.0001**	0.9999	**<0.0001**	0.0537
wt_d4 vs wt_d12	0.8438	0.9503	0.9995	0.9335	**<0.0001**	**0.0077**
wt_d4 vs wt_d16	0.1047	**0.0098**	0.6853	0.7619	0.5510	0.9092
wt_d4 vs ko_d4	0.1735	**0.0093**	0.5347	0.9994	0.6146	0.9880
wt_d4 vs ko_d8	0.0716	**0.0191**	**0.0082**	0.1370	**0.0372**	0.0924
wt_d4 vs ko_d12	**0.0423**	0.9999	0.9995	0.9940	0.3304	**0.0205**
wt_d4 vs ko_d16	>0.9999	0.0791	0.9997	0.9997	0.3418	0.8460
Atresia	***ctsba***	***casp3***	***bcl2***	***baxa***	***becn***	***fst***
wt_d4 vs wt_d8	0.9996	0.9997	0.7939	**0.0298**	>0.9999	0.1300
wt_d4 vs wt_d12	**<0.0001**	**0.001**	**<0.0001**	0.7917	0.4162	0.9977
wt_d4 vs wt_d16	**<0.0001**	**0.0006**	**<0.0001**	**<0.0001**	0.8555	**<0.0001**
wt_d4 vs ko_d4	**<0.0001**	**<0.0001**	**<0.0001**	0.0712	0.0737	0.0979
wt_d4 vs ko_d8	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
wt_d4 vs ko_d12	**<0.0001**	0.0897	**<0.0001**	**0.0015**	**<0.0001**	**<0.0001**
wt_d4 vs ko_d16	>0.9999	**0.0037**	**<0.0001**	**0.0025**	0.2498	0.9407

Bold signifies that the *P* value is below the significance threshold of 0.05.

there could be a peripherally regulated component via direct signaling to the ovaries, consistent with the aforementioned presence of Lepr on follicle cells. To test the latter, we performed a GVBD assay *in vitro* using primary cultures of stage 4 oocytes from WT and *lepr*^{sa1508} LOF animals treated with mammalian leptin and/or DHP, an ovarian hormone which is secreted when Lh acts on ovaries to promote follicular maturation (Levavi-Sivan *et al.* 2010). We first showed that mammalian leptin induced zebrafish oocyte maturation compared to untreated control conditions (Fig. 5A, two-way ANOVA shows an effect of leptin exposure F(1,12)=79.35, $P < 0.0001$) similar to the recently reported effect of carp leptin in a zebrafish GVBD assay (Chen *et al.* 2018). In contrast to Chen *et al.* (2018), we did not observe an increase of the GVBD rate as a factor of the incubation time (Fig. 5A, two-way ANOVA shows no effect of time of exposure F(2,12)=1.388, $P=0.2870$). We next tested whether the effect of leptin is abolished in *lepr*^{sa1508} LOF animals. Of note, GVBD rates of WT ovaries could be increased by 13% upon *in vitro* treatment with leptin, whereas GVBD rates of *lepr*^{sa1508} LOF oocytes remained completely unaffected by such exogenous leptin (Fig. 5B, ANOVA F(3,28)=35.34, $P < 0.0001$). Similarly, *in vitro* administered DHP increased the maturation of WT oocytes by 37%, which was significantly blunted by 10% in oocytes from *lepr*^{sa1508} LOF animals (Fig. 5C, ANOVA F(3,28)=127.4, $P < 0.0001$). When we tested leptin and DHP together, we found that at least partially, leptin shows an additive effect to DHP, increasing maturation by an additional 7% beyond the effect of DHP alone in WT oocytes (Fig. 5D, ANOVA F(2,21)=174.5, $P < 0.0001$). Together, this indicates that peripheral leptin signaling in ovaries themselves can stimulate oocyte maturation, thus, the progression through oogenesis.

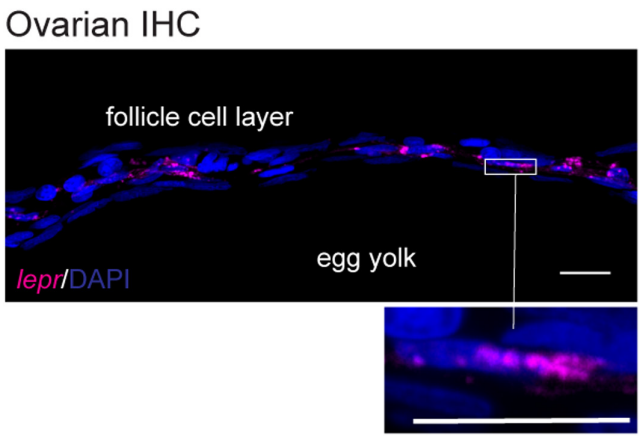

Figure 4
Evidence for ovarian *lepr* expression by immunohistochemistry; expression of *lepr:lepr–mCherry* in the follicle cell layer of mature oocytes labeled using rabbit anti-RFP antibody, counterstained with DAPI. Data shown is a representative image of $n = 4$ fish. A full color version of this figure is available at https://doi.org/10.1530/JOE-22-0026.

The oocytes isolated for the GVBD assay are immature oocytes that are arrested at the prophase I stage. These cells typically remain immature *in vitro*, however, under proper conditions, the cell cycle arrest can be released, and they undergo GVBD or atresia (Selman *et al.* 1993, 1994). Since we saw an effect of Lepr LOF on atresia histologically, we wondered whether DHP and/or leptin also affected oocytes undergoing atresia *in vitro*. We found that DHP as well as leptin significantly reduced the numbers of dying WT oocytes *in vitro*. Strikingly, however, this pro-survival effect of DHP was partially, and the pro-survival effect of leptin

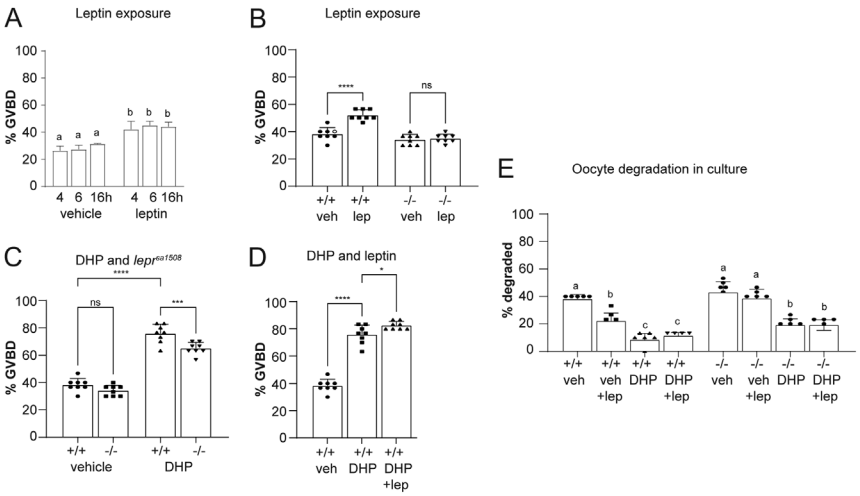

Figure 5
Evidence for the peripheral action of leptin in ovarian modulation in an *in vitro* germinal vesicle breakdown (GVBD) assay, (A) germinal vesicle breakdown in control, vehicle exposed and leptin exposed oocytes as a function of culture time, two-way ANOVA followed by Šídák's MCT, statistical difference between a, b and c at least $P < 0.05$, $n = 8$ culture experiments at each timepoint; (B) leptin induces GVBD in WT compared to Lepr LOF animals; (C) the effect of DHP in WT and Lepr LOF animals; (D) the effect of leptin and DHP is partially additive and (E) atretic oocytes in culture which underwent degradation in contrast to germinal vesicle breakdown or prophase I arrest are also modulated by leptin and DHP; (B, C and D) $n = 8$ culture experiments each group, one-way ANOVA was followed by Šídák's MCT, asterisks indicate significant effects against vehicle-injected control (ns, no significant difference, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

completely blocked in *lepr*^{sa1508} LOF oocytes. Lastly, the effect of DHP and leptin do not appear to be additive in this aspect (Fig. 5E, $F(7,56)=50.08$, $P < 0.0001$). Together, this indicates that in addition to promoting oocyte maturation, direct leptin signaling in ovaries can also block oocyte atresia.

Conclusion

Both in mammals and in fish, there has been a long-standing debate to which extent leptin regulates oocyte biology indirectly via the HPG axis, thus centrally, and to which extent it does so via direct signaling to ovarian cells, thus peripherally. Consistently, we found the leptin receptor to be present on Pomc neurons in the arcuate nucleus of the hypothalamus and found decreased *fshb/lhb* mRNA levels in the pituitary of *lepr*^{sa1508} LOF zebrafish (Fig. 1), most likely reflecting the central POMC-GNRH-LH/FSH-Progesterone HPG path. In addition, we found Lepr in follicle cells of the ovaries themselves (Fig. 4), consistent with comparable data for mammalian granulosa cells (Karlsson *et al.* 1997, Spicer & Francisco 1997, Ruiz-Cortes *et al.* 2003, Ryan *et al.* 2003), most likely constituting the direct/peripheral path. We also present functional data for this peripheral leptin path, performing *in vitro* GVBD and oocyte survival studies data of cultured fully grown follicles isolated from WT and *lepr*^{sa1508} LOF zebrafish treated with recombinant leptin protein or with DHP, an oocyte maturation-stimulating steroid hormone that is normally secreted by granulosa cells of the follicle upon stimulation by Lh from the pituitary/central leptin path (Levavi-Sivan *et al.* 2010). In this GVBD assay, we found no effect of time of incubation in the control medium over 4–16 h arguing that there are no endogenous factors that were carried over into the control medium. In contrast, recombinant leptin protein exogenously administered to such cultures led to an increase in GVBD rates of cultured WT follicles, consistent with recent data reported in the context of studies on growth hormone transgenic carp (Chen *et al.* 2018), whereas most strikingly, this gain-of-function effect was completely abolished in our *lepr*^{sa1508} LOF follicles. Therefore, this effect is unequivocally peripheral. Interestingly, the effect of DHP was also partially blocked by *lepr*^{sa1508} LOF and exposure to DHP as well as leptin was at least partially additive. This suggests that peripheral leptin signaling may at least in part elicit its effect by making oocytes more susceptible to DHP signals from the follicle cells, thus, for mediators of central leptin signaling via the HPG axis. The interplay and relative contributions of central vs peripheral leptin remain unclear and a subject for further studies.

Another aspect we found was the increase in oocyte atresia in the *lepr*^{sa1508} LOF animals post purging. The biology of atresia is not well understood. Initially described as a mechanism of oocyte resorption (Bretschneider & De Wit 1947), atresia is mostly seen as indicative of reproductive capacity of a population of fish (Witthames *et al.* 2013) and/or indicative of endocrine disruption (Molina *et al.* 2018). However, to our knowledge, there is little literature as to the central or peripheral regulation of atresia. We therefore carefully looked at cell death in the GVBD assay and found that both exposure to DHP and leptin reduced the amount of cell death in the GVBD assay. It is important to note that the cultured oocytes are arrested in prophase 1 and a good proportion of the oocytes remain immature and arrested

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throughout the culture period. The reason a proportion of oocytes die is unclear and could be a fate choice toward maturation or degradation based on the culture conditions and has been described as atresia (Selman *et al.* 1994). This is hard to establish since as the cells die, the RNA fragments and it is hard to get the timing right to collect RNA in order to test whether autophagy and/or apoptosis genes are activated similar to atresia (Gonzalez-Kother *et al.* 2020). However, the fact that leptin reduces the number of oocytes dying in culture is blocked in *lepr*^{sa1508} LOF oocytes is indicative of a controlled mechanism. Further, similar to the observation that the effect of DHP is partially blocked by *lepr*^{sa1508} LOF during the maturation of oocytes, we observed that DHP also reduced the number of dying oocytes in a culture which is similarly partially blocked by *lepr*^{sa1508} LOF. Therefore, we seem to have uncovered a role of leptin in directly blocking follicular and oocyte atresia which has interesting implications in fish biology and needs to be elucidated in further studies.

