COMPARATIVE STUDIES OF ENERGY HOMEOSTASIS IN VERTEBRATES

EDITED BY: Maximilian Michel
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COMPARATIVE STUDIES OF ENERGY HOMEOSTASIS IN VERTEBRATES

Topic Editor:
Maximilian Michel, University of Cologne, Germany

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A brief glimpse into new insight driving the comparative biology of energy homeostasis in vertebrates with a focus on non-mammalian vertebrates. What are the key conserved mechanisms and what aspects of feeding behavior and energy allocation are different between species?

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Table of Contents

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EDITORIAL
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Editorial: Comparative Studies of Energy Homeostasis in Vertebrates

*Maximilian Michel**

Institute of Zoology, University of Cologne, Cologne, Germany

Keywords: energy homeostasis, melanocortins, leptin, food intake regulation, energy expenditure

Editorial on the Research Topic

Comparative Studies of Energy Homeostasis in Vertebrates

Energy homeostasis of an organism is the sum of processes integrating energy intake with resource allocation. Its central control mechanisms are essential to an animal's life history and govern daily activity, such as searching for food, reproduction, etc. The first major inroads into our understanding of the genetic basis of energy homeostasis were made by the fortuitous finding of five monogenic obesity strains in mouse breeding experiments (1) *obese*, (2) *diabetes*, (3) *agouti*, (4) *fat*, and (5) *tubby* (1). The genes underlying these strains were eventually cloned, laying the groundwork for the analysis of the neural circuitry which underlies energy homeostasis in mice [for recent reviews, please see Ref. (2–4)]. This research quickly led to the discovery of the core genes involved in energy homeostasis in obese human patients such as the leptin system (5, 6) which is mutated in the *obese* and *diabetes* mouse strains or the melanocortin 4 receptor system (7, 8) which is impacted in the *agouti* mouse strain. By and by, the key genes involved in mammalian energy homeostasis were cloned and found to be conserved not only across mammals but also across all vertebrates. However, while the genes are conserved, the anatomical and functional data on these genes varies between species from comparable to variable. In contrast, research into the biochemical basis of energy acquisition and utilization showed clearly that large swaths of the underlying building blocks are highly conserved (9). It follows that the basic building blocks which play a role in energy homeostasis are largely conserved, for example, lipid handling by adipose tissue or glucose handling by the liver. In contrast, the central regulation of these processes differs between species. An argument can be made that these differences in regulation are likely dependent on the ecological niche of an individual species [see for example, Ronnestad et al. in this issue]. Understanding what exactly constitutes a conserved building block in energy homeostasis and how these are centrally regulated to allow an animal to exist in a given ecological niche will allow us to (1) gain insight into the evolution of biological diversity; (2) modulate growth behavior in farm animals (domestication as an ecological niche); and (3) ameliorate abnormal homeostasis patterns in humans (from metabolic syndrome on one extreme to anorexia and cancer cachexia on the other). This research topic comprises reviews and a research article focused on recent insight. Of note—this topic is complemented by a concurrent research topic on the "Neuroendocrine Control of Feeding Behavior" (10), which has a strong emphasis on mammalian systems.

The first set of contributions covers lipid and glucose handling in zebrafish. The first review by Steven Farber's group (Quinlivan and Farber) introduces the mechanisms governing embryonic and larval lipid dynamics in zebrafish around the time of the transition from egg yolk feeding to prey feeding. The authors highlight that the gut is colonized by microbiota following the animals first feeding. They show that during this time, gut microbiota has an immense impact on host absorptive processes. Finally, the authors point out the benefits of the zebrafish model system to carefully study the dynamics of lipid absorption and processing (Quinlivan and Farber). Amnon Schlegel (Schlegel) focuses the discussion on lipoprotein biology. He argues for the use of zebrafish as a model system,

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Edited by:
Cunming Duan, University of Michigan, United States

Reviewed by:
Chunyang Zhang, Harvard Medical School, United States

Correspondence:
Maximilian Michel
maximichel@gmail.com

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since cholesteryl ester transfer proteins, which predispose people as well as zebrafish to atherogenic lipoprotein profiles, are not conserved in rodents. This allows for the study of plaque formation in order to identify dyslipidemia and atherosclerosis modifiers—both chemical and genetic (Schlegel). Staying on the topic of adipose biology, James Minchin's group (Wafer et al.) reviews adipogenesis, in particular its master regulator peroxisome proliferator-activated receptor gamma (PPARG) in fish compared to mammals. Not only is this gene highly conserved, but surprisingly, the authors find that only a single ortholog is present across fish species which suggests a critical role in adipogenesis. Astonishingly, while target genes of PPARG seem to be conserved, ligands are more diverse. This suggests that specifically control of adipose tissue evolved but not the role it plays within the organism (Wafer et al.). Veering away from the control of lipid levels, the next contribution focuses on the control of systemic glucose levels by the pancreatic endocrine cells (Maddison and Chen). The authors showcase the utility of the zebrafish as a model system for beta cell proliferation, differentiation, and regeneration (Maddison and Chen). Taken together, these four contributions argue not only for the utility of the zebrafish as a model system but also that the basic building blocks underlying energy homeostasis are fundamentally conserved across vertebrates.

Conde-Sieira and Soengas expand this in their review on peripheral nutrient sensing systems. Importantly, the authors show that the basic glucosensory and fatty acid sensory mechanisms are conserved from different fish species to mammals. However, they see species-specific differences by how central regulation of energy homeostasis is achieved (Conde-Sieira and Soengas). The topic of central mechanisms is further developed in a paper in the concurrent research topic written by Delgado et al., where the authors specifically look at the hypothalamic integration of signals from glucose and fatty acid sensing systems as well as integration of circadian information and the stress axis to achieve food intake regulation (Delgado et al.). Boswell and Dunn reviewed the central melanocortin system in birds and present data for the conservation of the key aspects of central melanocortin action. Interestingly, the authors point to a little understood connection between the melanocortin system, growth, and food intake in birds (Boswell and Dunn), This effect, also seen in platyfish (11), mice and humans and may be mediated *via* the somatostatin axis (12). Hélène Volkoff in her contribution summarizes what we know to date on the impact of different endocrine systems in fish. While most mammalian peptides and hormones either have an effect on fish metabolic state (when injected) and/or are regulated by metabolic state, the phenotype strongly varies between species. This again suggests that the building blocks supporting metabolic state are intact and conserved but the control mechanisms vary between species. The author points out that this is possibly not all too surprising given the widespread adaptations necessary for more than 30,000 species of fish to find ecological niches (Volkoff). This aspect is further developed by Ronnestad et al., where the authors bring up examples of central control differences in fish with vastly different life history and ecological niches. Specifically, the authors review metabolic control in long-term fasting species (the arctic charr, mouthbrooders, and fasting in aquaculture), throughout life transitions (first feeding to juvenile), in voracious feeders, and in cave-living fish (Ronnestad et al.). Ben Renquist's group introduces metabolic rate in fish, an essential underlying principle in energy homeostasis. The authors concentrate on variations in proton leak, calcium cycling, and membrane potential and relate this to differences in habitat and ecological niche of the investigated species. They point out that small changes in genes involved in ion gradients and their regulation can correlate with critical biological differences and advantages to the species—allowing the organism to adapt to particularly extreme environmental conditions by changes in metabolic rate (Geisler et al.). The subsequent contribution by Marnix Gorissen (van de Pol et al.) further develops the concept of life history, focusing on what is required for an organism to live and survive in an aquatic niche. With a keen eye on the developments in the mammalian literature, the authors look at insulin and leptin biology in fish, from appetite regulation *via* sexual maturation, salt water adaptation, and glucose/adipose homeostasis to stress (van de Pol et al.).

The next set of reviews focus on mechanisms specifically controlled by leptin signaling. Russell Borski's group (Deck et al.) starts with an overview of leptin structure and points out that leptin has an anorexigenic effect throughout vertebrates. However, this overall effect is mediated by recruiting different building blocks in different species—mobilization of lipid or glucose stores. The authors then look at the mobilization of energy after osmotic stress, hypoxia, disease, and fasting in fish (Deck et al.). Richard Londraville's contribution (Londraville et al.) introduces the recent discovery of leptin in birds, where evidence suggests that leptin may not have an endocrine but rather may have an autocrine/paracrine function. The authors embark on an up to-date analysis of leptin receptor and endospanin (an overlapping transcript with modulatory function) evolution and structure, probing databanks based on recent structural insight. This insight can be used to inform future research into leptin function (Londraville et al.). The last contribution in this leptin-specific set of reviews is an original research article from the Denver lab which provides evidence on a scarcely studied aspect of leptin biology: leptin's role during development (Bender et al.). In *xenopus*, the authors show that leptin levels (secreted by adipose tissue) rise just prior to metamorphosis and stay elevated throughout metamorphosis. They find evidence that leptin mediates cell proliferation and conduct a microarray study which provides a basis for further investigation of leptin's role and regulation of neurogenesis (Bender et al.). These four articles provide a good overview on what is known about non-mammalian leptin biology and highlight the complex nature of this hormone more than 50 years after the identification of the first leptin phenotypes (13).

The last contribution forms a bridge between nonmammalian energy homeostasis research with the mammalian realm. Here, Stewart Nicol discusses energy homeostasis in monotremes (Nicol). Similar to evidence from birds and fish, leptin does not appear to play a role in adipostasis in monotremes. The author further looks at special life history adaptations, such as seasonality, hibernation, and torpor (Nicol). This is of particular note in relation to the contribution of Ronnestad et al. who discuss the role of seasonality in the arctic charr. An organism's life history

needs to be taken into account when translating insight gained from one organism (for example, a fish or a mouse) to another (such as humans). It is imperative to keep Krogh's principle of comparative biology in mind (for each problem there will be an animal where it can be most conveniently studied) and the harvest this has brought to our understanding of biology. While historical examples range from action potentials in squid *via* learning in mollusks or the shark rectal gland; relevant examples in the context here could be the study of: (1) metabolic control in voracious feeders and seasonal animals; (2) the thermogenic role of muscle in birds lacking brown adipose; (3) the role of the melanocortin system in body growth in platyfish size morphs and birds; (4) metabolic control of migrating animals (eels, salmon, birds). These contributions clearly show the variety of mechanisms which a single regulatory hormone can control. However, these reviews also point out that (a) a hormone can have one general role (such as the anorexigenic effect of leptin) throughout all vertebrates, while recruiting different building blocks to achieve this effect and (b) that different building blocks (such as control of adipose through PPARG) can be centrally controlled by different ligands depending on the species and likely its ecological niche.

AUTHOR CONTRIBUTIONS

The author listed has made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Lipid Uptake, Metabolism, and Transport in the Larval Zebrafish

*Vanessa H. Quinlivan[1,2] and Steven A. Farber[1,2]**

[1] Carnegie Institution for Science (CIS), Baltimore, MD, United States, [2] The Johns Hopkins University, Baltimore, MD, United States

The developing zebrafish is a well-established model system for studies of energy metabolism, and is amenable to genetic, physiological, and biochemical approaches. For the first 5 days of life, nutrients are absorbed from its endogenous maternally deposited yolk. At 5 days post-fertilization, the yolk is exhausted and the larva has a functional digestive system including intestine, liver, gallbladder, pancreas, and intestinal microbiota. The transparency of the larval zebrafish, and the genetic and physiological similarity of its digestive system to that of mammals make it a promising system in which to address questions of energy homeostasis relevant to human health. For example, apolipoprotein expression and function is similar in zebrafish and mammals, and transgenic animals may be used to examine both the transport of lipid from yolk to body in the embryo, and the trafficking of dietary lipids in the larva. Additionally, despite the identification of many fatty acid and lipid transport proteins expressed by vertebrates, the cell biological processes that mediate the transport of dietary lipids from the intestinal lumen to the interior of enterocytes remain to be elucidated. Genetic tractability and amenability to live imaging and a range of biochemical methods make the larval zebrafish an ideal model in which to address open questions in the field of lipid transport, energy homeostasis, and nutrient metabolism.

Keywords: lipid metabolism, zebrafish, lipoproteins, comparative physiology, enterocytes

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Edited by:
Maximilian Michel, University of Michigan, United States

Reviewed by:
Mingyu Li, Vanderbilt University, United States
Satoshi Ogawa, Monash University Malaysia, Malaysia
James Minchin, University of Edinburgh, United Kingdom

***Correspondence:**
Steven A. Farber
farber@carnegiescience.edu

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INTRODUCTION

The developing digestive system of the embryonic and larval zebrafish is a well-established model system for the study of vertebrate gastrointestinal physiology and metabolism. Metabolic and regulatory pathways for gastrointestinal system development, intestinal and liver cell differentiation, digestion, and nutrient uptake and transport are highly conserved between zebrafish and humans (1–7). The functional regionalization of the intestine also appears to be conserved among vertebrates including zebrafish with respect to transcription factor expression in epithelial cells over the length of the intestine (8). Additionally, the transparency of the developing larva makes it ideal for live imaging experiments: The larval zebrafish has a functional and visible liver, pancreas, gallbladder, intestine, and intestinal microbiota by 5 days post-fertilization (dpf) when it begins to feed. The zebrafish is also suitable for large-scale and high-throughput experiments due to its small size and high fertility (a single pair can produce hundreds of embryos in a day). Finally, as the importance of the gut microbiome to studies of nutritional physiology is becoming increasingly clear; the larval zebrafish microbiota are well-characterized, and germ-free and gnotobiotic models are available (9).

The zebrafish zygote contains a large yolk cell which is absorbed over the first 5 days of life and supplies the developing embryo with nutrients. The yolk consists of a lipid and protein rich core with a cellular syncytium at its periphery, called the yolk syncytial layer (YSL). The YSL exports amino acids, hydrolyzes complex lipids to release fatty acids, and synthesizes lipoproteins, which export lipid to the developing embryo until it is able to feed independently (10). The intestine of the larval zebrafish is open at both ends and ready to absorb exogenous food at 5 dpf, though the non-enterocyte secretory cell populations do not differentiate until later larval stages (11). Once the intestinal tract is open, the gut microbiota are acquired from the media. At this time, colonization occurs essentially immediately and is maintained throughout life with the main source of variation in bacterial community composition being changes in diet (12).

Both the embryonic and larval zebrafish are valuable models of lipid uptake and trafficking, respectively, from the yolk cell and the diet. This review encompasses the roles of lipid remodeling, lipoproteins, intestinal lipid transport proteins, and the gut microbiota in lipid processing during zebrafish development.

YOLK LIPID UPTAKE IN THE EMBRYONIC AND LARVAL ZEBRAFISH

Lipoproteins Transport Yolk Lipids to the Body of the Developing Zebrafish Embryo

The majority of the mass of a zebrafish zygote consists of the yolk, a lipid-rich structure that is gradually depleted by transport of its contents to the embryo as it develops into a free-feeding larva. Yolk lipids are packaged into lipoproteins in the YSL before being exported to the body of the developing zebrafish. Lipoproteins are lipid-transporting structures consisting of a neutral lipid interior bounded by a phospholipid (PL) and cholesterol monolayer, carrying one or more apolipoproteins. Apolipoproteins mediate interactions among lipoproteins, cellular receptors, and lipid-processing enzymes. The zebrafish genome contains analogs of every major human apolipoprotein, but there are some differences in patterns of expression and function. Due to the teleost genome duplication, zebrafish have multiple paralogs of each apolipoprotein gene. Human lipid metabolism genes with corresponding zebrafish paralogs discussed in this review are summarized in **Table 1**. There are 11 apolipoprotein genes in the *apoB*, *apoA-IV*, *apoE*, and *apoA-I* families, and all are expressed in the YSL (13) (**Figure 1**). Whole-mount *in situ* hybridization reveals that expression of some apolipoprotein genes is localized to subregions of the YSL, suggesting a previously uncharacterized compartmentalization of this structure. For example, mRNA encoding *apoA-IV* appears to be specific to the yolk extension at earlier stages (though different paralogs in this family are concentrated here at different points in development), while members of the other apolipoprotein families are expressed more evenly throughout the YSL (13). The significance of these potential YSL subdomains has yet to be described, but it is possible that there is a relationship to the regionalization of the developing intestine.

TABLE 1 | Zebrafish express multiple paralogs of human lipid metabolism genes.

Product class	Human gene	Zebrafish gene (paralogs)	Expression in digestive tissues
Apolipoproteins	*apoA-I*	*apoA-Ia*	Yolk syncytial layer (YSL), larval intestine
		apoA-Ib	YSL, larval intestine, and liver
	apoA-II	*apoA-II*	YSL (14), larval, and adult liver (15)
	apoA-IV	*apoA-IVa*	YSL(extension), larval intestine
		apoA-IVb.1	YSL(extension), larval intestine
		apoA-IVb.2	YSL(extension), larval intestine
		apoA-IVb.3	YSL, larval intestine and liver
	apoB	*apoBa*	YSL, larval liver
		apoBb.1	YSL, larval intestine and liver
		apoBb.2	YSL, larval liver
	apoC1	*apoC1*	Larval and adult liver (15)
	apoC2	*apoC2*	Larval and adult liver (15)
	apoE	*apoEa*	YSL(extension), larval intestine
		apoEb	YSL, larval intestine
Cholesterol transporter	*npc1l1*	*npc1l1*	Adult intestine and liver (16)
Fatty acid transporters	*fatp3/ acsvl3*	*fatp3/slc27a3*	
	fatp1/ acsvl5	*slc27a1a*	
		slc27a1b	
	fatp2/ acsvl1	*slc27a2a*	Adult liver
		slc27a2b	
	fatp4/ acsvl4	*slc27a4*	Anterior larval intestine
	fatp6/ acsvl2	*slc27a6*	
	cav1	*cav1*	Basal border of enterocytes
	cd36	*cd36*	YSL, larval intestine (17)
Long-chain Acyl-CoA synthetases	*acsl1*	*acsl1a*	Adult liver and intestine
		acsl1b	YSL, larval gut
	acsl2	*acsl2*	Adult liver
	acsl3	*acsl3a*	Adult liver and intestine
		acsl3b	Adult liver and intestine
	acsl4	*acsl4a*	YSL, larval gut, adult gut
		acsl4b	YSL, adult liver and intestine
	acsl5	*acsl5*	YSL, adult liver and intestine
	acsl6	*acsl6*	Adult liver and intestine

Although the expression of apolipoprotein genes in the developing embryo and larva has been thoroughly characterized, the lipoprotein profile at these stages is less well defined. Most work on fish lipoproteins has focused on adults, likely due to the

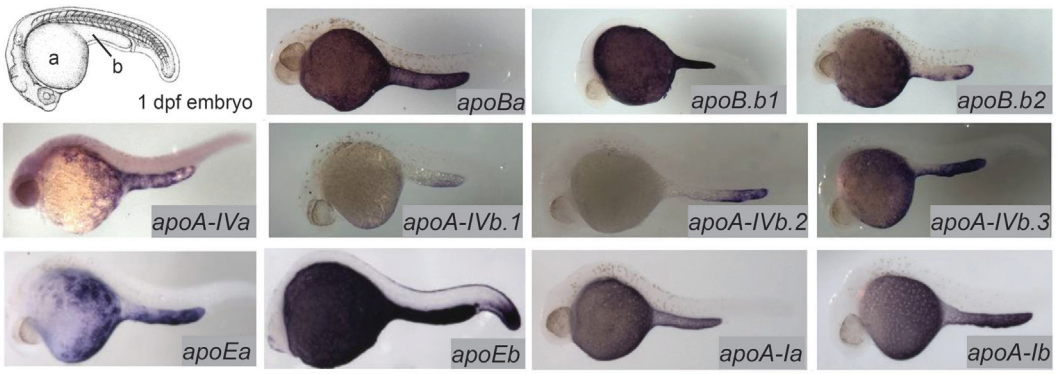

FIGURE 1 | Zebrafish apolipoprotein genes are expressed in the yolk syncytial layer (YSL). The developing zebrafish embryo gradually absorbs lipids from its yolk (a), which is surrounded by the YSL. At 1–5 dpf, the yolk ball is lengthened along the tail of the embryo forming the yolk extension (b). *In situ* hybridization reveals expression of all 11 zebrafish apolipoprotein genes in the *apoB*, *apoA-IV*, *apoE*, and *apoA-I* families in the YSL at 1 day post-fertilization. Adapted and reprinted from Miyares et al. (18), and Otis et al. (13), under a CC-BY license.

difficulty of obtaining adequate blood samples from larvae (19). Secretion of very low-density lipoprotein (VLDL) particles from the yolk has been demonstrated by electron microscopy (20, 21). The YSL also expresses *apoA-I* and *apoA-II*, which are found in HDL (high-density lipoprotein) particles and chylomicrons but not LDL (low-density lipoprotein) or VLDL (13, 14). ApoB, which is a component of chylomicrons, LDL, and VLDL, has a vital role in the export of yolk lipids. Microsomal triglyceride (TG) transfer protein (MTP) packages TGs into ApoB-containing lipoproteins. ApoB is degraded if it is not associated with lipid so, in the absence of MTP function, ApoB is not functional (22). In $mtp^{-/-}$ mutant zebrafish larvae, lipids are trapped in the yolk (characterized by retention of yolk volume, an increase in yolk opacity, and a reduction in neutral lipid in the body) and larvae do not survive beyond 5 days (23). Additionally, unlike their wild-type siblings, $mtp^{-/-}$ embryos retain fluorescent fatty acid injected into the yolk and do not export it or its fluorescent products to the circulation (18). The ability of $mtp^{-/-}$ larvae to grow and survive to 5 dpf suggests that some lipid must be transported out of the yolk in order for membranes to be synthesized, possibly through the synthesis of HDL-like particles that contain ApoA-I and do not require MTP for their assembly.

Lipid Composition of the Embryo Changes over the Course of Yolk Absorption

According to a recently published developmental study of lipid composition performed by liquid chromatography-mass spectrometry (LC-MS), at the time of fertilization, embryo lipids are approximately 40% cholesterol, 35% PL, and 9% TG, with less abundant species, including mono- and di-glycerides, cholesterol esters (CE), ceramides, and lysophospholipids, making up the remainder (24). Over the first 5 days of life, a linear decrease in the molar amount of most lipid species is observed in the yolk with a corresponding increase in the embryonic/larval body. Some exceptions have been observed: TG in the body remains consistently low as it is depleted from the yolk, suggesting that yolk TGs are primarily broken down and either oxidized for energy or resynthesized into other lipid products. Interestingly, CE, the other "energy storage" lipid class, is exchanged evenly from the yolk to the body during this period of development with the total amount remaining the same (24). Cholesterol synthesis in animal cells is tightly controlled in response to the cholesterol content of membranes *via* regulation of HMG-CoA reductase expression, and esterification is a major mechanism by which excess cholesterol is neutralized (25). One possible reason that CE is not depleted during the lecithotrophic (yolk-feeding) period of development is that breaking down CE for fatty acid oxidation would result in an overabundance of cholesterol. Favoring glycerolipids as an early energy source, therefore, would be important for cholesterol homeostasis, while CE from the yolk could be repackaged into intracellular lipid droplets for later oxidation or storage in adipocytes. Free cholesterol in the yolk and the body decrease and increase, respectively, at the same apparent rate between 24 h and 5 days of development, but the cholesterol content of the body at 5 dpf is less than the initial amount in the yolk (24). It is likely that this portion of the cholesterol is directed to synthesis of steroid hormones and bile, though these compounds were not measured in this study.

Phospholipid dynamics in the developing embryo also appear to be more complex than simple yolk to body trafficking: while other PL classes seem to move gradually from the yolk to the body, phosphatidylcholine (PC) levels in the yolk increase over the first 24 h, then decrease over the next 4 days while remaining relatively constant in the body (24). Though the specific lipid composition of zebrafish embryonic lipoproteins has not been investigated, one possible explanation is that the initial increase in PC goes to building the outer monolayer on lipoproteins exported from the yolk. It is possible that when this lipoprotein-associated PC reaches the body, it is in excess and is either oxidized or remodeled.

Although Fraher and colleagues' published analysis of their LC-MS data set was limited to discussion of developmental changes in lipid classes, quantitation of all individual lipid species was published as a supplement to the manuscript. These data provide an opportunity to examine the changes in individual lipid

species that occur during the first 5 days of zebrafish development. For example, the major PL classes are defined by head group (e.g., PCs, phosphatidylethanolamines, phosphatidylserines, etc.), but each of these classes comprises thousands of different molecules with different types of fatty acid "tails." Modern mass spectrometry technologies optimized for lipidomics can differentiate between individual lipid species at this level of resolution because they can precisely determine the mass to charge ratio (*m/z*) of each analyte in a mixture and because they employ a second step in which the molecules are fragmented and the subsequent *m/z* values of these fragments are also determined. Complex lipids such as PLs are identified using *m/z* values calculated from molecular formulas and expected fragmentation patterns, and are annotated in Fraher's supplemental data and other lipidomics data sets as "Head Group (FA 1/FA 2)." The most abundant PL in animal cell membranes, for example, is PC with the saturated 16-carbon fatty acid palmitate and the monounsaturated 18-carbon fatty acid oleate and is annotated as PC(16:0/18:1). When the specific fatty acid composition of a complex lipid cannot be determined, only the total fatty acid carbon chain length and number of unsaturated carbon–carbon bonds is given [e.g., PC(34:1)].

When trends in the amounts of individual lipids in Fraher's data set are examined, results suggest that changes in the PL profile are consistent with an increase in membrane PL in the larval body that is expected to occur with increasing growth. However, the trends in total amounts of PL present in the yolk and body are skewed by changes in individual PL species. Specifically, PC(18:2/20:4) is a major PL in the body at the start of development and shows a large decrease by 5 dpf. However, the expected major PC components of cell membranes including PC(34:1) and other PCs with total chain lengths in the low 30s increase over the course of larval development as expected. It is possible that longer-chain PLs predominate in lipoproteins but are a minor species in cell membranes, a model supported by a large increase in the amount of PC(18:2/20:4) in the yolk over the course of development (this species is the only PL in the yolk whose total molar amount increases over 1–5 days, though other PL species increase in the yolk in terms of percentage of total lipid). PLs containing the fatty acid arachidonic acid (20:4) are the precursor of eicosanoids, a class of signaling molecules with roles in regulating inflammation, vascular physiology, and stem cell activity (26, 27).

This finding suggests eicosanoids as an important area of interest in the ongoing characterization of yolk utilization in the zebrafish. Although the physiological implications of changes in individual lipids were not within the scope of this published work, the rich MS data set that was produced highlights the importance of examining behavior of individual lipids in studies of metabolism and transport.