Yet, despite this effect on oogenesis and oocyte survival, and in contrast to mammals, *lepr*^{sa1508} LOF zebrafish do not show impaired fertility (Michel *et al.* 2016). In support of this, we did not see overt differences in ovarian histology when we looked at fish directly out of our holding tanks. There are several zebrafish lines that do not show impaired fertility after a central reproductive pathway is lost (Trudeau 2018, Li & Ge 2020), in particular the *gnrh2*, *gnrh3* and *gnrh2/3* LOF fish (Spicer *et al.* 2016, Marvel *et al.* 2018, 2019). Interestingly, the *gnrh2* LOF animals do not show a reduction in pituitary *fshb/lhb* expression, and the neuropeptide is compensated by a central mechanism. However, more careful analysis revealed that reproduction was significantly impaired in fasted LOF fish compared to fasted WT siblings (Marvel *et al.* 2021). We instead challenged our fish by synchronizing ovarian development and found that ovarian maturation was severely delayed in *lepr*^{sa1508} LOF fish compared to WT siblings. We saw a reduced number of mature oocytes as well as increased signs of atresia in H&E histology which was supported by qPCR of a set of maturation and atresia genes. The reproductive phenotype in these animals therefore consists either of a decline in the maturation rate, an increase in the rate of oocyte degradation (atresia), or both. Since this data is in the *lepr*^{sa1508} LOF animals, this predicts that conversely leptin signaling would increase maturation, decrease atresia or both. In fish, leptin is generally considered to increase during fasting which is suggestive of leptin being a signal that leads to energy mobilization (Deck *et al.* 2017). However, there is significant variability in fish life histories and mechanisms of energy homeostasis (Ronnestad *et al.* 2017) and fish with a reduction in hepatic leptin gene expression have been reported such as the large yellow croaker which shows a 3.4 fold downregulation of *lepa* during fasting (Qian *et al.* 2016, Supplemental material). In zebrafish, it has been shown that *lepa* is upregulated by overfeeding zebrafish larvae (Löhr *et al.* 2018) while postprandial fasting in zebrafish juveniles led to an immediate upregulation of *lepa* but a protracted decrease (Tian *et al.* 2015). This suggests that feeding similar to mammals appears to lead to elevated leptin levels in zebrafish. This could then signal sufficient energy to oocytes and promote maturation and/or reduce follicular atresia. In female fish which invest up to 20% of their body weight into oocytes, this is a significant energetic pathway as opposed to mammals where oocytes in females are formed *in utero* from the germline and are carried arrested in the prophase of the first meiotic division until a set of oocytes is released during every cycle. However, some of these mechanisms appear to still be present in rodents where leptin appears to attenuate follicular atresia (Almog *et al.* 2001) and LEP or LEPR LOF increases granulosa cell apoptosis (Hamm *et al.* 2004, Serke *et al.* 2012).

While this paper was under review, the Schmitz lab published that a different Lepr LOF line – *lepr*^{sa12953} – shows centrally mediated subfertility (Tsakoumis *et al.* 2022). The authors also observe reduced *lhb* expression in *lepr*^{sa12953} LOF females. However, the authors also see subfertility which they were able to largely overcome with the administration of human chorionic gonadotropin (Tsakoumis *et al.* 2022). In contrast, we did not see subfertility in the *lepr*^{sa1508} LOF animals (Michel *et al.* 2016) and uncovered a central as well as a peripheral component here. The differences are reminiscent of the diversity of body growth phenotypes seen in zebrafish leptin system LOF – with some authors seeing data indicative of (compared to mammals mild) obesity, and other studies not seeing a phenotype (discussed in Bagivalu Lakshminarasimha *et al.* 2021). The Zohar lab found that energy status (fasting) uncovered a phenotype in the *gnrh2* LOF line (Marvel *et al.* 2021). At this stage, the etiology of the differences is unclear and requires further study. It is possible that the difference is due to genetic backgrounds in these lines (since zebrafish are highly outbred (LaFave *et al.* 2014)), rearing and analysis conditions (Bagivalu Lakshminarasimha *et al.* 2021), age (He *et al.* 2021) or different compensatory responses in the different LOF lines. It is also possible that some of the leptin phenotypes that are seen in mammals are uncoupled in zebrafish. A further, intriguing possibility is the dependence of these phenotypes on the nutritional and energy status of the fish.

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Based on this and the Schmitz lab study (Tsakoumis *et al.* 2022), we hypothesize that Lepr signaling leads to increased maturation and reduced atresia during times of plenty, whereas decreased hepatic leptin production upon fasting would lead to decreased oogenesis and oocyte maturation, thus saving energy, as well as increased oocyte atresia, thus re-mobilizing energy formerly spent into oogenesis. Thereby, energy will only be irreversibly invested into reproduction when the energy balance of the body is positive. However, it remains unclear how such a system will allow oocyte maturation and reproduction to occur in the absence of energy in species such as the anadromous trout and salmon which need to retain reproductive competence in the face of dwindling energy. In this case, compensatory mechanisms need to be postulated, similarly to how it has been suggested for lack of reproductive phenotypes in adult teleost models after knockouts of central neuropeptides (Trudeau 2018).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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6.4 Publication 4

“Loss of Agrp1 in zebrafish: Effects on the growth and reproductive axis”

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Amrutha Bagivalu Lakshminarasimha: Data curation (Figure 2 and 3), Formal analysis, Investigation, Writing – review & editing.

Roger D. Cone: Conceptualization, Funding acquisition, Writing – review & editing.

Maximilian Michel: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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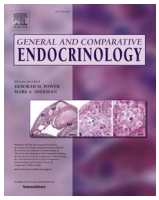

Loss of Agrp1 in zebrafish: Effects on the growth and reproductive axis

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ABSTRACT

Loss of agouti related neuropeptide (AgRP) does not lead to overt phenotypes in mammals unless AgRP neurons are ablated. In contrast, in zebrafish it has been shown that Agrp1 loss of function (LOF) leads to reduced growth in Agrp1 morphant as well as Agrp1 mutant larvae. Further, it has been shown that multiple endocrine axes are dysregulated upon Agrp1 LOF in Agrp1 morphant larvae. Here we show that adult Agrp1 LOF zebrafish show normal growth and reproductive behavior in spite of a significant reduction in multiple related endocrine axes namely reduced expression in pituitary growth hormone (*gh*) follicle stimulating hormone (*fshb*) as well as luteinizing hormone (*lhb*). We looked for compensatory changes in candidate gene expression but found no changes in growth hormone and gonadotropin hormone receptors that would explain the lack of phenotype. We further looked at expression in the hepatic and muscular insulin-like growth factor (Igf) axis which appears to be normal. Fecundity as well as ovarian histology also appear largely normal while we do see an increase in mating efficiency specifically in fed but not fasted AgRP1 LOF animals. This data shows that zebrafish can grow and reproduce normally in spite of significant central hormone changes and suggests a peripheral compensatory mechanism additional to previously reported central compensatory mechanisms in other zebrafish neuropeptide LOF lines.

1. Introduction

The melanocortin signaling system is at a crossroads for energy partitioning in mammals (Cone, 2006). Mutations in the melanocortin receptor gene (*MC4R)* are the single most common cause of genetic obesity in humans. The core melanocortin genes comprise the melanocortin receptor (*MC4R*), the agonist α-melanocyte stimulating hormone α-MSH which is coded for by the *POMC* gene (proopiomelanocortin, from which α-MSH is cleaved) and the antagonist/inverse agonist agouti related peptide (AgRP) coded for by the *AgRP* gene (Farooqi, 2021).

These genes are well conserved on a sequence level across all vertebrates, including fish (Soengas et al., 2018). Headway has been made in fish to elucidate whether the function of these genes is conserved as well (Volkoff, 2019). The antagonist AgRP is known to be orexigenic in mammals, that is it induces appetite upon injection and its expression rises upon fasting (Ollmann et al., 1997). In fish, expression of *agrp1* was shown to rise in fasted zebrafish (Song et al., 2003) and goldfish (Cerda-Reverter and Peter, 2003). Further, injection of the agonist α-MSH was shown to inhibit food intake (Cerda-Reverter and Peter, 2003), suggesting that melanocortin function is conserved. Overexpression of *agrp1* under the control of a ubiquitous promoter leads to obesity, increased linear growth and adiposity (Song and Cone, 2007). In contrast, blocking *agrp1* expression with a morpholino oligonucleotide was shown to decrease linear growth in zebrafish early development (Zhang et al., 2012). An important approach to elucidate the function of a gene in a given organism is to look at the loss of function *in vivo*. We previously reported on the zebrafish line *agrp1*^{vu623}, where we caused a 34 bp insertion using CRISPR mutagenesis, which disrupts the cysteine knot of Agrp1 and thereby disrupts core Agrp1 function. We showed with immunostaining that truncated Agrp1 does not enter the axons and

Abbreviations: LOF, loss of function; AgRP, agouti related neuropeptide; LH, luteinizing hormone; FSH, follicle stimulating hormone; GnRH, gonadotropin-releasing hormone; GH, growth hormone; CGA, glycoprotein hormone alpha polypeptide; IGF, insulin-like growth factor; MC4R, melanocortin 4 receptor; MSH, melanocyte-stimulating hormone; POMC, proopiomelanocortin; CRISPR, clustered regularly interspaced short palindromic repeats; HPG, Hypothalamic–pituitary–gonadal axis; HE, Hematoxylin and eosin; SL, standard length; ANOVA, Analysis of variance; PV, pre-vitellogenic; EV, early vitellogenic; MV, mid-vitellogenic; LV, late vitellogenic; FV, full vitellogenic; dpf, days post fertilization.

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instead immunoreactivity remains in the cell bodies. We showed phenotypically that *agrp1*$^{vu623-/-}$ animals are hypophagic and significantly shorter than their wildtype siblings at 8 days post fertilization (dpf) (Shainer et al., 2019).

The previous study focused on the early phenotype at the larval stage (4-8 dpf). Here, we studied Agrp1 loss of function (LOF) in adult zebrafish. In order to further characterize the LOF model, we investigated central gene expression in endocrine axes known to be impacted by the melanocortin system. We found that gene expression in the growth axis and the hypothalamic–pituitary–gonadal axis (HPG axis) was significantly reduced in the Agrp1 LOF animals. We further investigated the phenotype of the Agrp1 LOF fish in growth to adulthood, as well as reproductive function/fertility. Surprisingly we saw no overt phenotypes in spite of the significant reduction of pituitary gene expression, showing that Agrp1 LOF zebrafish can compensate for the loss of Agrp1 through a peripheral mechanism.

2. Materials and methods

2.1. Zebrafish strains and maintenance

The mutant strain *agrp1*^{vu623} (Shainer et al., 2019) was bred as a heterozygous incross and mixed genotype embryos were raised in 28 °C incubators on a 14:10 h light–dark cycle until 5 dpf, after which they were transferred to a standard diet and maintained in an Aquatic Habitats system on a 14:10 h light–dark cycle at a controlled density of five fish per liter. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee, the University of Michigan Institutional Animal Care and Use Committee or the national animal care committee (LANUV Nordrhein-Westfalen) and the University of Cologne.

Body Growth: Animals were raised until the day of testing in mixed genotype tanks at a controlled density, isolated, anesthetized with tricaine solution and blotted dry. Standard length and weight were measured, a piece of the fin-clipped and animals were placed in a recovery tank before being returned to their home tank. Animals were genotyped using the primers F 5′CCATAACACAGGATCTGGGC3′ and R 5′ CTGTGGATTCTCTGTGCGAGT3′ on a heteroduplex mobility shift assay according to (Ota et al., 2013).

RNA extraction and qPCR: Whole liver, pituitary, ovary and testis were dissected, then immediately placed on dry ice and stored at −80 °C until RNA extractions were performed. Total RNA was extracted using Trizol (Invitrogen) and purified using a PureLinkTM RNA mini kit with on column DNAse treatment (Thermo Fischer) following the manufacturer's protocols. Final RNA concentration was determined by optical density reading at 260 nm using NanoDrop 2000c. A 1 μg of RNA with a 260/280 ratio > 1.8 was used for reverse transcription using a high-capacity cDNA kit (Applied Biosystems). Primers were designed using Primer-BLAST (NCBI) with similar melting temperatures. The sequences are as listed in Table 1. Oligos were ordered from IDT. The qPCR was performed with SYBR Select Master Mix (Life Technologies, Thermo Fisher Scientific) on an ABI-Prism 7500 Fast Detect system under the following conditions: initial PCR activation at 50 °C for 20 s, 95 °C for 2 min; PCR cycles at 95 °C for 15 s for denaturation, 60 °C for 1 min for annealing and 72 °C for 30 s for extension with repeating for 40 cycles. Each sample was performed in duplicate. Relative expression levels were calculated following a modified ΔΔCt method to include primer efficiency according to (Pfaffl, 2001). Efficiencies and R^2 were assessed using a five-point cDNA serial dilution and efficiencies of all the primers ranged between 96 and 102%. Primer melt curves of every experiment were assessed to have a single clear peak. Data are presented as fold change relative to WT and standardized to *ef1α* expression. The reference was stably expressed between all groups. Primers for *ef1α* and *gh* were from (Zhang et al., 2012) and the remaining primers are listed in Table 1.

Ovarian histology analysis: Whole zebrafish were fixed in 10% buffered formalin for two days and whole ovaries were dissected and embedded in paraffin wax using standard protocols. A section of approximately 5 μm thickness was stained with hematoxylin and eosin (HE) to evaluate morphology. Images were captured using an Olympus microscope (BX53, Tokyo, Japan). For quantification, histological slides were carefully chosen to be in a comparable anatomical plane. After fixation, we dissected the abdominal cavity with ovaries and head before embedding the tissue, so it is easy to orient the ovaries in the same way between individual fish and pick comparable planes of sectioning. This was ascertained on the H&E Histology based on the hepatic and gut tissue. Individual oocytes were staged and counted using Fiji (ImageJ) software.

Statistical analysis: Statistics were carried out in GraphPad Prism version 9.1.1 for Windows, GraphPad Software, San Diego, California USA. Unpaired, two tailed t-tests were carried out to compare LOF and wildtype data. One-way ANOVA was followed by Dunnett's multiple comparisons test. P-values less than 0.05 were considered significant.

Table 1
Real-time PCR primer sequences.

Gene	Forward/Reverse	Primer	Fig.
cga	Forward	TGGACAAGATACGCTGAAGCA	3a,c,d
cga	Reverse	CGGGTTTGGAGAAACGTTCG	3a,c,d
lhb	Forward	GGTGGAAAAAGAGGGCTGTCC	3a,c,d
lhb	Reverse	CACACTGTCTGGTGGACGGT	3a,c,d
fshb	Forward	CGACTCACCAACATCTCCATC	3a,c,d
fshb	Reverse	ACTCGATCCATTGTCCAGCAT	3a,c,d
ghra	Forward	CTCCCAGCAGCAGAGGTTGATG	2b
ghra	Reverse	GAATTCTTCTTATCTGCAGGATCGTC	2b
ghrb	Forward	GAAAAGGATCCAAAGAAAACTTACGG	2b
ghrb	Reverse	CTACAGGTGGGTCTGGAAACACAATA	2b
igf1	Forward	GCATTGGTGTGATGTCTTTAAGTGTA	2c
igf1	Reverse	GTTTGCTGAAATAAAAGCCCCT	2c
igf2a	Forward	GAAACACGAACAACGATGCG	2c
igf2a	Reverse	AGTACTTCACATTTATGGTGTCCTTG	2c
igf2b	Forward	ACAGACAGTTTCGTAAATAAGGTCATAA	2c
igf2b	Reverse	CAACACTCCTCCACAATCCCAC	2c
asip1	Forward	TGGGGGCCTCAGTACTCACA	3e
asip1	Reverse	CAGAGCAGCACACAGCACAG	3e
agrp1	Forward	GTGAATGTTGTGGTGATGG	3e
agrp1	Reverse	TTCTTCTGCTGAGTTTATTTC	3e
agrp2	Forward	GCTCTTCATCTGCTTGTTCTT	2f, 3e
agrp2	Reverse	CTCCTGATTCCACACTCCT	2f, 3e
fshr	Forward	AACCTGACCTACCCCAGCCA	3b
fshr	Reverse	CGCAAGGGTTGAATGCGTCT	3b
lhcgr	Forward	AGTCACTGCTGCGCTCTGAT	3b
lhcgr	Reverse	CCTCGACGGATGACGATCCA	3b
ghra	Forward	GCCCTGACTACACGCAGACT	2d
ghra	Reverse	GCAGAGTCCAGTTCAGCCCA	2d
ghrb	Forward	ACGGCTGTGCTGGTCTTTACT	2d
ghrb	Reverse	CAAGTCCGGGTGTGTCCCTA	2d
igf1	Forward	GTGTGTGGAGACAGGGGCTT	2e
igf1	Reverse	GATGCCACGGTTGTGTGACC	2e
igf2a	Forward	TGTTTCCTCCACCTCGCTGC	2e
igf2a	Reverse	GGGCCAACAGAATGGATGGGA	2e
igf2b	Forward	GTCATTCCAGTGATGCCCGC	2e
igf2b	Reverse	CTCCGTAGCCTCTGAGCAGC	2e

3. Results

3.1. Agrp1 LOF body growth

We previously showed that early Agrp1 loss of function using morpholino antisense oligonucleotides directed against *agrp1* results in reduced linear growth at 5 days post fertilization (dpf) as well as a reduction in growth hormone (*gh*), insulin-like growth factor 1 (*igf1a*) and thyroid stimulating hormone (*tsh*) expression at 4dpf (Zhang et al., 2012)). To further support this data, we established an Agrp1 LOF line - *agrp1*^{vu623}, which also showed reduced growth at 8 dpf (Shainer et al., 2019).

In order to elucidate this model further, in the present study we

tested two independent cohorts of adult animals. We kept the mixed-genotype first cohort offspring of a heterozygous incross in a tank under normal feeding conditions, measured standard length and body weight at 79 dpf and genotyped according to (Bagivalu Lakshminarasimha et al., 2021). Surprisingly, animals appear to compensate in adulthood for the Agrp1 LOF. Neither male nor female fish showed a difference in standard length (Fig. 1a, ANOVA F (2,50) = 0.6586, p = 0.5220 for male and F(2,15) = 0.6917, p = 0.5160 for female fish) or weight (Fig. 1b, ANOVA F(2,51) = 1.764, p = 0.1816 for male fish and F (2,43) = 2.660, p = 0.0814 for female fish). A second independent cohort of fish similarly raised as mixed genotype fish under normal conditions confirmed that the *agrp1* LOF does not result in a reduction in standard length (Fig. 1c, ANOVA F(2,31) = 1.200, p = 0.3147 for male and F(2,15) = 0.6917, p = 0.5160 for female fish) nor weight in adults at 88 dpf (Fig. 1d, ANOVA F(2,32) = 0.4321, p = 0.6529 for male and F (2,15) = 0.3455, p = 0.7134 for female fish).

3.2. Agrp1 LOF and gene expression in the growth axis

The *gh/igf* axis is the primary axis controlling growth in vertebrates (David et al., 2011). Since we found normal adult growth in Agrp1 LOF fish, we reasoned that the reduction in *gh* gene expression in Agrp1 morphant larvae at 4 dpf (Zhang et al., 2012) would be normalized. Surprisingly, in the adult Agrp1 LOF pituitary, gene expression of *gh* was still significantly downregulated (Fig. 2a, *gh* t(25) = 4.667, p < 0.0001). Complete lack of GH leads to dwarfism (McMenamin et al., 2013). We therefore examined hepatic *ghr* and *igf* expression in adults in order to determine whether a compensation at this level could explain the normal growth in Agrp1 LOF fish. We found that hepatic expression of *ghra* (Fig. 2b, t(9) = 1.014, p = 0.3372) or *ghrb* (Fig. 2b, t(9) = 0.08217, p = 0.9363) was unaltered in adult *agrp1* LOF zebrafish compared to wildtype siblings. Similarly, expression of hepatic *igf* was not altered (Fig. 2c, *igf1a:* t(9) = 0.6251, p = 0.5474; *igf2a:* t(9) = 0.3880, p = 0.7070; *igf2b:* t(9) = 0.06250, p = 0.9515). Therefore, the reduction in *gh* expression at larval and adult stages and *tsh* expression at larval stages does not induce compensatory *ghr* expression, and somehow does not lead to a reduction in hepatic *igf* expression. Approximately 75% of serum *igf* is considered to be liver derived, but extrahepatic sources also play a role in GH signalling (David et al., 2011). We therefore verified expression in muscle tissue, another target of growth hormone. We found a reduction in *ghra* (Fig. 2d, t(8) = 2.986, p = 0.0174) as well as *ghrb* (Fig. 2d, t(8) = 8.235, p < 0.0001) in *agrp1* LOF zebrafish compared to wildtype siblings, which is indicative of reduced muscle growth. Even though the reduction in *ghrb* expression in mutants was 3-fold compared to siblings, we saw no changes in *igf1a* or *igf2a* and only a slight reduction in *igf2b,* by 1.4-fold (Fig. 2e, *igf1a:* t(8) = 0.7253, p = 0.4889; *igf2a:* t(8) = 1.338, p = 0.2176; *igf2b:* t(8) = 3.6231, p = 0.0066).

We then tested for a possible compensation through Agrp2*,* but found no difference in *agrp2* expression in *agrp1* LOF hypothalamus compared to wildtype siblings (Fig. 2f, *agrp2* t(9) = 0.03696, p = 0.9713). Taken together, we found a reduction in central *gh* signalling in *agrp1* LOF mutants which is not reflected in hepatic or muscle *ghr/igf* gene expression changes, and is not compensated by *agrp2* expression.

3.3. Agrp1 LOF and gene expression in the HPG axis

In mammals, AgRP is known to interact with the reproductive axis (Israel et al., 2012; Padilla et al., 2017). However, the interaction is complicated and *AgRP -/-* mice remain fertile (Qian et al., 2002). In fish, *agrp1* was found to be dysregulated in gonadotropin releasing hormone (Gnrh) 2 LOF mutants as well as with Gnrh2/Gnrh3 double LOF zebrafish adults (Marvel et al., 2018; Marvel et al., 2019). We therefore wondered whether the reproductive axis is dysregulated in adult, sexually competent Agrp1 LOF zebrafish. We examined gene expression in the HPG axis, specifically of follicle stimulating hormone (*fshb*), luteinizing hormone (*lhb*) and the gonadotropin alpha subunit (*cga*). In adult *agrp1* LOF pituitaries, expression of all three genes was significantly reduced compared to WT siblings (Fig. 3a, *fshb:* t(27) = 3.193, p

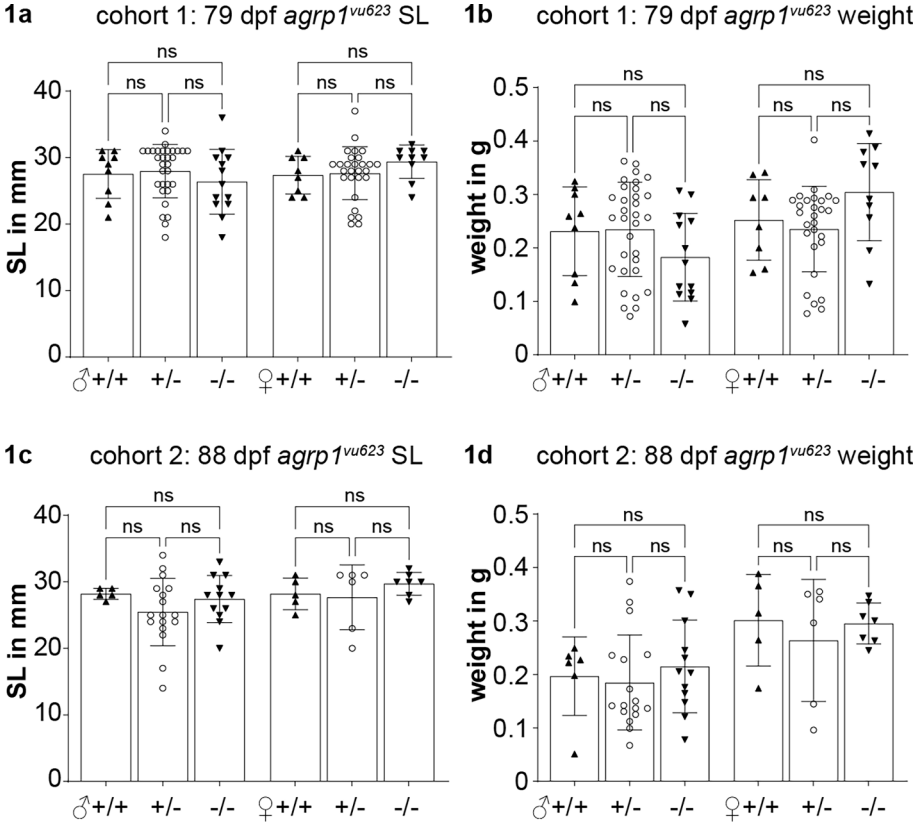

Fig. 1. Agrp1 LOF does not lead to an adult growth phenotype in the *agrp1*^{vu623} allele (A) cohort 1 animal size in standard length at 79 dpf, male n = 9/31/13 and female 8/28/10 and (B) cohort 1 animal weight in grams at 79 dpf; (C) cohort 2 animal size at 88 dpf, male n = 5/17/12 and female n = 5/6/7 and (D) cohort 2 animal weight at 88 dpf. (ns = no significant difference, One-way ANOVA was carried out to compare between genotypes followed by Šídák's multiple comparisons test, +/+ wildtype; +/- heterozygous and -/- loss of function).