Complex Lipid Synthesis and Remodeling Occurs in the Embryonic and Larval Zebrafish Yolk

The embryonic and larval zebrafish yolk is metabolically active not just in lipid transport, but also in the synthesis and remodeling of complex lipids, as was demonstrated through the injection of radioactive and fluorescently labeled lipids into the larval yolk followed by thin layer chromatography (TLC) analysis of the products of these metabolic tracers (18). Fatty acids labeled with BODIPY-FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene; a green fluorescent small molecule tag) or radioactive fatty acids injected into the yolk of 3 dpf larval zebrafish were both metabolized into complex lipids including PL, CE, and TG and transported throughout the developing body. Furthermore, injection of radioactive oleate showed that the yolk synthesizes complex lipids at the earliest stages of development, as radioactive TG and PL products were found in embryos injected as early as 0.75 hours post-fertilization (hpf). While the rate of incorporation of radioactive oleate into each PL class was consistent in embryos and larvae aged 0.75–3 dpf, larvae injected at 3 dpf were the only group to synthesize labeled CE, and there was a large increase in the amount of radioactive TG at later stages as well (18). When BODIPY-C12 was injected into the yolk of 24 hpf zebrafish embryos and yolk and body lipids were analyzed separately by TLC 1–6 h post injection (hpi), fluorescent complex lipids including TG, CE, and several unidentified species were produced in the yolk at early time points. Some fluorescent complex lipids were detected in the body at 6 hpi (24). (It is not known whether fluorescent PL was synthesized in this experiment as the assay only detected nonpolar lipids.) Injection of fluorescent PL into the yolk at 24 hpf resulted in fluorescent diglyceride and unidentified complex lipid species in the yolk, but no identified products in the body up to 6 hpi (24). Taken together, this and other evidence shows that the yolk is metabolically active throughout development and can both break down and synthesize complex lipids (18, 24, 28) (**Table 2**).

DIETARY LIPID UPTAKE IN THE LARVAL ZEBRAFISH

Digestion and Absorption of Dietary Complex Lipids

The larval zebrafish undergoes a switch from a lecithotrophic state to a free-feeding animal during its fifth day of development,

TABLE 2 | The larval zebrafish is a versatile model system for metabolic labeling of lipids.

Labeled lipid substrate	Developmental stage/delivery method	Assay	Reference
Radioactive FA	1 dpf/yolk injection	Thin layer chromatography (TLC)	Miyares et al. (18)
	3 dpf/yolk injection	TLC	Miyares et al. (18)
	6 dpf/feeding	HPLC	Quinlivan et al. (29)
Fluorescent FA	1 dpf/yolk injection	TLC	Fraher et al. (24)
	3 dpf/yolk injection	TLC	Miyares et al. (18)
	6 dpf/feeding	TLC	Carten et al. (28)
		HPLC	Quinlivan et al. (29)
Fluorescent PL	1 dpf/yolk injection	TLC	Fraher et al. (24)
Fluorescent CE	6 dpf/feeding	HPLC	Quinlivan et al. (29)

so by the time its yolk supply is depleted it must be able to digest and absorb nutrients from exogenous food sources. The ability to precisely control timing of the first meal is an advantage of this model as processing of dietary lipids by enterocytes can be observed without interference from lipids absorbed from previous meals. Additionally, because the larva retains its transparency for several weeks after it becomes free-feeding, it is possible to perform live imaging experiments with either single meals or ongoing defined diets in the same system.

Most dietary lipid consumed by animals enters the intestine not in the form of free fatty acids, but in complex lipids. Dietary TGs, PLs, and CE must be broken down by intestinal lipases in the lumen before the components of these molecules can cross the enterocyte membrane. As the fatty acids in these molecules are all linked by ester bonds, the intestinal lipases secreted by the exocrine pancreas are versatile and process a wide range of dietary lipids so that they can be absorbed (30). Following lipolysis, dietary lipid products form micelles in the intestinal lumen, which are emulsified in this aqueous environment by bile. The composition of bile varies between species and there are significant differences between teleost fish and humans, but its function is conserved (31, 32).

Enteroendocrine Cells in the Intestine Regulate Digestion and Are Influenced by the Microbiota

As they do in mammals, enteroendocrine cells in zebrafish secrete a wide range of hormones including serotonin, which influences motility and appetite, and cholecystokinin (CCK), which stimulates gall bladder contraction and release of digestive enzymes from the pancreas (33, 34). The zebrafish genome contains two CCK paralogs; *ccka* is expressed in the digestive system of adults (no data are available for larvae at this time) and both *ccka* and *cckb* are expressed in the brain starting at 24 hpf (35, 36). In mammals, CCK promotes lipid digestion by stimulating the gall bladder to secrete bile, but does not increase lipase activity (37, 38). Similarly, larval zebrafish treated with a CCK receptor antagonist show reduced protease activity while intestinal phospholipase activity is unaffected (30). Enteroendocrine cells expressing serotonin begin to appear in the larval zebrafish intestine at 5 dpf. They may be detected by immunohistochemistry for serotonin, and are distinguished from the enteric neurons (which also express serotonin) by their shape and location in the epithelium. By 8 dpf, 10–18 enteroendocrine cells per larva may be observed in the distal intestine (posterior to the swim bladder) (11). A notable difference is that the larval zebrafish intestine does not have crypts, where enteroendocrine cells would be located in mammals.

The intestinal microbiota is required for normal enteroendocrine cell development (11). In germ-free larval zebrafish, 0–6 enteroendocrine cells were observed at 8 dpf (the total number of cells in the distal intestinal epithelium did not vary between germ-free and conventional groups). Larvae raised germ-free until 5 dpf, and then colonized with the conventional microbiota, developed normal numbers of enteroendocrine cells, suggesting that the yet-unidentified signal from the microbiota that promotes enteroendocrine cell development is not required before 5 dpf. Higher gut motility was observed in zebrafish larvae raised germ-free, suggesting a possible connection to digestive problems (including irritable bowel disease) observed in humans when the gut microbiota is disrupted (11). The lower number of serotonin-positive cells could explain this physiological effect as serotonin regulates gut motility in humans (33).

Lipid Transport into Enterocytes

Dietary lipids are imported from the intestinal lumen across the apical enterocyte membrane by several different mechanisms depending on their class. After complex lipids (including both glycerolipids and CE) are digested to yield fatty acids, monoglycerides, and/or lysophospholipids, these products may cross membranes by a variety of transport processes conserved among zebrafish and mammals.

Cholesterol is taken up by enterocytes by a mechanism that requires the Niemann-Pick C1-Like 1 (NPC1L1) transport protein (39, 40). This membrane-associated protein is located at the brush border of enterocytes and is translocated to an intracellular compartment when cells are exposed to cholesterol; current models postulate a clathrin-dependent endocytic mechanism in which NPC1L1 is internalized along with a cholesterol cargo, which then moves through endosomes to the endoplasmic reticulum where it can be packaged into membranes or used to synthesize cholesterol ester (41, 42). NPC1L1 is encoded in the zebrafish genome, and several lines with point mutations in this gene have been created through the Sanger Institute Zebrafish Mutation Project (43). Ezetimibe, an inhibitor of NPC1L1-mediated cholesterol absorption that is used to treat hypercholesterolemia in humans, also blocks dietary cholesterol absorption in larval zebrafish (44–46). This creates an opportunity to use the zebrafish model to study physiological effects of modulating metabolic availability of a single component of a mixed-lipid diet. Regulation of NPC1L1 activity remains largely uncharacterized, although there is evidence from studies in humans given statins (inhibitors of cholesterol synthesis) that NPC1L1 expression levels increase in response to low intracellular cholesterol levels, suggesting that there may be an unidentified genetic mechanism that regulates NPC1L1 expression that could counteract the effects of statins by upregulating import of dietary cholesterol (47).

Fatty acid transfer proteins (FATPs) are a family of integral membrane proteins that facilitate transport of fatty acids into cells, including transport of dietary fatty acids into enterocytes. FATPs act in concert with acyl-coA synthetases (ACSs), which activate the newly imported fatty acids so that they are ready to form ester bonds and be incorporated into complex lipids (48, 49). There is evidence from mammalian and cell culture models that both the FATP and ACS families play roles in regulating preferential uptake of some dietary fatty acids over others, and in the partitioning of dietary fatty acids among complex lipids (48, 50). The zebrafish genome encodes 9 ACSL (ACSs specific to long-chain fatty acids, the type of fatty acid most abundant in animals including zebrafish) gene paralogs in six families. Expression of this class of genes is ubiquitous in adults, with proteins corresponding to seven of nine paralogs detectable by Western blot in most tissues including the gut (51). Expression

of ACSL genes in the larva is more regionalized: in the *acsl1* family, *acsl1b* mRNA is detectable in the YSL and gut in early larval stages. The *acsl1a* paralog is not expressed in the YSL, and no expression data are available for early-gut development (17). Only *acsl1a* is expressed in the gut in adults (51). *Acsl4a* mRNA is present in both the YSL and the larval gut (52). Expression of *acsl4b* and *acsl5* is detectable in the YSL, but expression data are not available from larval stages after the gut has begun to develop (17) (**Figure 2**). Expression data are unavailable for the other *acsl* paralogs at any embryonic or larval stage, but what is known about expression of *acsl* genes in this model suggests potential division of function among paralogs similar to that suggested by regionalized apolipoprotein gene expression.

Compared with the ACSs, there is far less coverage of zebrafish FATPs in the current literature. As of now, no studies of FATP function in this model system have been published and only one genomic sequence is annotated as a FATP in the Ensembl database; FATP3/ACSVL3/SLC27A3 [with 7 paralogs, all annotated as members of solute carrier family 27 (*slc27*)]. The other six putative FATP paralogs are annotated as SLC27A1A and B {both with 65% protein sequence identity to human SLC27A1/FATP1/ACSVL5 [a mitochondrial long-chain FATP (54)], using the NCBI protein BLAST tool}, SLC27A2A (47% protein sequence identity to human SLC27A2/FATP2/ACSVL1), SLC27A2B (55% protein sequence identity to human SLC27A2/FATP2/ACSVL1), SLC27A4 (70% protein sequence identity to human SLC27A4/FATP4/ACSVL4), and SLC27A6 (57% protein sequence identity to human SLC27A6/FATP6/ACSVL2). The chromosomal locations of all of these putative *fatp* genes are conserved between the human and zebrafish genomes (syntenic analysis by ZFIN). Zebrafish SLC27A2A is expressed in the adult liver (55), and SLC27A4 is expressed in the anterior gut at 5 dpf (53) (**Figure 2**). No expression data are available for other adult organs, earlier larval stages, or the other putative FATPs at this time. However, as FATP4 is the primary fatty acid transporter on the apical brush border of human enterocytes, the similarity in expression between zebrafish and humans supports the larval zebrafish as a model in the investigation of FATP function in dietary fatty acid absorption (56).

The relative contributions of FATP4, other membrane-associated fatty acid-binding proteins, and passive diffusion to uptake of dietary fatty acids by enterocytes in larval zebrafish are not known. A recent review proposes a model in which the transmembrane receptor protein CD36, Caveolin 1 (Cav1), and FATP4 all act as fatty acid transporters at the enterocyte brush border, and in which passive diffusion of long-chain fatty acid salts across the enterocyte membrane plays a major role in adsorption (57). Larval zebrafish express CD36 and Cav1 in the intestine as well as FATP4 and, therefore, present an opportunity to apply live whole-animal imaging tools toward investigations of the roles of each of these proteins in dietary fatty acid processing (58, 59). [Cav1 is located on the basolateral membrane of enterocytes in zebrafish and not at the brush border and, therefore, is unlikely to participate directly in uptake of fatty acids from the intestinal lumen (59).] In sum, despite tight conservation of FATPs and other fatty acid transporters, and their intestinal expression throughout the vertebrates, their physiological role in the intestine remains unclear.

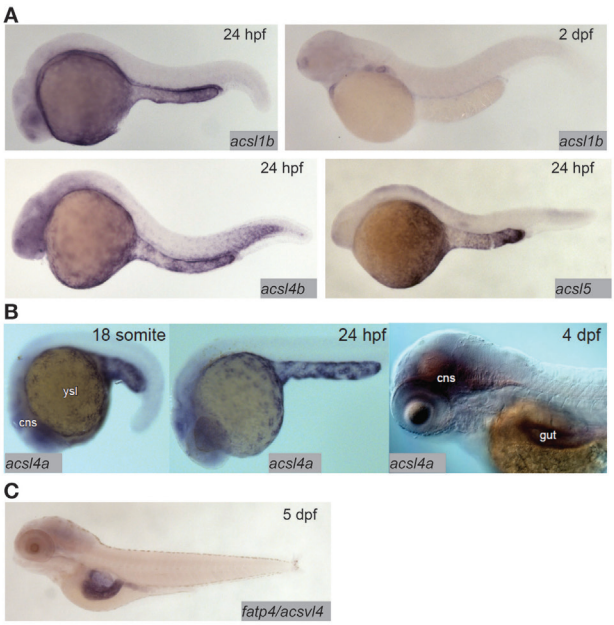

FIGURE 2 | Acyl-CoA synthetases are expressed in the larval zebrafish yolk syncytial layer (YSL) and intestine. **(A)** *In situ* hybridization reveals expression of *acsl1b*, *acsl4b*, and *acsl5* in the YSL at 24 hpf, and *acsl1b* in the developing gut at 2 dpf. Adapted from Ref. (17). **(B)** acsl4a is expressed in the gut and central nervous system (cns) of the 4 dpf larval zebrafish, and in the YSL at 24 hpf and earlier. Reprinted from Ref. (52), Figure S1E in Supplementary Material, under a CC-BY license. **(C)** *fatp4/acsvl4* is expressed in the gut (especially the anterior bulb) of the 5 dpf larval zebrafish. Adapted from Ref. (53).