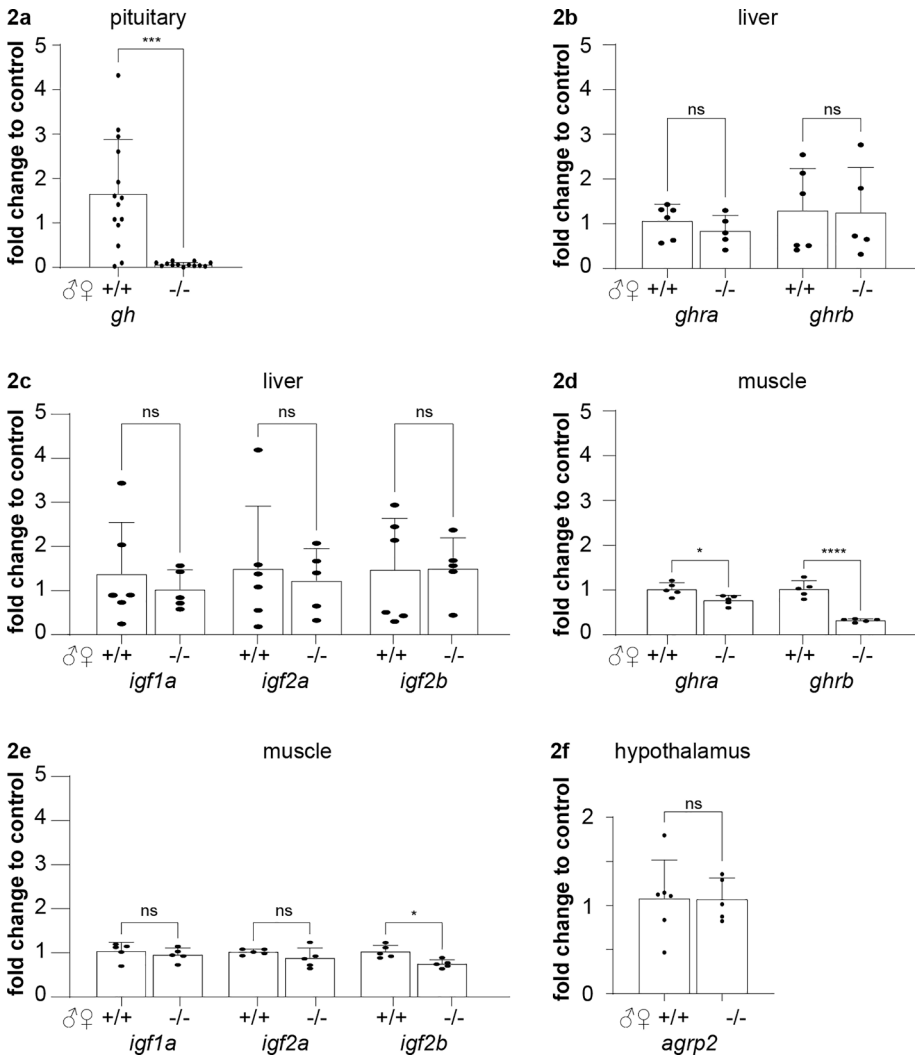

Fig. 2. Growth axis gene expression in Agrp1 LOF animals (A) significant reduction of pituitary growth hormone expression in adult *agrp1* LOF animals compared to wildtype siblings, n = 14/13; (B) growth hormone receptor a and b expression in the liver are normal, n = 6/5,6/5; (C) hepatic insulin-like growth factor gene expression is not altered, n = 6/5,6/5,6/5; (D) the growth axis expression in muscle tissue, *ghra* and *ghrb*, n = 5; (E) as well as *igf1a, igf2a* and *igf2b*, n = 5 and (F) lack of a hypothalamic *agrp2* compensatory response, n = 6/5. Expression data shown as fold change relative to controls. Unpaired t-tests were used to compare between genotypes. Asterisks indicate significant effects against control (* $P < 0.05$; *t*-test).

= 0.0036; *lhb:* t(28) = 4.802, p < 0.0001; *cga:* t(22) = 4.458, p = 0.0002).

Reduced circulating gonadotropins could be compensated for by changes in gonadotropin hormone (GtH) receptors. However, we found no change in *fshr* expression (Fig. 3b, t(8) = 0.08762, p = 0.9323) and only a slight (1.3 fold) reduction in *lhcgr* (Fig. 3b, t(8) = 3.617, p = 0.0068) in mutant ovaries compared to WT siblings.

In addition to expression in the brain and the pituitary, expression of gonadotropins (*fshb, lhb, cga*) has also been reported peripherally, which likely plays a role in calibrating HPG function (Levavi-Sivan et al., 2010). Consequently, we also evaluated expression in either ovaries or testes of Agrp1 LOF fish, and found that again *fshb, lhb* and *cga* expression levels were significantly downregulated in Agrp1 LOF ovaries compared to WT siblings (Fig. 3c, *fshb:* t(13) = 2.408, p = 0.0316; *lhb:* t(13) = 2.549, p = 0.0242; *cga:* t(12) = 3.232, p = 0.0072); however only *fshb* and *lhb* were downregulated in testes (Fig. 3d *fshb:* t(12) = 2.802, p = 0.0160; *lhb:* t(13) = 3.246, p = 0.0064; *cga:* t(13) = 0.6813, p = 0.5076).

3.4. Potential peripheral modulators

Central compensation would lead to regular pituitary hormone levels, and we have shown that pituitary hormone expression is abnormal in the Agrp1 LOF adults. However, recent evidence has suggested that the role of melanocortins (Navarro et al., 2022) as well as leptin (Bagivalu Lakshminarasimha et al., 2022) could play a peripheral role aside from their central role. Therefore, an increase in, for example, leptin levels could compensate for the loss in central *fsh/lh*. We therefore tested ovarian expression of agouti signalling protein 1 (*asip1*), agouti signalling protein 2b (also called *asip2b* and the same gene as *agrp2* above, see also (Braasch and Postlethwait, 2011; Vastermark et al., 2012)) as well as leptin a (*lepa*) and leptin b (*lepb*). In our assay, *lepa* was not expressed significantly in the ovary and we found no expression differences in either *asip1*, *agrp2* or *lepb* (Fig. 3e, *asip1:* t(8) = 1.738, p = 0.1204; *agrp2:* t(8) = 0.9015, p = 0.3937; *lepb:* t(8) = 2.008, p = 0.0796) in *agrp1* LOF zebrafish compared to wildtype siblings. Thus, peripheral expression of these genes does not compensate for the loss of central *fsh/lh*.

3.5. Agrp1 LOF and reproduction

After finding significant differences in reproductive gene expression in both adult pituitary and gonadal tissues in *agrp1* LOF adults, we decided to look for a breeding phenotype specifically in females. Zebrafish are income breeders and as such can breed every day provided they have sufficient energy (McBride et al., 2015). We bred animals every day, or separated males and females for increasing time periods. Egg mass reaches a plateau after 9–11 days (Eaton and Farley, 1974; Niimi and LaHam, 1974). We found that *agrp* genotype did not affect the number of eggs per mating at any given time point, while the fecundity increased with increasing time of separation as expected, providing evidence that Agrp1 LOF fish do not have an overt breeding phenotype

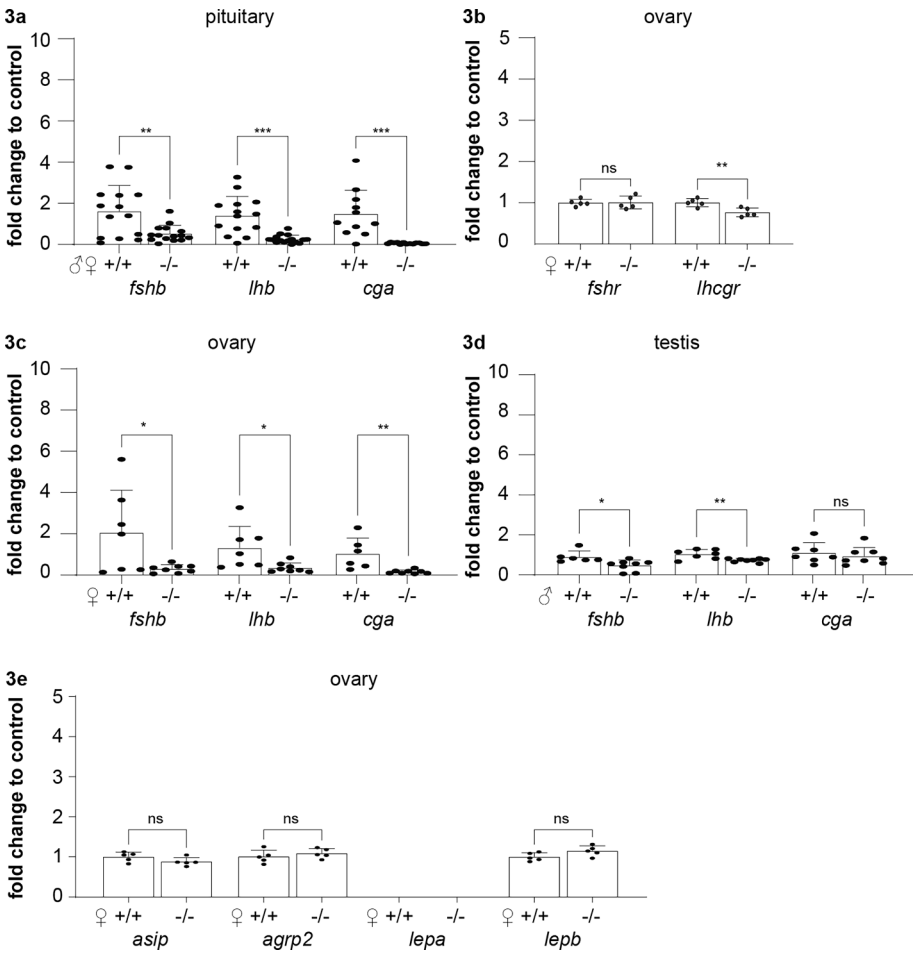

Fig. 3. Expression of genes in the reproductive axis in Agrp1 LOF animals (A) *fshb*, *lhb* and *cga* gene expression in the pituitary of mixed gender fish, n = 14/16, 14/15 and 11/13; (B) Gonadotropin hormone (GtH) receptors in the ovary of Agrp1 LOF animals; *fshr* and *lhcgr*, n = 5; (C) reproductive gene expression in the female ovaries n = 7/8, 7/8 and 6/8; (D) reproductive gene expression in the testis of male animals n = 6/8, 7/8 and 7/8 and (E) potential peripheral modulator expression in the ovary, *asip, agrp2, lepa* (below the limit of detection) and *lepb* n = 5. Expression data shown as fold change relative to controls. Unpaired t-tests were used to compare between genotypes. Asterisks indicate significant effects against control (* $P < 0.05$; *t*-test).

(Fig. 4a, the Two-way ANOVA shows a significant effect of separation time ($F(9, 367) = 18.12 p < 0.0001$) but not genotype ($F(1, 367) = 1.703 p = 0.1927$)). When we evaluated mating efficiency however, we found that as opposed to an impairment, Agrp1 LOF females were more successful in mating and yielding fertilized oocytes when mated with a wild type male (Fig. 4b shows 74.25% mating efficiency in fed *agrp1*$^{vu623\ +/+}$ versus 85.96% in fed *agrp1*$^{vu623\ -/-}$ animals, χ^2 (1, N = 461) = 9.902, p = 0.0017). In contrast, when the females were fasted before breeding, we found that mating efficiency equalized based on genotype (Fig. 4c shows 74.19% mating efficiency in fasted *agrp1*$^{vu623\ +/+}$ versus 77.42% in fasted *agrp1*$^{vu623\ -/-}$ animals, χ^2 (1, N = 62, p = 0.76668).

3.6. Histological changes after breeding in Agrp1 LOF

The observation that individual breeding events were more likely to be successful in animals with a significant reduction in reproductive hormone gene expression was very surprising to us. We therefore purged the oocytes of female fish by breeding with wildtype males, separated the female fish, and allowed oocytes to mature for one to 12 days in order to carefully evaluate oocyte maturation. We collected, fixed, and paraffin-embedded ovaries of *agrp1*$^{vu623\ +/+}$ and *agrp1*$^{vu623\ -/-}$ fish, prepared coronal sections at comparable anatomical locations, and quantified numbers of oocytes according to developmental stage (representative histology one day after purging see Fig. 5a). Counting oocytes in the primary growth stage revealed no significant effect of *agrp* genotype on oocyte maturation (Fig. 5b, Two-way ANOVA $F(1, 64) = 0.03405$ p = 0.8542), yet we saw a significant effect across days after purging as expected (Fig. 5b, Two-way ANOVA F (0.5290, 8.463) = 20.43 p = 0.0042). We grouped oocytes at the pre-vitellogenic (PV), early vitellogenic (EV) and mid-vitellogenic (MV) stages and again found no significant differences between genotypes for most time points, (Fig. 5c, Two-way ANOVA $F(1, 65) = 3.787$ p = 0.0560), however Šídák's multiple comparisons test did reveal significantly increased numbers of PV/EV/MV stage oocytes at three and nine days after purging in Agrp1 LOF females compared to wild type controls. As expected, we also found a significant effect of time of separation (Fig. 5c, Two-way ANOVA F (1.090, 17.72) = 18.32 p = 0.0004). Lastly, counting mature oocytes at the late vitellogenic (LV) and full vitellogenic (FV) stages again showed no overall differences between mutants and control females across all time points, (Fig. 5d, Two-way ANOVA F (1, 65) = 2.747 p = 0.1023), however Šídák's multiple comparisons test picked up a significant difference at 3 and 9 days, with Agrp1 LOF females having more mature oocytes at 3 days after purging and fewer mature oocytes at 9 days after purging. Once again, we observed a significant effect of time of separation (Fig. 5d, Two-way ANOVA F (1.644, 26.71) = 21.69 p < 0.0001). Consequently, we did not see significant histological changes between genotypes that would be expected downstream of a significant drop in central gonadotropin expression, or which could explain the observed increase in breeding success in fed *agrp1* LOF female fish.

4. Discussion

Here we show that adult Agrp1 LOF fish have reduced central expression of growth axis (*gh*) and reproductive axis (*fshb, lhb, cga*) genes. This is consistent with the animal's central nervous system being in a fasted or starving state. Upon fasting, various endocrine axes are downregulated in order for the animal to conserve energy (Ahima et al.,

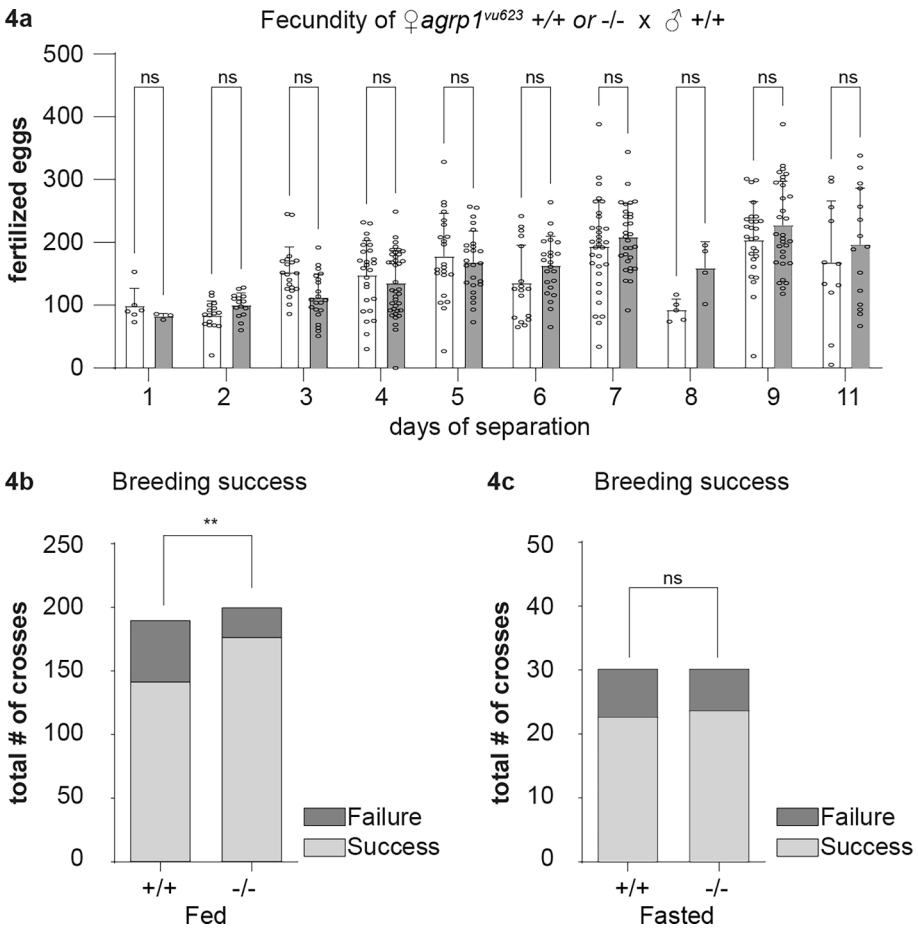

Fig. 4. Agrp1 LOF phenotype *in vivo* (A) Female *agrp1*^{vu623} +/+ (open columns) and *agrp1*^{vu623} -/- female fish (gray columns) were purged and allowed to recover individually for the indicated amount of days (x axis) before being bred with a wildtype male fish, n = 6/3, 16/15, 20/21, 26/38, 22/25, 17/23, 32/30, 5/4, 27/31, 11/15 pairings; (B) mating efficiency as the number of successful mating events leading to fertilized oocytes with a wildtype male shows a statistically significant relationship in fed animals, *agrp1*^{vu623} +/+ n = 173/60 and *agrp1*^{vu623} -/- n = 192/32, p = 0.0017; (C) mating efficiency success of Agrp1 LOF females is normalized upon fasting, *agrp1*^{vu623} +/+ n = 23/8 and *agrp1*^{vu623} -/- n = 24/7. (ns = no significant difference); (A) Two-way ANOVA carried out to compare for the effect of genotype versus the effect of days of separation followed by Šídák's multiple comparisons test; (B&C) χ^2 test was carried out to compare between genotypes, * P < 0.05).

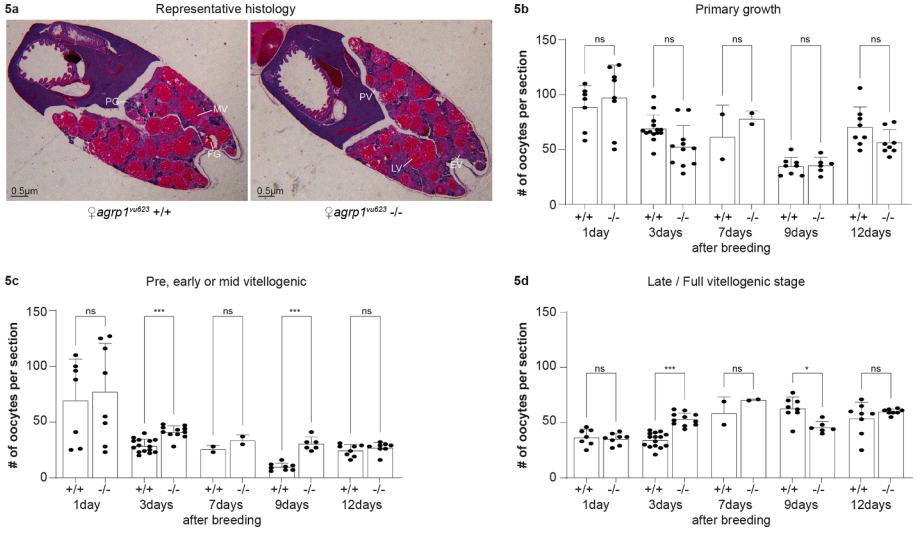

Fig. 5. Histological analysis of Agrp LOF female fish after breeding (A) Representative H&E stained histological slides of *agrp1*^{vu623} +/+ and *agrp1*^{vu623} -/- oocytes; PG = Primary growth; PV, EV, MV and LV are pre, early, mid and late vitellogenic stages and FG represents fully grown oocytes; (B-D) counts of single representative slides for each fish at a comparable cutting plane at indicated days after breeding, n = 7/8, 15/11, 2/2, 8/8 for 1 day, 3, 7, 9 and 12 days of separation respectively; (B) shows early stage oocytes of the primary growth stage; (C) shows oocytes of the pre, early and mid vitellogenic stages and (D) shows mature late vitellogenic and fully grown oocytes. (ns = no significant difference, * P < 0.05; a Two-way ANOVA was carried out to compare the effect of genotype versus the effect of time of separation followed by Šídák's multiple comparisons test).

1996). Paradoxically, in contrast to what pituitary gene expression suggests, we did not observe an *in vivo* impairment either of growth or reproductive capacity in adult fish suggesting compensatory responses downstream of pituitary gene expression.

It is plausible that a predicted LOF mutation does not actually lead to an amorph and loss of function of the protein, but instead to a hypomorph. While this would not explain the altered central gene expression we observed, it could explain the lack of a peripheral phenotype. The *agrp1*$^{vu623\ -/-}$ mutation is predicted to disrupt a cysteine motif in the core of the bioactive mini-AgRP (Wilczynski et al., 2005) and therefore, translation of a functional Agrp peptide is very unlikely. However, the *agrp1*$^{vu623\ -/-}$ transcript does not undergo nonsense-mediated decay, and instead expression is even significantly increased in *agrp1* LOF hypothalamus (data not shown). We previously examined Agrp1 subcellular localization and found that the mutated Agrp1 does not undergo axonal transport and instead accumulates in cell bodies (Shainer et al., 2019). Hence, *agrp1*$^{vu623\ -/-}$ animals are *bona fide* Agrp LOF animals.

In larvae, a morpholino oligonucleotide against *agrp1* has been

shown to lead to reduced *tsh* and *gh* expression, as well as reduced somatic growth (Zhang et al., 2012). We previously replicated this phenotype in *agrp1*$^{vu623\ -/-}$ larvae, which shows reduced growth as well as reduced food intake at 6 dpf (Shainer et al., 2019). The larval phenotype would be congruent with hypothyroidism (Deal and Volkoff, 2020), however when we investigated *gh* expression in adult Agrp1 LOF fish pituitaries, we found that *gh* expression is still significantly reduced, replicating the previous larval findings. While adult hypothyroidism would not be expected to lead to growth deficiency, adult lack of growth hormone would. The surprising lack of a reduced growth phenotype *in vivo* in adult fish suggests that compensatory mechanisms allow LOF fish to grow normally. In our previous paper we showed that Agrp2 might act in a neuroendocrine or hypophysiotropic manner (Shainer et al., 2019). It is therefore plausible that Agrp2 could compensate for the loss of *agrp1*, however we found that hypothalamic expression levels of Agrp2 were unchanged. This data suggests that the Agrp1 LOF is not compensated for centrally. When we measured peripheral markers of the growth axis, we found no sign of reduced *gh* activity. For example, hepatic *igf* expression, a critical target of somatic growth-related *gh* activity (Won et al., 2016), was not significantly different in Agrp1 LOF animals. One caveat is that we did not measure blood levels of GH in zebrafish as this is technically difficult in such small animals. It is therefore plausible that reduced expression of *gh* does not lead to reduced pituitary secretion of *gh* but this is unlikely. Therefore, this data points to *gh* being compensated for between the pituitary and the liver. This data is reminiscent of several neuropeptide knockouts in fish which did not lead to deficits in the reproductive axis (Trudeau, 2018).

What about the reproductive axis in Agrp1 LOF zebrafish? We identified significant reductions in pituitary *fshb, lhb and cga* gene expression in Agrp1 LOF fish. This reduction in reproductive gene expression suggests that Agrp1 plays a role in reproductive function in fish, similar to mammals. In mice, a role of AgRP was found upstream of gonadotropin-releasing hormone (GnRH) neurons (Israel et al., 2012; Sheffer-Babila et al., 2013). Indeed, in zebrafish, *gnrh2* LOF animals (Marvel et al., 2019) as well as *gnrh2/gnrh3* double knock outs (Marvel et al., 2018), show decreased *agrp* expression levels as well as increased food intake. Loss of function of the main *gnrh* isoform *gnrh3* surprisingly did not result in changes in *fshb, lhb or cga* expression (Spicer et al., 2016). Loss of *gnrh2* only led to a significant reduction of *lhb* in male pituitary but not female, and no significant changes in *cga* or *fshb* in either sex (Marvel et al., 2019). The double loss of function line did not exhibit expression changes in these genes (Marvel et al., 2018). Consequently, in the absence of changes in *fsh* or *lh* expression, the *gnrh* LOF lines do not show evidence of a loss of reproductive capacity (Marvel et al., 2018; Marvel et al., 2019; Spicer et al., 2016). The authors argue that non-cell-autonomous compensatory changes occur in the brain peptidergic neurons in order to ascertain pituitary expression of these neuropeptides (Marvel et al., 2018). In contrast, we did observe a loss of pituitary expression of these neuropeptides in the Agrp1 LOF fish, which led us expect an *in vivo* reproductive phenotype. However, these animals are reproductively normal. Further, *lh* and *fsh* are also locally expressed and we did not detect compensatory responses to the central loss of *fsh* and *lh* expression in the ovary. In contrast, we also found a significant reduction of reproductive gene expression in the ovary similar to the reduction in pituitary gene expression. Further, even after purging the existing mature oocytes from the female ovary and evaluating oocyte maturation over time, we only saw slight differences between genotypes. As opposed to an impairment of oocyte maturation, our data suggest that there may even be an increase in maturation in Agrp1 LOF oocytes in select time windows. Similarly, we found an unexpected increase in mating success in normally fed *agrp1* LOF females compared to wildtype siblings. Therefore, this data suggests that zebrafish not only exhibit evidence for compensatory mechanisms at the level of the central nervous system (Marvel et al., 2018), but in the case of Agrp1 LOF, compensatory mechanisms at the peripheral level that allow the animal to be reproductively competent even though central expression of reproductive genes is disrupted.