The Intestinal Microbiota Influences Dietary Lipid Uptake

The bacterial population of the intestine also plays an important role in dietary lipid uptake and metabolism. Fermentation by the gut microbiota allows host animals to utilize dietary plant polysaccharides that would otherwise be indigestible by converting them to metabolizable short-chain fatty acids and monosaccharides (60). Multiple studies over the last decade have shown effects of changes in composition of the gut microbiota on adiposity, serum lipids, and tissue lipids in mammals (61–66). However, determining mechanisms by which bacteria may cause global changes in vertebrate host physiology has been difficult as the composition of the gut microbiota also changes in response to changes in diet (67, 68). The larval zebrafish model was recently used to investigate aspects of the relationship between gut bacteria and lipids involving processes other than short-chain fatty acid synthesis: when larvae raised germ-free were given a high-fat meal labeled with fluorescent fatty acids, less fluorescence accumulated in the intestinal epithelium when compared with conventionally raised larvae, showing that at least some members of the microbiota are necessary to promote uptake of dietary lipids. Monoassociated larvae (larvae raised

germ-free and then inoculated with a single bacterial species) colonized with the Firmicutes strain *Exiguobacterium* sp. were used to demonstrate that this bacterial strain alone was sufficient to promote intestinal fatty acid uptake to a point where fluorescence could be observed in extra-intestinal tissues. Furthermore, experiments using conditioned media from this strain and two others also revealed significant increases in enterocyte lipid droplet number over untreated germ-free larvae, suggesting that a factor secreted by these species is involved in promoting dietary lipid uptake (69). The exact mechanism for this host–microbe relationship is currently uncharacterized, as is the evolutionary advantage of promoting host lipid uptake for these microbial species.

Lipid Processing in Enterocytes for Storage and Export

Fatty acids taken up by enterocytes are repackaged into complex lipids at the endoplasmic reticulum and are subsequently stored in enterocyte lipid droplets or directed to lipoprotein synthesis for export. Lipid droplets are composed primarily of TGs and CE in the interior, and bounded by a PL monolayer with associated proteins such as perilipins (70). Though the mechanisms by which lipid droplets grow and shrink are well characterized, the regulation of lipid droplet size and number in various tissues is not as well understood, and most current research efforts focus on adipose and hepatic lipid droplets (71). As the intestine is not a site of long-term lipid storage in vertebrates including larval zebrafish, enterocyte lipid droplets are highly dynamic, temporary structures that respond with high sensitivity to the nutritive state of the animal. This property combined with the relative ease of live imaging in the larval zebrafish intestine compared with other animal models makes for an ideal system for the study of lipid droplet dynamics and regulation. When 5 dpf larvae are fed a high-fat/high-cholesterol meal of chicken egg yolk, both the average lipid droplet number per enterocyte and total area of the cell covered by lipid droplets increase significantly by 1 h post-feeding. Lipid droplet number peaks at 1 h and then gradually decreases, while total lipid droplet area is maintained up to 3 h following the meal, suggesting that smaller lipid droplets fuse as they mature (72). The gut microbiota also influence enterocyte lipid droplet number and size. Intestinal lipid droplets are both larger and more numerous in conventionally raised larvae after feeding than in germ-free larvae. Furthermore, conditioned media from a Firmicutes bacterial strain found to promote dietary fatty acid uptake and export to the liver was sufficient to increase enterocyte lipid droplet number but not the average lipid droplet size (69). These results have begun to reveal the diverse mechanisms by which different members of the gut microbiota influence lipid droplet dynamics and dietary lipid metabolism.

Lipoproteins are essential for the export of the products of dietary lipid from enterocytes into the circulation. Expression and function of apolipoproteins in the zebrafish is similar to that observed in mammals; at least one paralog from each of the ApoA-I, ApoB, ApoE, and ApoA-IV families is expressed in the larval zebrafish intestine (13). There is evidence that division of apolipoprotein function among organs is regulated by different mechanisms that achieve the same end in zebrafish and mammals: while different variants of ApoB are produced in the mammalian intestine and liver *via* RNA editing, larval zebrafish produce mRNA for the ApoB paralog b.1 in the intestine and liver and ApoBb.2 in the liver only. Similar compartmentalization of paralog expression between the liver and intestine is observed in the other apolipoprotein families as well (13) (**Figure 3**). Intestinal lipid accumulation in animals treated with an MTP inhibitor shows that as in the larval

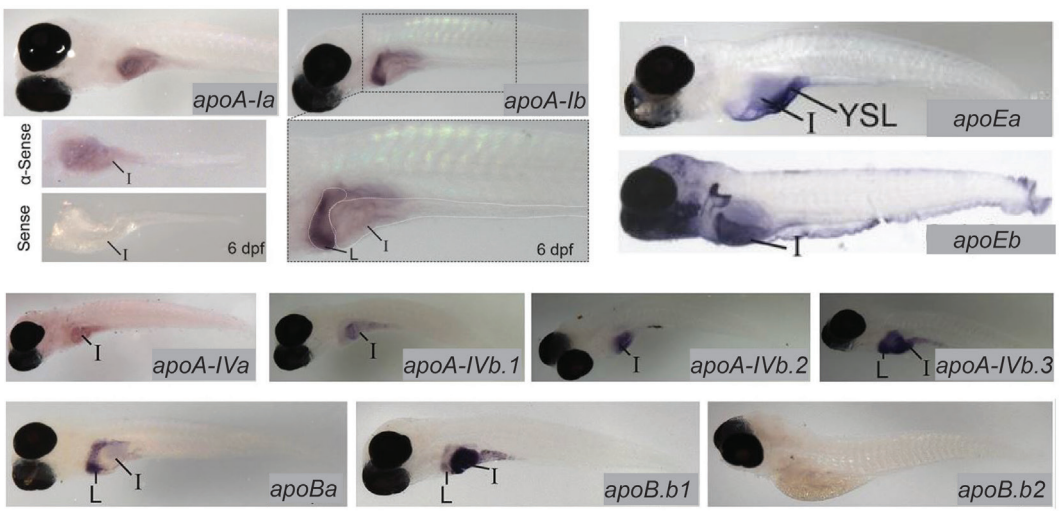

FIGURE 3 | Zebrafish apolipoprotein genes are expressed in the larval digestive system. *In situ* hybridization reveals expression of 10 of the 11 zebrafish apolipoprotein genes in the *apoB*, *apoA-IV*, *apoE*, and *apoA-I* families in the liver and/or intestine of the 6 dpf larva. Dissected intestines probed for *apoA-Ia* are shown, and the gut of a larva probed for *apoA-1b* is magnified below the image of the whole larva. L, liver, I, intestine. Adapted and reprinted from Ref. (13), Figures 2–5, under a CC-BY license.

zebrafish yolk, availability of functional ApoB in necessary for normal rates of lipid export from the intestine, and that enterocyte lipid droplets are the destination of excess dietary fatty acids when export is slowed (73–75). The MTP inhibitor lomitapide is effective in larval zebrafish (72). It has also been observed that in mammals as the dietary fat content increases, chylomicron number reaches a plateau but average chylomicron size continues to increase, suggesting that apolipoprotein expression is the limiting factor in the rate of lipid export from the intestine (76).

Total Lipid Biochemistry of the Larval Zebrafish Reveals Global Effects of Diet on Lipid Composition, and Facilitates Metabolic Labeling Studies

The larval zebrafish intestine is not only an excellent model for the study of lipid droplet and lipoprotein packaging, but also a site of differential channeling of dietary fatty acids depending on their chemical properties. The amenability of this model to biochemistry due to the ease of obtaining large numbers

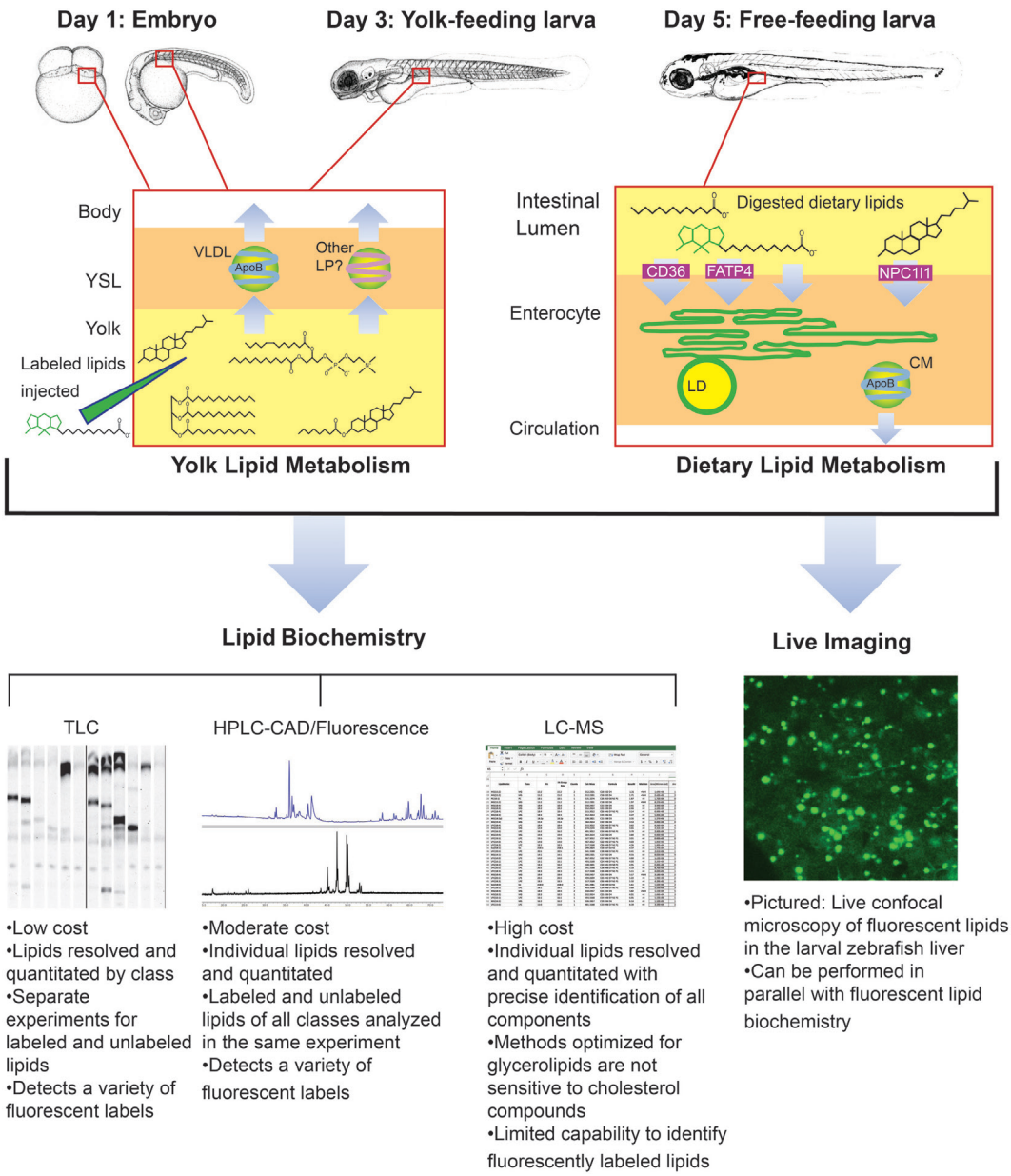

FIGURE 4 | Metabolic labeling with fluorescent fatty acids is performed in the context of zebrafish development, yolk absorption, and dietary lipid metabolism. Fluorescent fatty acids (BODIPY-FL-C12 depicted) are trafficked and metabolized along with native yolk or dietary lipids when delivered to the developing zebrafish by yolk injection or feeding. LD, cytoplasmic lipid droplet, LP, lipoprotein, VLDL, very low-density lipoprotein. Embryo and larva illustrations adapted from Ref. (18).

of embryos and larvae and performing lipid extractions from them, combined with the transparency of the larva, provides an opportunity unique among vertebrates to perform live imaging and metabolic labeling experiments in parallel using the same fluorescent lipid reagents (28, 77–79). Additionally, the whole-body lipid composition of the larval zebrafish is highly sensitive to changes in diet: the TG content of the 6 dpf larva increases 10-fold 24 h after a single high-fat meal (compared with a standard low-fat diet, and allowing time for the intestinal lumen to clear) (29). (In these experiments, the high-fat meal was chicken egg yolk; ~50% lipid dry weight, and the low-fat meal was SERA Micron larval growth food; 7% lipid. The lipid content of "standard chow" for zebrafish larvae is typically 5–15%.) Working at developmental stages before adipose tissue appears (~14 dpf) avoids signal to noise problems that may occur when the neutral lipid stored in adipose is included in the whole-body lipid profile. Also, at these early developmental stages examination of dietary lipid processing in the intestine can be isolated from potential regulatory influences from adipose tissue.

Though it was beyond the scope of our recent metabolic labeling study (29), the biochemical techniques described therein could be applied to later-stage larvae in order to examine potential crosstalk between adipose tissue and the enterocytes that could influence dietary lipid partitioning. We have also developed methods for using fluorescent fatty acids as metabolic labels in the context of standard and lipid-enriched diets in larval zebrafish (**Table 2**). In addition to exploring the metabolic labeling potential of fluorescent lipids whose product profiles were not previously described, we have also applied HPLC with charged aerosol (total lipid detection) and fluorescence detection to obtain a greater depth of information than previous studies using fluorescent TLC (28). Initial findings indicate that the partitioning of saturated fluorescent fatty acids among complex lipid classes varies with carbon chain length, the total fat and cholesterol content of the diet, and the type of fluorescent tag (29). Metabolic labeling with fluorescent fatty acids in the context of lipid metabolism by the larval zebrafish is summarized in **Figure 4**.

Potential mechanisms regulating the rate of lipid export from the intestine beyond lipoprotein levels, the regulation and physiological effects of the size of enterocyte lipid droplets, and the channeling of newly absorbed dietary fatty acids into the different classes of complex lipids are currently largely uncharacterized. The optically clear and genetically tractable larval zebrafish model presents an ideal system in which to investigate these questions relating to energy homeostasis with a combined live imaging and biochemical approach.