In zebrafish, complete loss of *fsh* function leads to delayed ovarian development, but fertile adult females. Loss of *lh* results in a failure to spawn (Zhang et al., 2015). In our case, we observed a 3–25 fold reduction in *lh, fsh and cga* in *agrp -/-* females compared to sibling wildtype females. This reduced expression however does not seem to interfere with reproductive function. Indeed, calorically restricted female fish prioritize energy allocation towards ovarian growth from a certain (adult) size onwards (Leibold and Hammerschmidt, 2015). The gene expression changes seen in Agrp1 LOF are similar to a fasting state in wildtype fish. In normally fed animals, we observed an increase in mating efficiency in Agrp1 LOF females compared to wildtype sibling females. When the two groups were fasted, we did not see a difference between wildtype fish (mating efficiency was 74% in fasted as well as fed animals), but instead observed a reduction in mating efficiency in Agrp1 LOF fish, from 87% in fed animals to 77% in fasted animals. Therefore, we conclude that fasting does not modulate mating efficiency in wildtype females but feeding specifically allows Agrp1 LOF females to be more successful. It appears that Agrp1 LOF uncovers a mechanism which in some way increases mating efficiency independent of neuropeptides, specifically in fed fish. During revisions of this manuscript, we found a report of melanocortins modulating steroidogenesis, and therefore potentially ovarian function peripherally (Navarro et al., 2022), and indeed we recently reported a peripheral component of leptin signaling in ovarian function as well (Bagivalu Lakshminarasimha et al., 2022). Following the hypothesis that we have uncovered an unknown mechanism in our Agrp1 LOF animals for peripheral compensation in reproductive function, we also tested whether ovarian leptin or melanocortins other than Agrp1 could be compensating, but found no changes that would explain the phenotype.

The mutant Agrp1 protein gets trapped in the cell bodies in Agrp1 LOF fish (Shainer et al., 2019). However, *agrp1* expression is upregulated in the CNS of Agrp1 LOF fish (data not shown). It is plausible that these neurons undergo other unknown compensatory changes, possibly leading to increased neurotransmitter release which could affect sympathetic neural activity, as Agrp1 afferent neurons have been found in the spinal cord (Reinoss et al., 2020). In addition, there could be entirely peripheral feedback circuits supporting reproductive function in fish in the absence of central input. Further research into compensatory mechanisms will help explain why animals can compensate for such a drastic loss of central gene expression in Agrp1 LOF fish, as well as many other neuropeptide LOF models which only have mild reproductive phenotypes *in vivo*.

CRediT authorship contribution statement

Zehong Wei: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. **Amrutha Bagivalu Lakshminarasimha:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Roger D. Cone:** Conceptualization, Funding acquisition, Writing – review & editing. **Maximilian Michel:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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7 GENERAL DISCUSSION

The optimal allocation of energy resources for the basic maintenance of an organism's metabolism and its daily activities such as foraging, somatic growth, reproductive investments, and energy storage are critical to its development and survival. The regulation of food intake and energy metabolism are vital processes that ensure these needs are met. Two main models have been proposed to explain this regulation. As explained by Speakman et al., in his review [237], the set-point paradigm proposes that an active biological feedback system uses a set point that is encoded in the brain to relate energy storage to energy intake and consumption [238]. The discovery of leptin and other genetic mutations in individuals leading to obese conditions, supported this theory [16, 17, 84]. Although this model considers many biological factors of energy balance, it finds it difficult to explain the significant increase in the obesity epidemic. The set point model fails to explain the contribution of substantial environmental and social factors on obesity, dietary intake, and physical activity [239].

The alternative theory, known as the settling point model, postulates the existence of a self-regulated system, that does not include any set point, between the quantity of energy consumed, the quantity of bodily reserves, and the quantity of energy expended [240]. The energy balance is accounted for by this model in many social and environmental elements, such as influence of high fat diets, large portions of food consumed etc., but it fails to explain other genetic and biological features [241, 242]. The shortcomings of these two models come from their failure to take into account how genes and environment interact to regulate body weight. The set and settling point models of energy regulation reflect a broader divide in the way we conceptualize the obesity problem [237, 243]. While both of these models are partially correct, the interplay between genotypes and environment is critical to understand their effect on metabolic complexities.

In the past years, mammalian animal models have provided insight into the molecular and cellular mechanisms of energy homeostasis that control different pathways of energy expenditure [244]. This method, which relies on a single model or broadly extrapolates the findings of a small number of species to a wider group

of species, has a number of drawbacks. The functional variety and life histories of the organisms cannot be accurately modelled with a small number of model systems. This is particularly concerning because, depending on their ecological niche, various species use different physiological and behavioural techniques to regulate distinct components of energy expenditure [159]. Therefore, comparative studies could answer more complex questions regarding the role of certain genes or hormones in the regulation of energy and further aid in the development of new model systems for biomedical research [154, 157]. In this thesis, we systematically characterised the dietary effect of obesity hallmarks and further investigated the peripheral and central pathways that regulate endocrine axis using genetic mutations in zebrafish.

7.1 Publication 1

7.1.1 DIO zebrafish exhibit hallmarks of long-term obesity

Given that zebrafish are income breeders, upon giving excess food, they are said to invest energy in increased reproductive capacity and linear growth instead of fat storage, thus avoiding detrimental effects, such as lipotoxicity or hyperglycaemia [201]. In support of these findings, initial evidence from our lab indicated that adult zebrafish females prioritize energy allocation differently, focusing on reproduction first, followed by somatic growth and fat storage [3]. Based on this evidence, we conducted a more comprehensive investigation of diet-induced obesity in zebrafish by comparing different caloric intake conditions.

Similar to mammals, in which excess lipid deposition and lack of linear growth is seen in adipose tissue of obese individuals [245], we found that adult zebrafish showed positive allometric growth, with increased circumference rather than increased length, upon overfeeding. At 12 months, DIO fish exhibited a significant increase in subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) volumes. Additionally, lipid deposition in the hepatic cells and phenotypes of hepatic steatosis, comparable to the scarring associated with chronic obesity in mammals were observed [41, Fig 4 and fig 5]. In mammals, obesity leads to reduced core temperature, which results in a reduced metabolic rate [246]. In support of this, we observed a reduction in the routine metabolic rate (RMR) and standard metabolic

rate (SMR) in DIO fish due to the relative increase in adipose depots and decreased muscle mass. The RMR is the amount of energy required by an organism at rest to perform normal spontaneous activities, whereas the SMR is the amount of energy required to maintain healthy physiological functions [244]. However, this observation was not drastically different in comparison to the metabolic rates observed in CG fish ([41] Fig 6) . This lack of drastic difference in the metabolic rate between DIO and CG groups could be attributed to the fact that fish are poikilotherms, and the core temperature is largely controlled by the environment (water) [185], which was maintained the same across different groups under our experimental conditions. As previously shown in long-term overfed fish, reduced glucose metabolism was also observed in our DIO fish, with increased levels of *insa* (insulin a) transcripts ([41] Fig 8), suggesting that fish showed an adaptive response to overfeeding by increasing insulin production and thereby glucose resorption as opposed to impaired glucose handling, such as in type 2 diabetes mammals [247].

7.1.2 Early CR does not lead to exacerbated growth in adulthood

In order to study the importance of nutrition in regulating energy homeostasis and efficient growth in early life, in our study we calorically restricted zebrafish larvae from 5 dpf onwards, when they start to feed, and aimed to see whether undernourishment conditions during childhood, could predispose them to develop metabolic diseases later in adulthood. To our surprise, we found that CR groups did not exhibit compensatory growth in body length or weight. However, they showed catch-up mechanisms ([41] Fig 1). As discussed earlier in the 4.1.4 section, compensatory and catch-up growth are often considered as “two sides of the same coin” [40], where the former refers to above normal increase in growth rates, while the latter is growth at normal rates after a period of restricted growth [40]. Moreover, compensatory growth is associated with several harmful impacts on animal physiology, which could lead to metabolic ailments [248]. The CR fish when transferred to *ad libitum* conditions exhibited increased growth rates which only caught up to the growth rates of those in DIO conditions, and never exceeded to what we observed in their conspecific DIO groups of similar age and standard length. This lack of compensatory mechanism in fish could imply two things: Either zebrafish have a very critical time window in their embryonic life, during which changes to their nutrition could alter their growth rates or result in compensatory

growth and that this time window is even earlier than 5 dpf. Alternatively, they might not respond to food limitation with above normal growth rates at any time. According to mammalian literature, dietary restrictions in pre-term is primarily linked to compensatory growth and downstream metabolic complexities and post-birth caloric restriction with catch-up growth [249]. In support of this, the food restriction conditions we performed on 5dpf larvae, are comparable to post-birth or after weaning conditions in mammals, given that up until 5dpf, larvae feed only on the egg yolk provided by their mothers. Therefore, it would be interesting to look at the effects of maternal caloric restriction or yolk depletion on growth conditions. A recent study on partial yolk depletion in 2 dpf embryos demonstrated changes in gene expression, which are linked to metabolic syndromes [250]. Expanding these ideas to study long-term development effects in adulthood and further investigation of the neuronal pathways that sense and regulate energy during yolk deprivation are of great interest.

7.1.3 CR fish at younger ages catch-up to DIO phenotypes

The growth curves observed in CR fish upon relief of dietary restrictions, showed catch-up growth phenotypes. We observed the CR groups in the early shifts (CG1 and CG3 months) caught up with metabolic endpoints of corresponding DIO groups, whereas in the later shift at 9 months (CG9), fish never caught up to DIO9 in terms of growth and metabolic endpoints ([41] Fig 1 and fig 2). This suggests that the catch-up process could be age-dependant and hence when induced at 9 months, the older group (CG9) could not catch-up. While the CG growth curves approximated to DIO groups in younger fish, the metabolic endpoints still had significant differences between the groups. According to the ‘fat cell size hypothesis’, as seen in mammals [251], the adipose tissue first undergoes hypertrophy followed by hyperplasia, when the capacity of existing adipocytes increases [252]. In line with this, in the CG3 group, the adipocyte size caught up to DIO, but not the adipocyte number, indicating that the capacity of SAT adipocytes had increased, but the differentiation of adipocytes has not yet taken place. This suggests that CR zebrafish undergo a sequential development of metabolic phenotypes upon shift to DIO conditions. The lack of catch-up growth observed in CG9 might be due to sigmoidal growth patterns observed in fish. They exhibit an early exponential phase, followed by a linear phase and a late growth stagnation,

pointing to the differential energy allocation shift from linear growth to energy storage as fat in internal organs [253]. However, the lack of metabolic endpoints contradicts this hypothesis. It is possible that CG9 fish have not had enough time to be exposed to an obesogenic environment because fat storage is a long-term process that occurs at a much later timepoint.

Overall, in the Publication 1, we showed that zebrafish exhibit comparable phenotypes to mammals when exposed to obesogenic environment. Although our data provides new insights into catch-up growth phenotypes and lays groundwork for the future comparable studies in obesity, the high diversity between different fish strains must be kept in mind. It is important to note that, in mammals inter-individual responses have been observed when exposed to obesogenic environments [15]. As seen in case of twin studies, these variations are influenced by their genetic makeup [254]. Similar to mammals, a recent study in zebrafish showed significant differences in fasting response between two strains. The authors point out that these differences could be due to underlying genetic composition, possibly mediated by the melanocortin system, between the strains [255]. Therefore, it is important to include genetic studies which could help to elucidate the mechanisms of energy regulation.

7.2 Publication 2

Early gene discovery approaches were hypothesis-driven, which led to a set of candidate genes associated with body-weight regulation. Many of the candidate genes and signalling pathways involved in the regulation of body weight were originally found in mice. Reverse genetics approaches revealed the *ob* gene to encode Leptin (LEP), a hormone produced by adipose tissue in proportion to fat stores [254, 256]. Shortly after the cloning of *ob*, the *db* gene was cloned and identified as encoding the Leptin receptor (LEPR). Further, the complex obesity phenotype of Agouti ‘lethal yellow’, caused by a rearrangement in the Agouti gene’s promoter sequence, was also identified. These findings linked the melanocortin pathway to body weight regulation, revealing a whole set of candidate genes for obesity [254]. The melanocortin system is conserved between mammals and teleost

species. As a comparative study, we used zebrafish with mutations affecting the genes involved neuroendocrine regulation of energy homeostasis.