AUTHOR CONTRIBUTIONS

VQ wrote the manuscript. SF edited the manuscript and provided guidance on the topic and scope.

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Zebrafish Models for Dyslipidemia and Atherosclerosis Research

Amnon Schlegel[1,2,3,4]*

[1]University of Utah Molecular Medicine Program, School of Medicine, University of Utah, Salt Lake City, UT, USA, [2]Department of Internal Medicine, Division of Endocrinology, Metabolism and Diabetes, University of Utah, Salt Lake City, UT, USA, [3]Department of Biochemistry, School of Medicine, University of Utah, Salt Lake City, UT, USA, [4]Department of Nutrition and Integrative Physiology, College of Health, University of Utah, Salt Lake City, UT, USA

Atherosclerotic cardiovascular disease is the leading cause of death. Elevated circulating concentrations of lipids are a central pathogenetic driver of atherosclerosis. While numerous effective therapies for this condition have been developed, there is substantial unmet need for this pandemic illness. Here, I will review nutritional, physiological, genetic, and pathological discoveries in the emerging zebrafish model for studying dyslipidemia and atherosclerosis. The technical and physiological advantages and the pharmacological potential of this organism for discovery and validation of dyslipidemia and atherosclerosis targets are stressed through summary of recent findings. An emerging literature shows that zebrafish, through retention of a *cetp* ortholog gene and high sensitivity to ingestion of excess cholesterol, rapidly develops hypercholesterolemia, with a pattern of distribution of lipid species in lipoprotein particles similar to humans. Furthermore, recent studies leveraging the optical transparency of zebrafish larvae to monitor the fate of these ingested lipids have provided exciting insights to the development of dyslipidemia and atherosclerosis. Future directions for investigation are considered, with particular attention to the potential for *in vivo* cell biological study of atherosclerotic plaques.

Keywords: atherosclerosis, dyslipidemia, zebrafish, genetics, physiology and metabolism

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***Correspondence:**
Amnon Schlegel
amnons@u2m2.utah.edu

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INTRODUCTION

Atherosclerosis is the leading cause of death (1). This chronic, progressive build-up of cholesterol, cellular debris, and calcium can narrow the lumens of critical arteries supplying the heart, brain, limbs, and organs. Plaques are mechanically weak structures and are prone to rupture. Once ruptured, a rapid thrombosis cascade is activated at the site of the plaque, occluding the artery and causing ischemic death to the supplied organ. Persons who have sustained an ischemic event in any artery are at substantially increased risk of repeated plaque rupture and thrombosis. While the last several decades have witnessed a decrease in the incidence of myocardial infarction and ischemic cerebrovascular accident, cardiovascular death is predicted to remain the leading killer for decades to come. A confluence of cardiovascular risk factors including tobacco exposure, hypertension, obesity in children and adults, type 2 diabetes mellitus, and non-alcoholic fatty liver disease is to blame for this trajectory (2–6). Moreover, there is widespread underutilization of effective antiplatelet, antihypertensive, and lipid lowering therapies (3, 7).

In the face of this clinical reality, I will argue in this minireview that zebrafish is an excellent system to discover and characterize new diagnostic and therapeutic targets for atherosclerosis. Those properties that make the study of lipid physiology and atherosclerosis in zebrafish potentially

transformative will be reviewed, with an emphasis on original work as throughout the remainder of this article, stressing studies published since others and I last reviewed this topic (8, 9).

ZEBRAFISH MODEL OVERVIEW

The general strengths of zebrafish for biomedical research are well known, owing to its facile husbandry and low cost of housing and maintenance. This organism can be used to generate large numbers of externally fertilized embryos. These animals develop rapidly and are a mainstay of embryological, forward genetic, and pharmacological research (10–12). In the last decade, a full array of modern genome editing tools has been deployed in zebrafish, including very promising knock-in technologies (13–15). These advances have been married to progress in working in zebrafish late larvae, juveniles, and adults, where numerous aspects of physiology pertinent to atherosclerosis emerge.

GENERAL FEATURES OF LIPOPROTEIN METABOLISM IN ZEBRAFISH

Lipoprotein Biology in Zebrafish

Elevated serum cholesterol and non-fasting triacylglycerol (TG) are central drivers of atherosclerosis (16–18). Understanding how lipids are absorbed from the diet, metabolized in tissues, and modified in atherosclerosis are central areas of investigation in developing newer and more effective therapies to treat atherosclerosis. A highly conserved system for transporting water-insoluble lipids is present in all animals (19). In particular, the apolipoprotein B (APOB)-coated particles produced by the intestine (chylomicrons) and liver [very low-density lipoprotein (VLDL) particles] are the carriers of the bulk of absorbed and resynthesized neutral lipids, cholesteryl esters (CE), and TG. These "β-lipoprotein" particles also carry fat-soluble vitamins A, D, and E from their sites of absorption or synthesis to their sites of use or storage (**Figure 1**).

In amniotes, two different protein products are encoded by a single *APOB* locus. In enterocytes of reptiles, birds, and mammals, the *APOB* pre-mRNA undergoes cytosine deamination (catalyzed by APOBEC) to generate a transcript encoding a truncated protein (APOB48) that is found exclusively on chylomicrons (21). The full-length *APOB* transcript can be translated in both the liver and intestine (encoding APOB100). APOB48-coated chylomicron remnants are susceptible to rapid postprandial clearance by the liver, whereas APOB100-coated chylomicron remnants and VLDL remnants [intermediate density lipoprotein (IDL) particles] can mature into the long-lived and atherogenic low-density lipoprotein (LDL) particles (**Figure 1**). Thus, it is important to appreciate that Apob (operationally equivalent to "Apob100")-coated zebrafish chylomicrons are, most likely, not cleared rapidly. Furthermore, zebrafish chylomicrons carry the potential to mature into LDL stoichiometrically (22). This lack of Apob48 might contribute to the rapid dyslipidemia and atherogenesis seen in dietary and genetic studies of zebrafish that will be discussed in subsequent sections. Finally, there are two zebrafish *apob* paralogs (two *apob* genes on different chromosomes). The contribution (expression and incorporation into chylomicrons and VLDL) of these Apob paralogs to circulating β-lipoproteins and atherogenesis is not known; however, their larval expression patterns are different, and their encoded proteins are structurally dissimilar, raising the possibility that they have unique functions (23).

Immediately beyond these critical issues of Apob biology, zebrafish utilizes highly conserved β-lipoprotein assembly proteins and transport mechanisms. Gene expression survey and knockdown approaches confirmed that the central Apob-coated lipoprotein particle-producing enzyme microsomal triglyceride transfer protein (encoded by *mtp* and having orthologs in all species ranging from insects to mammals) is present and functional in zebrafish yolk cell layer, liver, and intestine (24–26). More recently, studies on the intracellular trafficking of nascent chylomicrons have confirmed that the zebrafish model is well suited to investigating the molecular and cellular machinery of dietary energy harvest: the enterocyte undergoes stereotypical changes in ultrastructure when absorbing fats, and its secretory apparatus uses proteins conserved in evolution to pack and traffic nascent chylomicrons (27, 28). Finally, the major determinant of clearance of LDL particles from the circulation, the LDL receptor (Ldlr), has conserved function in zebrafish (29). In short, zebrafish has a complement of conserved lipid trafficking genes that renders study of lipid transport in this model organism relevant to human physiology. The next section will consider one additional, critical circulating protein that makes zebrafish lipoprotein biology particularly useful for modeling human lipoprotein biology.

Cholesteryl Ester Transfer Protein (CETP)

Following release into the circulation, lipoproteins are modified in zebrafish blood by enzymatic machinery that is also highly conserved with humans. Specifically, zebrafish carries an ortholog of the human *CETP* gene (30). CETP encodes a circulating protein that transfers CE from HDL particles to LDL particles in exchange for TG (**Figure 2**). Once loaded with TG and subject to additional modification, HDL is rendered more prone to rapid clearance, decreasing its "ability" to engage in atheroprotective processes such as reverse cholesterol transport (i.e., retrieving cholesterol from tissue macrophages to delivery to the liver and intestine for elimination). Likewise, increased CE loading of and depletion of TG from LDL contribute to atherogenesis by producing readily modifiable (oxidizable) small dense particles that can enter the subintimal space and drive atherogenesis (31–33). The net effect of Cetp function is to leave the organism with a higher concentration of atherogenic LDL particles and a lower concentration of atheroprotective HDL particles in circulation (the so-called "β-dominant" lipoprotein profile). In zebrafish, the fasting lipoprotein profile is β-dominant (34). This similarity to human lipoprotein composition reflects retention of a *cetp* ortholog in the zebrafish genome. As discussed below, this conservation of a critical human lipoprotein-modifying enzyme

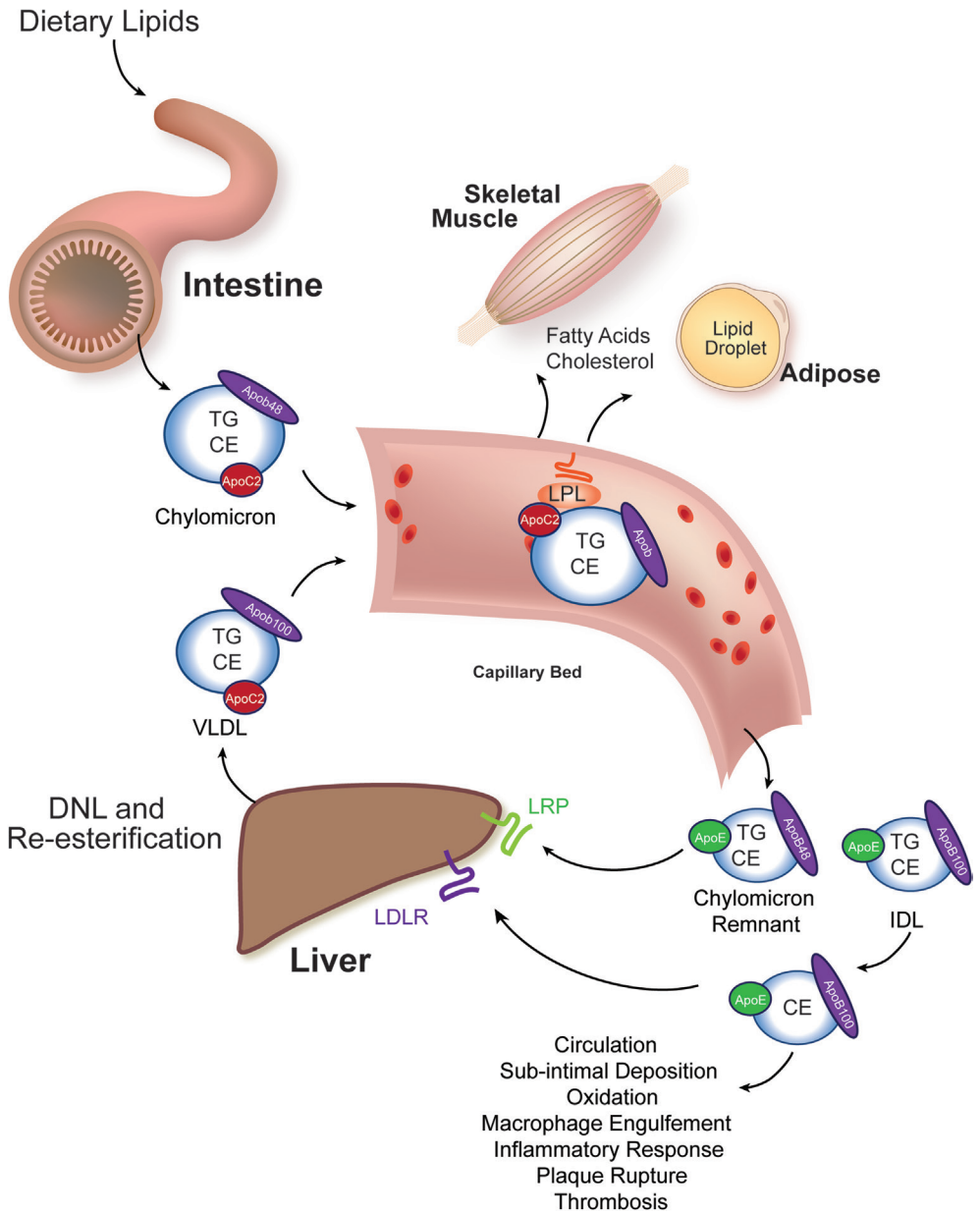

FIGURE 1 | **Intestinal and liver β-lipoprotein synthesis and vascular modification**. Ingested lipids are hydrolyzed in the lumen of the intestine to absorbable species, such as free cholesterol, free fatty acids, and monoacylglycerol. These molecules are re-esterified in the enterocyte of the small intestine to triacylglycerol (TG), cholesteryl esters (CE), and phospholipids (not shown) and packaged into chylomicrons, whose signature coat protein in humans is Apob48 (one molecule per particle). This particle enters the vasculature and acquires an Apoc2 molecule from an HDL particle (not shown). Apoc2 is a required binding partner for lipoprotein lipase (LPL), an enzyme tethered to the apical surface of capillary bed endothelial cells in muscle and adipose tissues (20). LPL liberates free fatty acids for use by these tissues. The partially lipid-depleted chylomicron remnant is rapidly cleared by the liver through the action of Apoe-binding LRP receptors and Apob-binding low-density lipoprotein receptors (LDLR). The liver synthesizes very low-density lipoprotein (VLDL) particles from *de novo* lipogenesis-derived fatty acids and re-esterified fatty acids that reach the liver after adipocyte hydrolysis (and has relatively less CE in it). Human VLDL's signature coat protein is Apob100. Following LPL-catalyzed lipid hydrolysis, VLDL remnants, intermediate density lipoprotein (IDL) particles, are either rapidly cleared by the liver or mature into LDL. LDL particles have a long circulating half-life, and they can deposit under vascular endothelial cells, undergo oxidation, and trigger an inflammatory atherosclerotic reaction with subsequent plaque rupture and thrombosis causing ischemia to the supplied tissue.

renders zebrafish susceptible to a dyslipidemia with short dietary interventions. This conservation of a critical aspect of lipoprotein biology also opens the door to pharmacological intervention studies in zebrafish: many other, non-rodent laboratory models also fall short of recapitulating human lipoprotein composition and atherogenesis (35).