7.2.1 *lepr* LOF alleles abolish leptin signal transduction

Several groups have reported differences in phenotypes involving mutations in the leptin pathway in fish, with some identifying a role of Leptin in body weight regulation and some not [214, 217-219, 221, 237]. These contrary results could be due to certain alleles being hypomorphs and therefore not abolishing the function. To test this, we conducted a functional analysis on body growth phenotypes in three *lepr* mutant alleles; the *lepr*^{sa1508} allele, the *lepr*^{vu624} allele and the *lepr*^{vu625} allele and one *lepa* mutant allele, *lepa*^{vu622} in zebrafish. We further tested if the canonical pathway downstream of Lepr signalling was functional, by measuring the *socs3a* transcript levels upon intra-peritoneal recombinant mouse leptin injections, in all LOF alleles. In order to make sure the response we were seeing was specific, we performed a detailed time course and selected a timepoint where *socs3a* is significantly elevated after heterologous Leptin injections and further optimised the minimum effective dose required to elicit these responses ([238] Fig 2).

Although, we confirmed that the canonical Lepr signalling pathway is blocked by mutations in *lepr* mutant strain, it is possible that some aspects of the leptin signalling system remains intact or pre-mRNA splicing occurs, that might lead to alternative splice product, which in turn may eliminate mutations. We confirmed that none of these lines have nonsense mediated decay (NMD) of *lepr* mRNA ([257] Fig 1). The predicted structure of leptin is highly conserved among vertebrates and several studies in the past have observed comparable effects after using heterologous leptin [162, 258]. The main problem with using heterologous leptin is that it may have a different dissociation constant and greater off-target activity than the species-specific source, potentially activating an entirely different receptor system. Therefore, future studies with homologous zebrafish leptin would help identify specific role of *lepa* and *lepb* paralogs [259]. Further, functional examination of Stat3 phosphorylation levels is required to evaluate the downstream pathways disrupted due to impaired Leptin signalling in the *lepr* mutants [260].

7.2.2 Obesity phenotype were not observed in *lepa* and *lepr* mutants

As mentioned before, the background variations between the different strains among fish can also lead to differences in the growth phenotypes seen across several gene mutation studies [154]. Studying genotypes by raising them separately from each other might lead to loss of competition between WT and LOF lines and this might further result in observation of growth-related phenotypes. To test whether the differences in results across studies was caused by background genetics or competition, we raised WT and LOF crosses separately, keeping their density and access to food the same. Although in this setup, we found a reduced trend in growth differences between the genotypes, the variations among them were high and we found no consistent obesity phenotypes in LOF fish ([257] Fig 3 and fig 4). Looking at the published *lepa* and *lepr* LOF studies in detail, the variations in growth phenotypes between these studies could result from the key differences between their experimental setup. Given such high variations among fish, the breeding scheme, and the way the fish are raised during the experiment can create differences in the results. Some studies used crossbreeding of heterozygous parents and raising the offsprings in tanks of mixed genotype. However, they observed no growth phenotype [229, 230]. Another strategy adapted was breeding the wild type to mutant line and raising the offsprings in separate tanks [225, 261]. In this experimental setup, the groups saw increase in somatic growth in the mutants. Although the two setups represent different cohorts of animals, the only difference is the way the animals were raised. Since zebrafish laboratory strains are highly outbred, we discovered significant growth difference among offsprings of different parental pairs, as well as among individuals of the same clutch [262]. This contrasts with the highly inbred mouse strains in which uniform growth phenotypes are observed.

In sum, our results confirm that none of the three *lepr* mutant alleles or the *lepa* mutant allele with loss of Leptin signalling show body weight or growth phenotypes in any sort of breeding scheme.

7.3 Publication 3

In fish, so far studies have identified a role of leptin in glucose metabolism, osmotic adaption, appetite regulation and stress regulation [225, 228-230, 232, 233, 263-265]. A very few studies have focused on its role in reproduction [198, 199, 236]. Therefore, it would be interesting to study the role leptin on regulation of endocrine and paracrine systems. The absence of detrimental body weight phenotypes in Lepr LOF zebrafish, at least up to a year of age, makes it an interesting model for studying other biological roles of leptin in energy homeostasis and metabolism. Reproduction and survival are the ultimate goals of any species to ensure the continuity of their generation. However, reproduction is an energy costing event and adequate energy stores are crucial to ensure reproductive success [189]. There exists a reciprocal relationship between nutritional cues, energy intake, and metabolic indicators in every organism [155]. The energy required for reproduction is found to be higher in females than in males, due to extra investment in the care of offspring [266, 267]. Leptin is known to be an important player in the endocrine regulation of reproduction. The leptin and its receptor mutations in female mice have been shown to result in infertility. Additionally, women with leptin deficiencies either due to genetic abnormalities, eating disorders, or hyper-athleticism show irregular or absence of menstruation. This comes from the fact that leptin has a permissive role in maintaining the hypothalamic-pituitary-gonadal (HPG) axis. Apart from its central role, several studies have reported expression of *LEPR* in the ovarian support cells, indicating an additional peripheral role [129, 131, 146, 236].

7.3.1 Leptin regulates ovarian development in adult females

In fish, a lot is not yet known about the role of leptin in reproduction. There has been a long-standing debate to which extent leptin regulates oocyte development, and whether this is regulated in the brain, centrally through the HPG axis, or via direct signalling to the ovarian supportive cells [268]. In our study we investigated the role of leptin in aspects of female reproduction using Lepr LOF zebrafish. We found evidence for both central and peripheral expression of *lepr*. Centrally, in the arcuate nucleus of the hypothalamus we saw co-expression of *lepr* on the neurons expressing *pomca*. Furthermore, we found reduced gonadotropins (*fsh* and *lh*) mRNA transcript levels in the pituitary of LOF zebrafish in comparison

to their sibling wild types ([269] Fig 1). These findings point to the central role of leptin in the regulation of HPG axis in zebrafish. Peripherally, we found sub-populations of follicle cells (granulosa and theca) with *lepr* expression in adult female zebrafish ([269] Fig 4). This is in line with the mammalian data where LEPR was found to be expressed in granulosa cells of the ovary in mice [270]. In order to investigate the direct effects of leptin on ovarian development, independent of the central regulation from the hypothalamus, we used an *in-vitro* Germinal vesicle breakdown (GVBD) assay in cultured oocytes, which is a readout of oocyte maturation, treated with recombinant mouse leptin. We found the GVBD rates increased in the oocyte cultures of WT strain upon treatment with heterologous leptin over 4-16 hours, suggesting the direct role of leptin in oocyte maturation. These results were completely abolished in the LOF line ([269] Fig 5), suggesting a direct peripheral effect of leptin on the follicle cells, aiding in maturation of the oocytes. We also tested the combined effect of leptin and an oocyte-stimulating hormone,17α,20β-dihydroxy-4-pregnen-3-one (DHP). DHP is known to be secreted by the granulosa cells of the follicle upon stimulation from Lh during maturation phase [213]. We found combination of recombinant leptin and DHP exhibited additive effects to GVBD rates. Interestingly, in our oocyte culture of Lepr LOF fish, the effect of DHP was partially reduced compared to the cultures from WT fish. From these results, we can infer that leptin elicits its effects centrally in release of gonadotropins from the pituitary and peripherally on the follicle cells, by supporting the oocytes and making them more accessible to maturation and ovulation signals. However, to understand the role of leptin in interplay of central and peripheral mechanisms, further studies are required.

7.3.2 Leptin signalling enhances oocyte maturation and reduces follicular atresia

Another interesting aspect we found in *lepr*^{sa1508} mutant line was the increase in follicular atresia rates. Follicular atresia is a highly regulated process that is required to ensure maintenance of ovarian homeostasis during reproductive cycle. Although, in our study, we initially observed normal reproductive behaviour and fertility rates in *lepr* mutant females [230], upon challenging the fish by synchronizing the oocyte development, we observed that the development of oocytes in the mutants was severely impaired. As analysed by H&E staining of ovarian cross sections, we found

reduced numbers of mature oocytes and increased signs of follicular atresia in mutant fish compared to WT siblings ([269] Fig 2). This suggests that Leptin signalling is required for oocyte maturation or attenuation of follicular atresia. There might as well be an interplay of both for which leptin signalling may be essential. In support of our data, we noticed a reduction in several genes related to maturation and an upregulation in genes related to atresia in the *lepr*^{sa1508} mutants ([269] Fig 3). The analysis of cell survival rates in our GVBD assay further demonstrated that exposure to DHP and recombinant leptin reduced the rate of non-viable oocytes in oocyte cultures. However, due to degradation of RNA fragments it is difficult to further confirm the markers of follicular atresia in cultured oocytes [271]. Several time points have to be tested in order to establish an assay to mimic/induce follicular atresia in cultures. To our knowledge, this is the first evidence of the central and peripheral role of leptin in oocyte maturation and follicular atresia in zebrafish. Around the same time as our study, Tsakoumis et al. also investigated the role of leptin using a different *lepr* mutant strain *lepr*^{sa12953}. The authors showed impaired leptin signalling as a result of centrally mediated subfertility, which they were able to overcome upon administration of human chorionic gonadotropin. In contrast, we did not observe subfertility in our strain. Although, both alleles carry a point mutation in *leptin* gene on chromosome 6*,* which result in a premature stop codon, they differ in the position of the substitution. This might lead to differences in the lengths of polypeptide truncation with sa12953 allele having more truncated polypeptide, affecting the functionality more severely in the *lepr*^{sa12953} allele. Both the groups observed reduction in *lhb* transcripts in the pituitary in the mutant lines [236]. In the *lepr*^{sa1508} mutants we also observed a downregulation in *fsh* levels suggesting oocyte development and vitellogenesis might further be disrupted. However, these needs be further confirmed with future studies by measuring the Estrogen levels and analysing the vitellogenin breakdown components in the mutant oocytes via immunosorbent assays or western blots.

These results bring back the variation aspects seen in fish, suggesting that the differences in results could be due to genetic backgrounds in these lines and rearing conditions. Another possibility could be that these phenotypes depend on the nutritional status of the fish and the feeding regimes might be different between

labs [255, 272]. Future studies with correlating the nutritional status with genetic mutations may be required to answer these questions.

7.4 Publication 4

7.4.1 Agrp1 mutants show dysregulated growth and reproductive axes

The melanocortin neuroendocrine system is one of the complex signalling systems and is crucial for energy regulation in all vertebrates [273]. Genetic mutations in the genes and proteins of melanocortin pathway are often associated with obesity in humans. In our study, we examined the effects of *agrp1* mutants on growth and reproduction in *agrp1*^{vu623}mutant zebrafish. We showed that adult *agrp1* mutants had reduced expression of genes in the growth axis, such as *growth hormone* (*gh*) ([274] Fig 2), as well as genes in the reproductive axis, such as *follicle stimulating hormone* (*fsh*), *luteinising hormone* (*lh*) and *glycoprotein hormone alpha subunit* (*cga*) ([274] Fig 3). Similar to the state of fasting, this reduction helps the animal to reallocate energy to carry out activities that would help to replenish its energy stores than investing in growth and reproduction.

7.4.2 Somatic growth rates were unaltered in *agrp1* mutants

However, to our surprise, the gene expression studies did not align with growth phenotype we saw *in vivo*. In adult *agrp1* mutants we did not observe any change in somatic growth ([274] Fig 1), despite reduced *gh* expression in the pituitary. We reasoned this could be due to compensatory mechanisms independent and downstream of pituitary gene expression. Additionally, it is possible that our LOF line is a hypomorph and the predicted mutation does not result in complete loss of function. This possibility has been disproved by previous studies, where they found that mutated Agrp1 did not undergo axonal transport and instead accumulated in cell bodies [222]. From these results, it confirms that *agrp1* LOF line is *bona fide*.