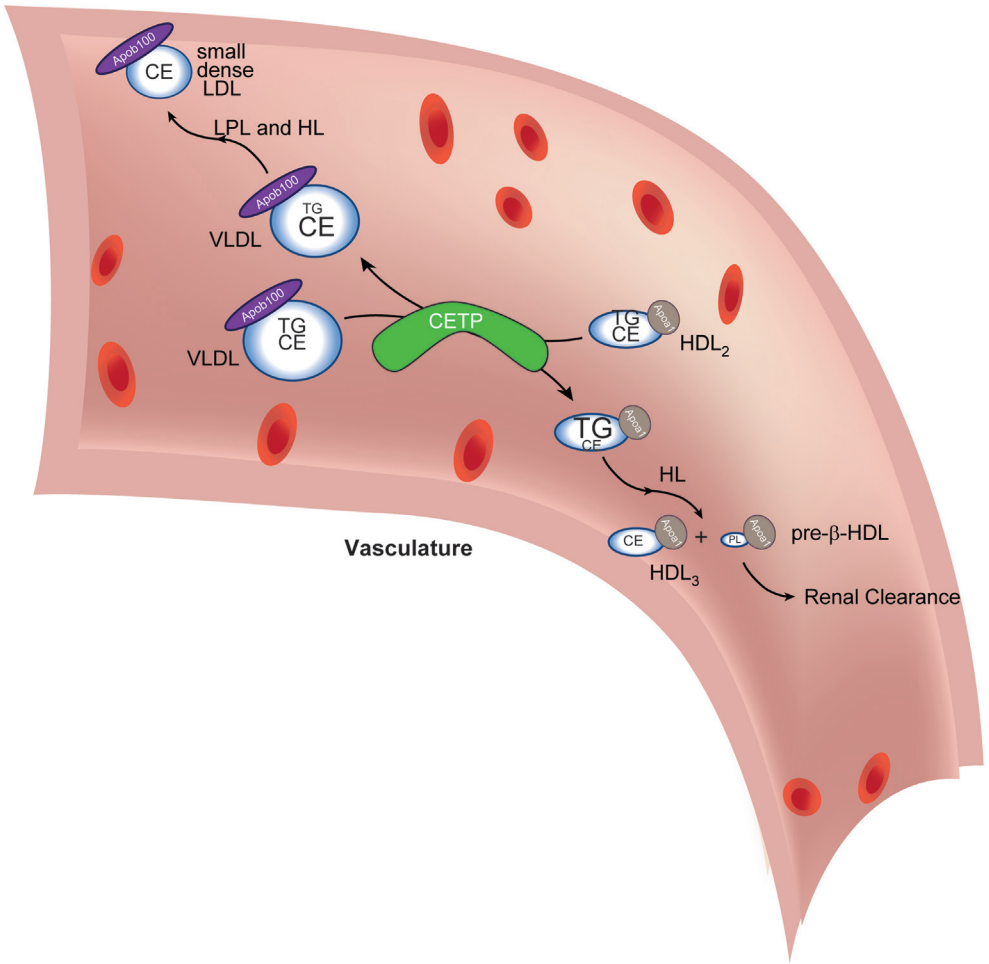

FIGURE 2 | Cholesteryl ester transfer protein (CETP) in lipoprotein lipid exchange. Very low-density lipoprotein (VLDL) particles and HDL_2 exchange triacylglycerol (TG) and cholesteryl esters (CE) in a reaction catalyzed by CETP. The depletion of TG and increase in VLDL CE (reflected in altered font sizes) coupled with lipoprotein lipase (LPL)- and hepatic lipase (HL)-mediated (further) depletion of TG (not shown) lead to the formation of small dense low-density lipoprotein (LDL), which is amenable to oxidative modification, a conversion central to driving subsequent atheromatous plaque formation. The transient increase in TG in HDL_2 (reflected in increased font size) delivers a substrate for HL-mediated hydrolysis (as it passes through the liver capillaries). This reaction generates small HDL_3 and pre-β-HDL, which contain scant amounts of phospholipids only. Pre-β-HDL is removed from the circulation *via* renal filtration. The net effect of CETP action, thus, is to cause maturation of VLDL into atherogenic, small, dense LDL and to decrease atheroprotective HDL concentration. Apoa1 is the signature coat protein of HDL.

A Cetp ortholog is absent in commonly used rodent models of dyslipidemia, rendering the study of atherosclerosis inherently difficult in these species. Specifically, rodents are resistant to atherosclerosis because, among other things, they lack this enzyme's action. Rodents have high circulating HDL concentrations and low LDL concentrations (the so-called "α-dominant" lipoprotein pattern) as a consequence of losing the *Cetp* gene (35). The most commonly used genetic strategy to trigger dyslipidemia in mice is to study dietary and genetic interventions in the context of deleting the *Apoe* or *Ldlr* genes (36–40). Although they are widely used, these models do not capture the full biology of the corresponding human Mendelian diseases, familial dysbetalipoproteinemia (in the case of *Apoe*), and familial hypercholesterolemia (in the case of *Ldlr*): the HDL-cholesterol in both $Apoe^{-/-}$ or $Ldlr^{-/-}$ mice is still higher than in humans with $APOE^{2/2}$ or $LDLR^{-/-}$ genotypes, and in both models, it is mainly the VLDL (and not the IDL and LDL, respectively) that increases. Furthermore, studies with only $Apoe^{-/-}$ or $Ldlr^{-/-}$ mouse models are often limited in generalizability: there is incomplete agreement in the findings with these two models (41). Beyond the limitations of standard mouse genetic models in driving atherogenic dyslipidemia, the atheromatous plaques that do form in mice lack features of "complex" human lesions. Indeed, to generate atheromatous plaques that appear more like human plaques, extreme physical stress is required in $Apoe^{-/-}$ animals deliberately maintained on a mixed genetic background (42). Even with this severe stress, plaque rupture (as in myocardial infarction) does not occur in rodents. Another supraphysiological approach to studying plaque rupture in mice that has met with some criticism because of its artificial nature involves causing a prolonged pharmacological hypertensive crisis in $Apoe^{-/-}$ animals;

this paradigm causes plaque rupture in the brachiocephalic artery, an uncommon site of rupture in humans (43).

ZEBRAFISH DYSLIPIDEMIA MODELS

High-Cholesterol Diet (HCD) Paradigm

The nutritional requirements of zebrafish are now known (44, 45). This knowledge has allowed several groups to establish conditions to induce metabolic stress by altering standard diets. For instance, zebrafish is susceptible to high-fat diet (HFD)-induced obesity, hyperglycemia, and dyslipidemia (46). The major breakthrough in applying zebrafish to the study of dyslipidemia was the development of HCD (34, 47, 48). Not only do larvae and adults readily ingest such diets but also these animals demonstrate a series of responses that firmly established the utility of this organism in studying dyslipidemia and atherogenesis. First, HCD-challenge caused β-dominant hypercholesterolemia in adults; second, vascular intimal lipid accumulation can be seen in larvae after short exposure to HCD, and these accumulated lipids attracted circulating monocytes; third, the extravasated LDL undergoes oxidation (to generate high-affinity ligands for innate immune receptors that are central for driving the inflammation of plaques); and fourth, the oxidized LDL particles can be tracked with live imaging (34, 47, 48). This last observation was made *via* transgenic overexpression of the human monoclonal antibody IK17, which binds to malondialdehyde-modified LDL. The sustained overexpression of IK17 prevented HCD-induced sub-intimal lipid accumulation (47). This is the first proof-of-principle demonstration that atherogenesis can be prevented in zebrafish through, presumably, accelerating immune complex-mediated clearance of modified LDL particles from the circulation (before they deposit in the walls of arteries). This constellation of findings sets the stage for future live imaging of atheromas *in vivo*, as discussed below.

CETP Pharmacology

Natural compound extracts of cinnamon, clove, grape skin, laurel, loquat, and turmeric contain inhibitors of zebrafish Cetp (30, 49, 50). These extracts protect zebrafish from high-cholesterol diet-induced dyslipidemia. Conversely, artificial sweeteners increase HDL particle-carried Cetp activity and drive hyperlipidemia (51, 52). Whether such zebrafish studies will translate into better inhibitors of human, CETP is difficult to predict; moreover, artificial sweeteners appear to exert multiple pathological effects, including triggering glucose intolerance by altering the gut microbiome (53). This pharmaceutical research space has been marked by several abandoned small molecules; one ongoing cardiovascular outcomes trial of a CETP inhibitor (http://ClinicalTrials.gov identifier NCT01252953) and a possible study of another inhibitor might provide opportunities for not only using this approach in humans but also to identify additional questions that might be answered with a zebrafish model (54, 55).

Zebrafish APOC2 Deficiency

A further advance in developing zebrafish dyslipidemia models comes from the targeted deletion of the *apoc2* gene. Humans lacking APOC2 have familial chylomicronemia, a condition marked by high serum TG concentrations and propensity to recurrent bouts of pancreatitis (56). Zebrafish *apoc2*$^{-/-}$ mutants were generated with genome editing tools (22). These mutants demonstrated the hallmark finding of human APOC2 deficiency: decreased plasma lipase activity and severe hypertriglyceridemia (**Figure 1**). Their lipoprotein pattern is predominantly large, β-lipoproteins, as quantified with size-exclusion chromatography and scanning electron microscopy techniques. Imaging of the vasculature in *apoc2*$^{-/-}$ mutants reveals accumulation of lipids and lipid-laden macrophages, both hallmarks of atherosclerotic plaques. This powerful dyslipidemia model might prove particularly useful in studying the steps of LDL extravasation, oxidation, and engulfment by vascular wall macrophages.

Zebrafish Liver X Receptor (LXR) Deletion

Lxrs are central inducers of cholesterol catabolism (57). These nuclear receptor transcription factors regulate metabolism through engaging oxysterol ligands and altering expression of functionally integrated genes involved in reverse cholesterol transport, lipoprotein modification, intestinal cholesterol absorption and excretion, liver fatty acid and TG regulation, bile transport, and immune and inflammatory signaling (57). There are two Lxr paralogs in mammals. Lxrα, which arose in fish, is mainly expressed in tissues involved in tissue macrophages, liver, and intestine, whereas Lxrβ, which arose in amphibians, is more widely expressed (58). Lxrα upregulates hepatic lipogenic enzymes and increases blood TG levels (59, 60). This seemingly self-defeating function—driving elimination of cholesterol while triggering fatty acid synthesis—has been a major impediment to developing Lxr-based therapeutics. Indeed, the LXRβ-selective agonist BMS-852927 not only promotes reverse cholesterol transport in humans but also induces hepatic *de novo* lipogenesis and attendant hypertriglyceridemia; BMS-852927 also causes a rapid decrease in circulating neutrophil counts in humans, but not in cynomolgus monkeys, underscoring the challenge of drug development (61).

Zebrafish bearing a targeted deletion mutation of the Lxrα gene *nr1h3* develop severe hypercholesterolemia and hepatic steatosis when fed HCD and HFD (62). Conversely, overexpression of *nr1h3* in enterocytes confers protection from dyslipidemia and hepatic steatosis when animals are fed a HFD; this metabolically beneficial effect of *nr1h3* overexpression is due to the induction of a transcriptional program resulting in temporary enterocyte storage of lipids, delaying an *en masse* delivery of atherogenic lipoprotein particles in the circulation. As noted above, zebrafish chylomicrons likely mature into LDL particles because of their full-length Apob-coat protein. As such, the *nr1h3* gene deletion and intestine-limited overexpression models might be useful for studying atherogenesis in that the increase in LDL-cholesterol seen in *nr1h3*$^{-/-}$ mutants is substantial. Furthermore, these studies may lead to the rational development of intestine-limited LXR agonists to blunt the development of dyslipidemia and atherosclerosis.

THE FUTURE: ATHEROMA CELL BIOLOGY, GENETIC AND PHARMACOLOGICAL SCREENS, AND CANDIDATE GENE ANALYSES

Cell Biology of Atherosclerosis *In Vivo*

There is no *a priori* guarantee that zebrafish atheromas will model the full natural history of the human disease more closely in terms of mechanisms of development, inflammatory response, and final architecture than available models. However, if the advances in studying lipoprotein biology are any guides, the natural history of atheroma progression—from simple lipid accumulation below the vasculature to organization into "complex," cell-rich, and debris-rich plaques—should be feasible in zebrafish. In particular, live fluorescent markers of various cell types that accumulate within plaques such as vascular smooth muscles, macrophages, and other immune cells are available. These live imaging reporters could be used to monitor atherogenesis in real time. Notably, such live imaging tools have been deployed with remarkable success in studying architecturally complex mycobacterial infection and host response in zebrafish (63–65). This success in modeling a human host response to mycobacteria in zebrafish—where other laboratory species have fallen short of producing human-like granulomas—is cause for hope that zebrafish atherosclerosis will reveal conserved inflammatory and immune mechanisms. In particular, the observation that zebrafish macrophages form granulomas in response to mycobacterial infection raises the hope that these cells may very well form lipid-laden "foam cells" in atherosclerotic plaques and, thus, drive an evolutionarily conserved inflammatory response that defines "complex" lesions (i.e., recruiting additional cells to the plaque and driving inflammation). Whether zebrafish will be useful in studying all aspects of more advanced atherosclerotic plaques biology is not clear. For instance, zebrafish have much lower blood pressure than terrestrial animals. Whether this organism will be useful for generating plaque rupture models is difficult to predict. Systematic histological examination of zebrafish arteries from dyslipidemia models will be required to determine what aspects of plaque biology can be studied in this model.

Genetic and Chemical Screens for Dyslipidemia and Atherosclerosis Modifiers

Beyond hemodynamic concerns regarding the natural history of zebrafish atherosclerosis, it remains uncertain whether genetic or pharmacological screens could be designed to look for modulators of dyslipidemia and atherogenesis in zebrafish. These complex phenotypes develop after a period of feeding in late larvae, raising the time and effort required to perform a large-scale screening project. Developing convenient reporters for the development of dyslipidemia and atherogenesis and their validation in already established zebrafish models would help guide screen design. Such an approach has proven feasible and informative in studying fasting glucose regulation in larvae (66, 67).

Candidate Genes

A large repertoire of genetic loci has been associated on a population genetics level with lipid parameters (68). For most, the molecular and cellular bases of the associations are unknown. While it is now experimentally tractable to rapidly overexpress and delete genes in zebrafish, phenotypic characterization for atherosclerosis is still limited in comparison to examination of alterations in glucose metabolism (for which larvae phenotypes develop more rapidly and do not require feeding). For instance, my group recently explored the mechanistic association of a single nucleotide polymorphism (SNP) in the *FOXN3* locus with fasting blood glucose (69). Through a blend of primary human hepatocyte, immortalized HepG2 hepatoma cell, and transgenic zebrafish approaches, we found that this SNP increases the expression of the FOXN3 protein and that this transcriptional repressor blunts a glucose utilization transcriptional program in the liver. Overexpression of both human *FOXN3* and zebrafish *foxn3* in liver increased fasting blood glucose in adults. Whether this gene acts in other tissues to modulate blood glucose is not known. Neither is the mechanism through which the risk allele increases FOXN3 expression. An approach similar to the one used to study FOXN3 could be used to examine the effect of overexpressing candidate genes (in liver or elsewhere) on zebrafish lipoprotein metabolism, again, with the caveat that the experimental window will need to be larger. Those "hits" showing changes in circulating lipids could be explored further using a large collection of null alleles (70) or through genome editing approaches (including conditional alleles).

CONCLUSION

Through a combination of genetic, developmental, and physiological advantages, the zebrafish has emerged as a major mechanistic discovery platform for studying dyslipidemia and atherosclerosis. Here, I highlighted individual areas of success using this organism, from dietary interventions, exploration of various aspects of lipoprotein production and processing, and genetic models for dyslipidemia and early atherosclerosis. Future work in the zebrafish system should include more thorough exploration of the composition and cellular architecture of atheromatous plaques, screenings effort to identify novel genes and small molecules that modulate atherogenesis, and candidate gene approaches to elucidating the functions of loci implicated in dyslipidemia and atherosclerosis through population genetic analyses. Collectively, this work in zebrafish may lead to the development of new and more effective therapies for dyslipidemia and atherosclerosis.

AUTHOR CONTRIBUTIONS

The author is the sole contributor to the conceptual development and writing of this minireview.

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The Role of Peroxisome Proliferator-Activated Receptor Gamma (*PPARG*) in Adipogenesis: Applying Knowledge from the Fish Aquaculture Industry to Biomedical Research

*Rebecca Wafer, Panna Tandon and James E. N. Minchin**

BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK

The tropical freshwater zebrafish has recently emerged as a valuable model organism for the study of adipose tissue biology and obesity-related disease. The strengths of the zebrafish model system are its wealth of genetic mutants, transgenic tools, and amenability to high-resolution imaging of cell dynamics within live animals. However, zebrafish adipose research is at a nascent stage and many gaps exist in our understanding of zebrafish adipose physiology and metabolism. By contrast, adipose research within other, closely related, teleost species has a rich and extensive history, owing to the economic importance of these fish as a food source. Here, we compare and contrast knowledge on peroxisome proliferator-activated receptor gamma (PPARG)-mediated adipogenesis derived from both biomedical and aquaculture literatures. We first concentrate on the biomedical literature to (i) briefly review PPARG-mediated adipogenesis in mammals, before (ii) reviewing Pparg-mediated adipogenesis in zebrafish. Finally, we (iii) mine the aquaculture literature to compare and contrast Pparg-mediated adipogenesis in aquaculturally relevant teleosts. Our goal is to highlight evolutionary similarities and differences in adipose biology that will inform our understanding of the role of adipose tissue in obesity and related disease.

Keywords: adipose, zebrafish, adipogenesis, pparg, aquaculture

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****Correspondence:***
James E. N. Minchin
james.minchin@ed.ac.uk

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Adipogenesis—the process of progenitor cell differentiation to generate mature, lipid-laden adipocytes (fat cells) is central to physiological homeostasis. Dysregulation of adipogenesis and a reduced capacity to sequester lipid within cytoplasmic lipid droplets (LDs) of adipocytes leads to lipodystrophy, ectopic lipid deposition, systemic metabolic dysfunction, and increased risk for developing diabetes and cardiovascular disease (1–3). Members of the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors have paramount roles in lipid metabolism; and, in particular, PPAR gamma (PPARG) is critical for adipogenesis. Much is known on PPARG-mediated adipogenesis in mammalian model systems; however, extensive research has also been conducted on adipogenesis in fish species relevant to the aquaculture industry. The aim of this mini-review is to integrate findings on Pparg-mediated adipogenesis from the aquaculture industry into the larger biomedical-centered literature. This review is focused on adipogenesis in white adipose

tissue (WAT); however, adipogenesis in brown adipose has also recently been reviewed (4).

PPARG: A MASTER REGULATOR OF MAMMALIAN ADIPOGENESIS

Peroxisome proliferator-activated receptor gamma is both necessary and sufficient for WAT adipogenesis in mammals, and is considered a "master regulator" of adipogenesis. In mouse, Pparg plays an important role in placental vascularization, monocyte differentiation, and cardiac development (5, 6); however, Pparg is also required for adipogenesis both *in vitro* (7) and *in vivo* (5, 7, 8). Naturally occurring mutations within the *PPARG* coding sequence can lead to PPARG loss-of-function (LOF), severe lipodystrophy, insulin resistance, and diabetes in humans (2, 3, 9). Further, adipocyte-specific deletion of *Pparg* in mouse results in the complete absence of WAT (8). Strikingly, expression of *Pparg*, together with provision of an activating ligand, is sufficient to initiate an adipogenic program and maintain an adipocyte phenotype in previously non-adipogenic cells (10, 11). Therefore, PPARG has a central role in mammalian adipogenesis, typified by PPARG LOF in humans, which is associated with severe lipodystrophy, and metabolic dysfunction and disease.

In mammals, *PPARG* exists as two isoforms, G1 (γ1) and G2 (γ2), derived from a single gene, and transcribed by distinct promoters (12, 13). *PPARG2* contains additional 30 amino acids at the N-terminal of *PPARG1* and is specific to WAT—whereas, *PPARG1* can be expressed at low levels in non-WAT tissues (12, 13). Both γ1 and γ2 isoforms can instruct a similar adipogenic gene expression program; however, *PPARG2* exhibits a quantitatively greater adipogenic ability (14). Structurally, *PPARG* contains six protein domains (domains A–F) (**Figure 1A**): the N-terminal A/B-domain contains the ligand-independent transactivation function 1 (AF-1); the C-domain is a highly conserved DNA-binding domain (DBD), consisting of two type II zinc fingers; the D-domain is a flexible hinge region; the E-domain contains the AF-2 ligand-binding domain (LBD); and at the C-terminus, a small F-domain has been shown to interact with cofactors (15).

The function of each PPARG domain has been extensively studied. The N-terminal AF-1 domain regulates the transcriptional activity of *PPARG* by (i) influencing Pparg ubiquitination and receptor turnover (16), (ii) controlling localization of Pparg to distinct cellular compartments (17, 18), (iii) facilitating communication with the LBD and enhancing ligand-dependent transcription (19), and (iv) recruitment of coactivators (20, 21) and corepressors (22). Importantly, many AF-1 focused regulatory mechanisms rely on posttranscriptional modifications of PPARG and can be both ligand-dependent or ligand-independent (23). Accordingly, inhibiting phosphorylation of serine 112 (S112) of Pparg2 in mouse results in improved insulin sensitivity when fed a high-fat diet (24). In addition, humans carrying a mutation blocking phosphorylation of an equivalent serine residue also have improved insulin sensitivity (18, 25). Together, these studies show that multiple diverse mechanisms converge on the AF-1 domain to regulate the transcriptional activity, and insulin sensitizing potential, of PPARG.

The transcriptional activity of PPARG is highly dependent on its DBD. Mutations within the DBD of human *PPARG* inhibit the transcriptional potential of PPARG and patients carrying such mutations exhibit severe insulin resistance and an increased risk for diabetes (3, 9, 26). The core DBD is highly conserved between different nuclear receptors; both within the PPAR family, and between distinct nuclear receptor families (27). Indeed, some nuclear receptors bind identical DNA motifs (28) and, in support, Pparg retains the ability to conduct an adipogenic program even when fused to alternative DBDs (29). These data suggest that the specificity of PPARG-mediated gene activation is not entirely contained within the DBD. Pparg generally binds DNA as obligate heterodimers with members of the retinoid X receptor (RXR) family of nuclear receptors (30), although some evidence suggests Pparg can also function as a homodimer (31). Strikingly, mutations within RXR DBDs have severe consequences for the transcriptional activity of PPARG:RXR heterodimers, suggesting the DNA-binding activity of RXR is also central to PPARG function (32). PPARG/RXR heterodimers bind to cis-acting peroxisome proliferator response elements (PPREs) containing direct repeats of 5′-AGGTCA-3′ separated by *n* nucleotides (DR*n*) (**Figure 1B**) (33–35). Along with an AAACT flanking sequence situated immediately 5′ to the core DR*n* motif, which helps guide selective PPAR binding (36–38). ChIP-Seq analyses for Pparg binding have identified DR1 as the canonical motif for PPARG binding (33, 39, 40), and binding is dependent on the sequence, and affinity, of specific DR1 motifs (40). Wider chromatin organization and accessibility also appear key for PPARG-mediated adipogenesis, as extensive chromatin remodeling occurs early in adipogenesis, prior to Pparg binding, and creates "hotspots" primed for future Pparg binding (41).

THE LBD OF PPARG, KNOWN LIGANDS, AND MODULATION OF TRANSCRIPTIONAL ACTIVITY

Ligand binding regulates the transcriptional activity of PPARG and, as such, the LBD is central to the ability of PPARG to direct adipogenesis and regulate insulin sensitivity. Numerous lipid metabolites have been identified as PPARG ligands; including, polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid and linoleic acid, eicosanoids, and 15-deoxy-Δ12,14-prostanglandin J2 [PGJ(2)] (10, 42–47). Many of these ligands bind PPARG with low affinity and are unlikely to be present at concentrations required to activate PPARG *in vivo* (48). However, derivatives of linoleic acid have been shown to potently bind Pparg, and may represent an endogenous ligand for PPARG (49, 50). Intriguingly, a cAMP-induced, transient Pparg ligand is produced by 3T3-L1 adipocytes during the early stages of adipogenesis (51) and drives Pparg-mediated progenitor differentiation. Furthermore, this transient Pparg ligand is suggestive of a positive feedback loop, which is autonomous to adipocytes and acts in a paracrine manner. Synthetic ligands