7.4.3 *agrp1* mutant zebrafish show normal reproduction

We identified significant reductions in pituitary *fshb, lhb and cga* gene expression in *agrp1* mutants. This reduction in reproductive gene expression suggests that Agrp1 plays a role in reproductive function. Surprisingly, the LOF fish showed normal reproduction ([274] Fig 5). In mammals, AgRP has been shown to act upstream of

gonadotropin-releasing hormone (GnRH) [94, 127]. Previous studies in zebrafish have shown that mutations in the *gnrh* isoforms, do not exhibit any major disruptions in reproductive function and behaviour, despite subtle changes in the genes along the reproductive axis [198-200]. The authors point out non-cell-autonomous compensatory changes as a reason for the absence of phenotypes in these fish. It is important to note that, both *gnrh2* and *gnrh2/gnrh3* double knockout lines showed reduction is *agrp* expression levels. Further, considering our learning from the Lepr LOF line, where we saw disruption in oocyte maturation upon synchronization of the oocyte development, we performed similar synchronization experiments in Agrp1 LOF fish. We did see obvious differences in oocyte development between Agrp1 LOF and WT. To our surprise, as opposed to impairment in oocyte development, in some Agrp1 LOF fish, we observed increased maturation rates. This was also supported by some fish exhibiting increased mating success in Agrp1 LOF fish. These results suggest some unknown compensatory mechanisms, both at central and peripheral level allow for normal reproductive function in Agrp1 LOF, despite changes in expression levels of the genes regulating reproduction.

In zebrafish, genome editing techniques have revealed the role of *fsh* in gonadal development and *lh* in maturation and ovulation. Consequently, *fsh* LOF leads to delayed ovarian development, but not in subfertility and *lh* LOF results in normal gonadal development, but failure to spawn [275]. Following such successful studies, in an attempt to elucidate the role of other players in the endocrine axis, several studies have looked at genetic manipulations in the teleost neuroendocrine systems, particularly with focus on reproduction. However, a large number of these knockouts have been less successful in blocking the reproduction function, despite dysregulated expression levels in the reproductive axis or exhibiting phenotypes of reduced fertility. One such example is the *gnrh* mutant lines from Zohar's lab [193, 198-200]. Although the Gnrh3 mutants made from TALEN based mutagenesis, in larval stage showed alterations in *fshb*, *lhb* and *cga*, they showed normal reproduction by adulthood. Accounting for the possible compensation by Gnrh2 system for the lack of effect observed in Gnrh3 knockout, they created Gnrh3/Gnhr2 double knockouts, which still remained fertile [198, 199]. Another study evaluated the role of kisspeptin in teleost reproduction, as it is also a key player in mammalian reproduction. Knockout studies in kisspeptin 1 and kisspeptin 2 showed that

spermatogenesis, folliculogenesis, as well as reproductive capacity was not impaired in these fish, suggesting they may not be dispensable for reproduction or may be acting via non-Gnrh system in teleost species [276, 277].

These differences suggest that the neuronal circuitry controlling reproduction may be very different in fishes compared to mammals. The hypothalamo-hypophysial portal systems, typical of mammals has been lost over the years of evolution in teleost species. Instead, the neurons of the hypophysiotropic system end at the anterior pituitary cells, which allow for the autonomous regulation of gonadotrophs by multiple neuropeptides [197]. However, it remains largely unknown which of these neuropeptides are essential for reproduction and growth regulation and which are evolutionarily conserved [278]. In addition, the absence of abnormalities in these gene knockout and gene knockdown investigations suggests that the teleost neuropeptide system contains a compensating network [279]. Often, the upregulation of other associated genes in the network following a knockout is viewed as a direct result of the loss of protein function [197]. Although evidence is still lacking to confirm this, genetic modification studies of multiple neuropeptide genes in the neuroendocrine axis would be necessary to elucidate this compensatory role.

8 CONCLUSION

The overarching goal of the thesis was to examine the aspects of energy intake and energy consumption using various systematic and functional analysis methods. Any imbalance in energy intake or output is known to lead to serious metabolic disorders. With obesity and other metabolic comorbidities on the rise worldwide, understanding the factors that contribute to these diseases is crucial in order to mitigate them. Here we propose zebrafish as a model organism to study the effects of a long-term obese environment on the development of obesity and other related metabolic endpoints. Furthermore, the functional role of the leptin and melanocortin system on somatic growth and reproduction was explored using genetic mutation studies.

The results suggest that DIO in zebrafish have characteristics of obesity and related metabolic endpoints as observed in mammals. In contrast, zebrafish exposed to CR in early life do not show enhanced growth or compensatory phenotypes in adulthood after relief of dietary restriction. In contrast to mammals, we observed growth at a normal rate, i. e. catching-up growth phenotypes rather than compensatory growth. For future studies, characterizing the neuronal signalling pathway involved in the transmission of these mechanisms would be crucial to gain a comprehensive understanding of energy allocation in zebrafish.

Next, in an attempt to define the role of peripheral hormones and the central mediators in regulating energy expenditure, particularly in growth and reproduction, we investigated the *lepr* and *lepa* mutant zebrafish lines. Our results showed that Lepa or Lepr LOF does not result in increased somatic growth despite loss of Leptin signalling in these alleles. Although we have validated these LOF lines and shown impaired *socs3* signalling at the transcriptional level as a readout for loss of Leptin signalling, functional analysis of *Stat3* phosphorylation at the translational level would provide further strong evidence confirming the loss of Lepa or Lepr function. Furthermore, the development of recombinant zebrafish leptin would give us more insight into the direct effects of leptin and limit any off-target effects that may result from heterologous leptin.

Considering that loss of Lepr function does not lead to aberrant obesity phenotypes in zebrafish, we further investigated its role in reproduction. We found a central dysregulation of gonadotropins, *fsh* and *lh* in the *lepr* LOF fish. Upon synchronization of oocyte development, we observed delayed maturation rates and increased follicular atresia in mutants compared to their wild-type siblings. This was supported by the downregulated maturational gene transcripts and upregulated atretic markers. Most strikingly, we found Lepr localization on subpopulations of follicular cells and confirmed that leptin also plays a direct and a peripheral role on the ovarian cells using an in vitro GVBD assay. However, it is still unknown which exact cell types of the follicular cells express leptin and what mode of action they have. This could shed light on the paracrine function of leptin. A comprehensive single-cell transcriptome analysis of ovarian cell types during different stages of oogenesis can help us understand the molecular pathways regulated by leptin during oogenesis in zebrafish.

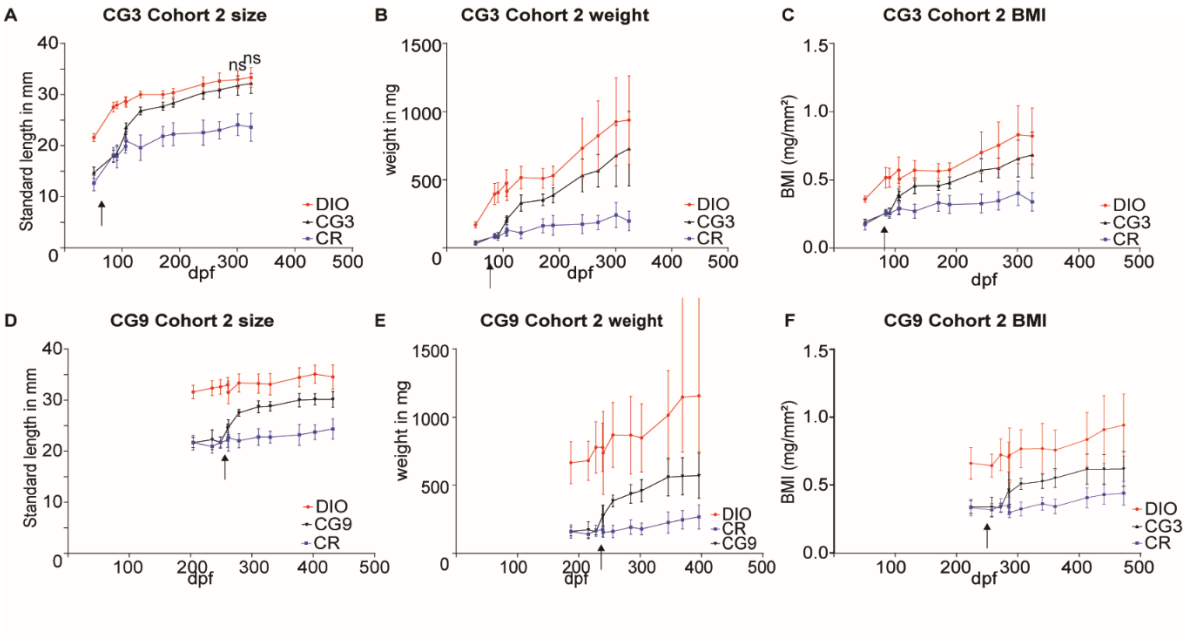
In the last part, we investigated the central role of the melanocortin system in regulating the HPG axis, specifically growth and reproduction in zebrafish. Interestingly, although Agrp1 LOF fish showed dysregulated gene expression of growth and reproductive axes, they did not show changes in somatic growth rates or reproductive potential. These findings imply that there may be other possible unknown compensatory pathways that can balance out the lack of Agrp.

Overall, the work described in this dissertation characterized the effect of long-term obesogenic diets on the development of obesity and other metabolic endpoints, as well as the effects of early life caloric restriction on catch-up growth. Further, it elucidated the role of Leptin and Agouti-related protein in the regulation of somatic growth and reproduction in adult zebrafish. This thesis serves as a basis for future studies on the endocrine regulation of growth and reproduction. Furthermore, the investigations gathered here lay the ground for future comparative studies across teleost and vertebrates.

9 SUPPLEMENT

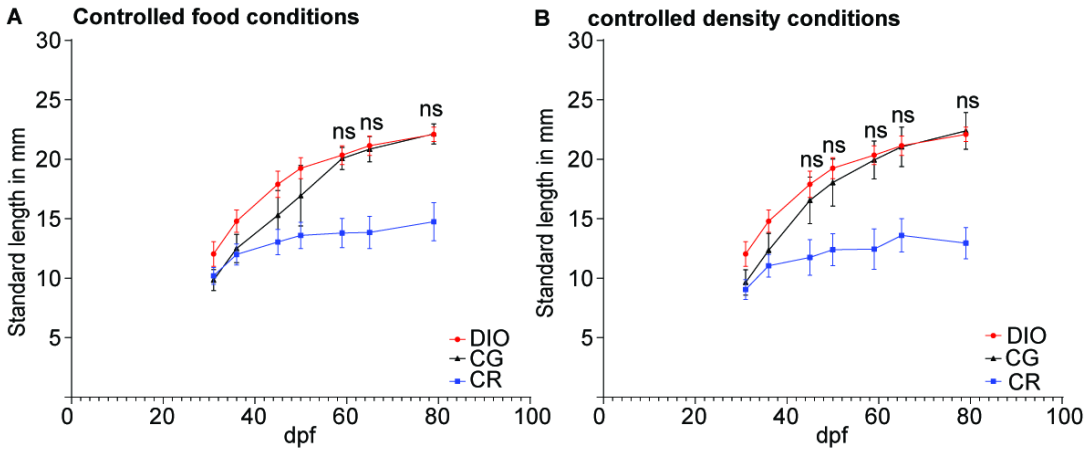
9.1 Supplement material SM1

1. S1 Fig. Compensatory growth can be induced by excessive feeding following caloric restriction: Cohort 2



Supplementary figure 1: (A, D) standard length; (B, E) fish weight and (C, F) body mass index of fish undergoing CG3 (A, B, C) of CG 9 (D, E, F); error bars indicate STDEV, ns indicates a lack of significant difference between the CG and the DIO group at that timepoint as indicated by a 2-Way ANOVA followed by Tukey's multiple comparison test.

2. S2 Fig. Compensatory growth (CG) can be induced by changes in feeding conditions and densities as well as with changes in feeding conditions only.



Supplementary figure 2: (A,B) Growth curves of fish kept at different feeding conditions and densities; (A) Body lengths of fish raised in low density (5 fish per tank) but different feeding regimes; fish raised with *ad libidum* feeding conditions which is able to induce diet-induced obesity (DIO) show significantly increased linear growth compared to fish raised under caloric restriction (CR); fish raised under caloric restriction before 1 month of age but with ad libitum conditions afterwards (CG) show briefly increased growth rates and compensate for differences in body length suggesting that these fish exhibit compensatory growth (CG); (B) Body lengths of fish raised with different densities while every tank received the same amount of food resulting in different feeding conditions; fish raised in low density (5 fish per tank) with *ad libidum* feeding conditions (DIO) show significantly increased linear growth compared to fish raised in high density (50 fish per tank) and therefore under caloric restriction (CR); fish raised in high density (50 fish/tank, caloric restriction) before 1 month of age but in low density (5 fish/tank, ad libitum conditions) afterwards (CG) show briefly increased growth rates suggesting that these fish exhibit CG; n = 10 for each condition; error bars indicate STDEV, ns indicates a lack of significant difference between the CG and the DIO group at that timepoint as indicated by a 2-Way ANOVA followed by a multiple comparison test

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12 ERKLÄRUNG ZUR DISSERTATION

gemäß der Promotionsordnung vom 12. März 2020

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Teilpublikationen:

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