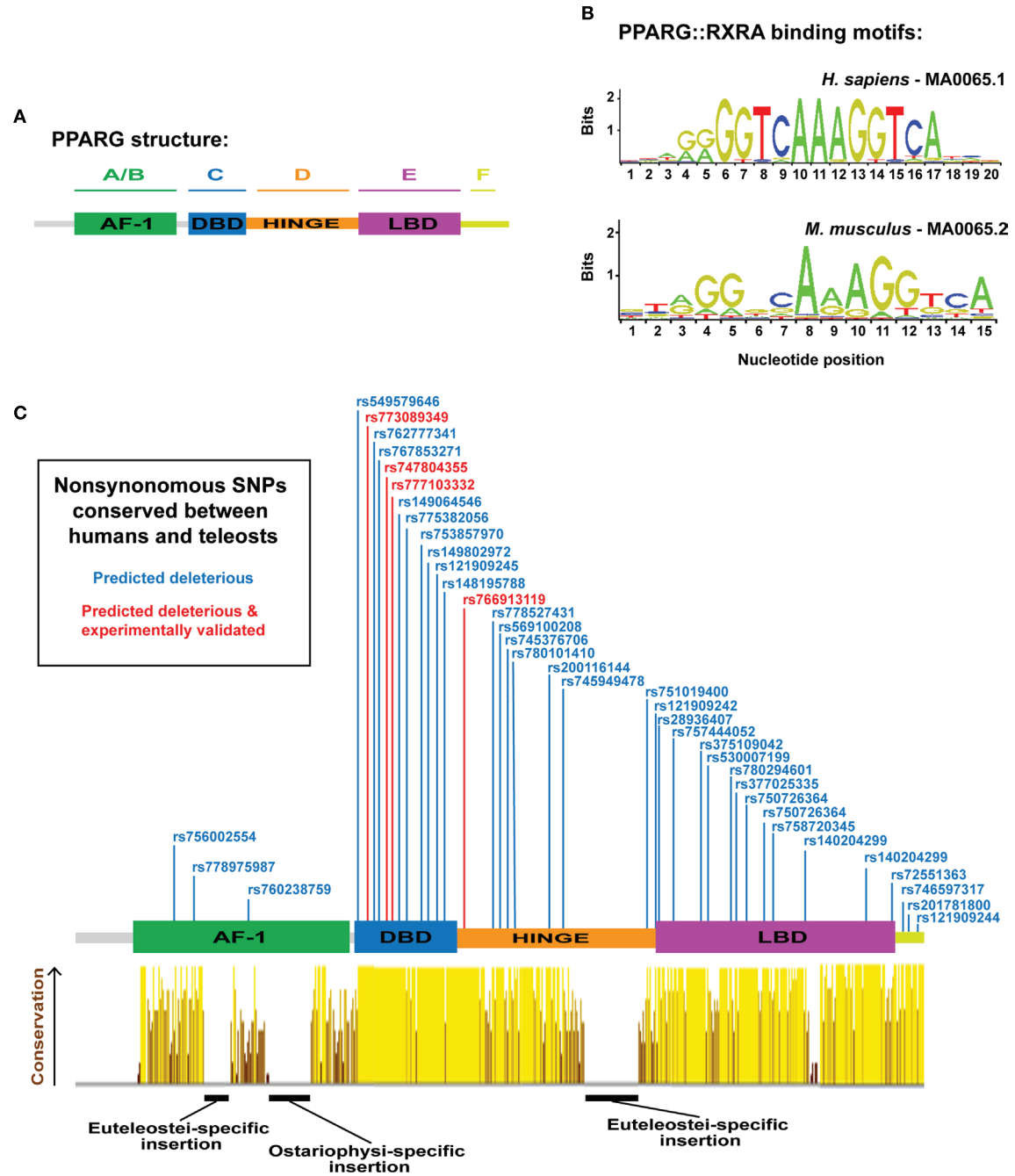

FIGURE 1 | Overview of peroxisome proliferator-activated receptor gamma (PPARG) structure, DNA-binding specificity, and identification of human *PPARG* genetic variation conserved to fish. **(A)** Schematic illustrating the domain organization of human PPARG. **(B)** PPARG:RXRA-binding motifs for human (upper motif) and mouse (lower motif). Motifs are derived from the JASPAR database (http://jaspar.genereg.net/). **(C)** PPARG domain structure with dbSNPS predicted to be deleterious using SIFT and Polyphen, and conserved to zebrafish, Nile tilapia, and fugu. Red single nucleotide polymorphisms (SNPs) indicate functional verification (9). Yellow–brown histogram indicates the degree of conservation in PPARG between human, mouse, coelacanth, spotted gar, zebrafish, fugu, and Nile tilapia. Height and color indicate the degree of conservation.

also bind and regulate PPARG activity. Most prominently, thiazolidinediones (TZDs) are potent PPARG agonists that lower hyperglycemia, decrease plasma triacylglycerides and free fatty acids, and increase insulin sensitivity (51). As such, TZDs have incredible potential to improve insulin sensitivity and glucose homeostasis in diabetic patients. However, many TZDs have been withdrawn from clinical use, or are under extensive review, owing to toxic side effects (52, 53). In particular, TZDs induce adipogenesis in patients and can lead to increased weight gain (51, 54, 55).

ZEBRAFISH AS A MODEL TO STUDY Pparg-MEDIATED ADIPOGENESIS

As a complement to mammalian model systems, the zebrafish has recently emerged as a tractable model for studying adipogenesis *in vivo*. Zebrafish possess adipose tissue that is morphologically similar to mammalian WAT (56–58), and which is deposited in anatomically homologous regions to mammalian WAT (56, 58, 59). Further, zebrafish adipose responds to nutritional manipulation, suggesting a conserved role for WAT as an energy store or supply during periods of caloric excess or restriction (56, 60, 61). Zebrafish possess a single *pparg* ortholog on chromosome 11 (47), which exhibits 67% overall similarity to human *PPARG* (47). The LBD and DBD of zebrafish *pparg* show especially high conservation to human *PPARG* (80.5 and 94.3% of amino acids are identical in LDB and DBD, respectively) (**Figure 1C**) (47, 62). The N-terminal AF-1 domain shows much less conservation between zebrafish and human (**Figure 1C**) (47, 62); however, this is unsurprising as the AF-1 domain is well known to exhibit low similarity even between more closely related species (23). Interestingly, zebrafish *pparg* contain multiple regions with amino acid insertions not present in mammalian *PPARG*, suggesting the potential for neo-functionalization of fish Pparg (**Figure 1C**) (47). Importantly, zebrafish *pparg* mRNA is detected in adipocytes (56, 58, 60). Moreover, many compounds known to stimulate mammalian Pparg also modulate zebrafish *pparg* mRNA; including, organotin compounds such as tributyltin (63, 64), halogenated analogs of bisphenol A (65), and PGJ(2) (66). Construction of a zebrafish transgenic line expressing the human PPARG LBD fused to a Gal4 DBD exhibits increased transcriptional activity after treatment with TZDs including rosiglitazone, pioglitazone, or troglitazone (67), thus suggesting that ligand-dependent coactivators of Pparg are conserved and functional in zebrafish. Intriguingly, recent work showed that treatment of zebrafish with the TZD rosiglitazone increased adiposity, suggesting that the role of Pparg in stimulating zebrafish adipogenesis may also be conserved to mammals (68).

ADIPOGENESIS AND THE AQUACULTURE INDUSTRY

The use of zebrafish as a biomedical model system to study adipogenesis is at a nascent stage, and many gaps exist in our understanding. However, the aquaculture industry has conducted extensive investigation into adipogenesis in closely related fish species, owing to the fact that adipogenesis affects meat quality, animal health, and harvest yields (69). Aquaculture is defined as the farming of aquatic organisms; including fish, crustaceans, mollusks, and plants. The aquaculture industry contributes ~50% of the world's aquatic food source (70); thus representing a significant proportion of all food consumed worldwide (71). For this review, we focus on teleost species most closely related to zebrafish. For a comprehensive review of teleost phylogeny, we refer you to the following articles (72, 73). The teleost lineage is divided into three branches; clupeocephalans (including the majority of teleosts); and the relatively minor elopomorpha (including eels and tarpons), and osteoglossomorpha (fish possessing toothed or bony tongues) (72, 73). For this review, we only consider clupeocephalans, which belong to two main lineages; ostariophysi and euteleostei. In 2010, freshwater fish production was dominated by ostariophysi such as silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), and the euteleostei, Nile tilapia (*Oreochromis niloticus*). The euteleostei Atlantic salmon (*Salmo salar*) was the most farmed saltwater fish (70). Extensive regional differences exist in the species of fish farmed; for example, Asian countries primarily farm ostariophysi carp species, accounting for 89% of world aquaculture (70). By contrast, Mediterranean countries farm euteleostei species including gilthead sea bream (*Sparus aurata)* (74), European sea bass (*Dicentrarchus labrax*), and flathead gray mullet (*Mugil cephalus*) (70). Northern European countries, primarily farm euteleostei salmonid species such as Atlantic salmon and rainbow trout (*Oncorhynchus mykiss)* (70). A characteristic of teleosts is a teleost-specific third whole genome duplication (Ts3R), which is estimated to have occurred ~225–333 million years ago (72, 75). Recent genome sequencing projects have revealed that, in addition to Ts3R, certain teleost lineages have undergone further extensive genome duplication; including, salmonids (76, 77) and common carp (78). Genome duplications are hypothesized to underlie the dramatic radiation of teleosts and often lead to multiple gene copies, under reduced selective constraint, and thus receptive to neo-, non-, and sub-functionalization of the ancestral gene role (73).

EXTENSIVE SYNTENIC CONSERVATION AT TELEOST *pparg* LOCI

As *PPARG* exerts such a central role in mammalian adipogenesis, we first wished to assess whether duplicated teleost *pparg* paralogs have been retained. Only a single *pparg* ortholog was identified in 9 (of 10) teleost fish species with genome data on Ensembl (79). These data are striking, as the other members of the PPAR family (*ppara* and *ppard*) have been extensively duplicated, with paralogs retained, in teleosts (Ensembl Gene Tree: ENSGT00870000136388) (47). Teleost species with a single *pparg* ortholog include, ostariophysi such as zebrafish (cyprinidae); and euteleostei such as, Atlantic cod (gadiformes), pufferfish (tetradontiformes, both fugu and tetraodon), stickleback (gasterodae), and Nile tilapia (cichlidae) (Ensembl Gene Tree: ENSGT00870000136388) (47). The single teleost species with a retained *pparg* paralog is the ostariophysi blind cavefish (*Astyanax mexicanus*) of the chariciformes order (**Figure 2**). The striking loss of duplicated *pparg* genes in the majority of teleosts suggest stringent selective pressures for retaining Pparg copy number and function. To construct a predicted ancestral *pparg* locus, we examined synteny at the *Pparg* locus in tetrapods (mouse and human), a basal sarcopterygian (coelacanth), an actinopterygian holosteian basal to teleosts (spotted gar), and a chondrichthyan, cartilaginous fish (elephant shark) (**Figure 2**). Following the Ts3R, two *pparg* loci can be identified which each share extensive synteny to the predicted ancestral locus (**Figure 2**). Remarkably, in both ostariophysi and euteleostei, *pparg* appeared to be retained at a specific single locus (locus 1) (**Figure 2**), with the

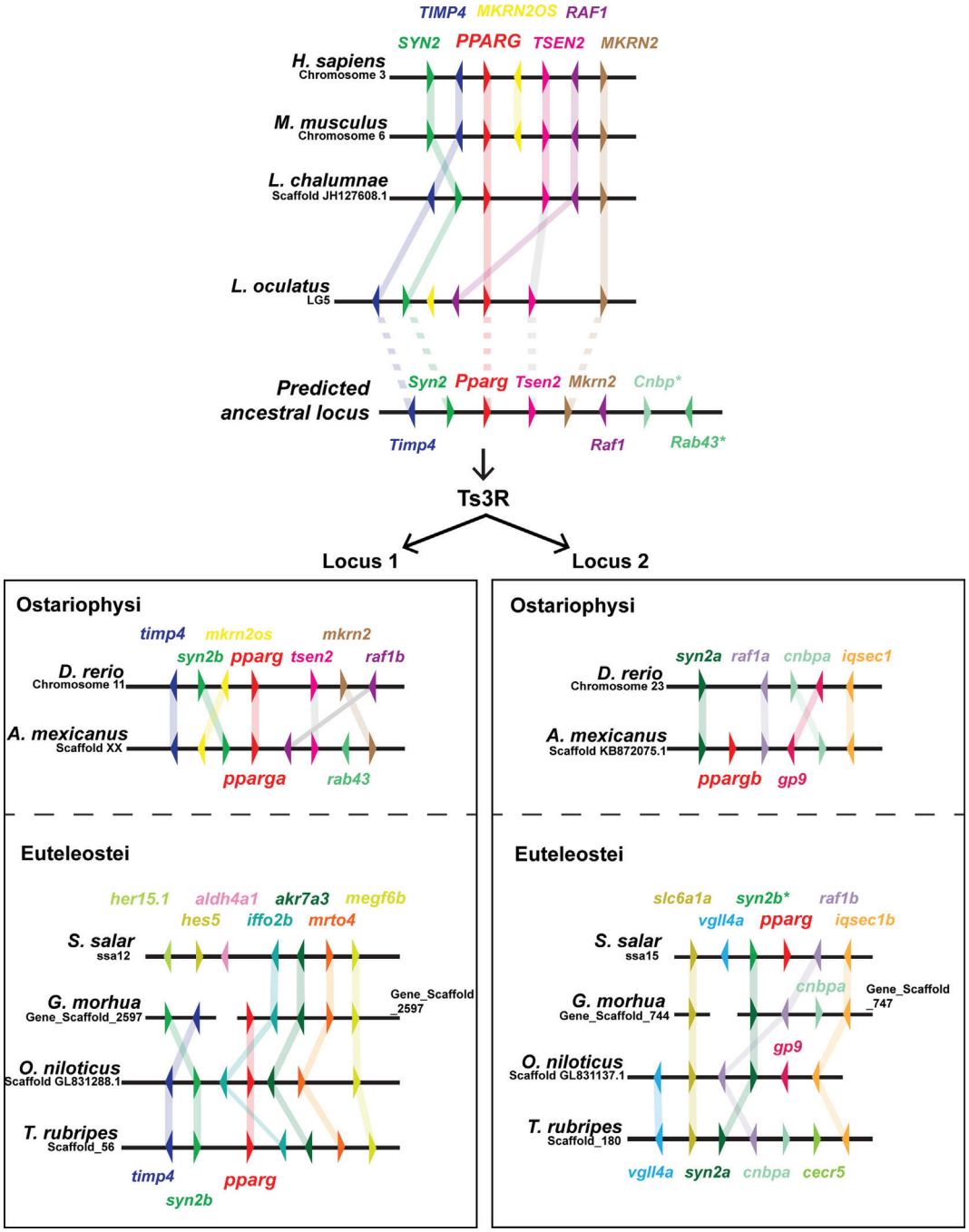

FIGURE 2 | Extensive shared synteny between mammals and fish at the *Peroxisome proliferator-activated receptor (PPARG)* locus. The predicted ancestral locus was inferred from comparing the loci documented in the figure, together with the chondrichthyan elephant shark (*Callorhinchus milii*) locus (not shown). The *C. milii* locus contained *Cenp* and *Rab43* genes (indicated with an asterisk). Ts3R indicates the teleost-specific genome duplication. Note the inversion of *TIMP4* and *SYN2* upstream of *PPARG* in the mammalian lineage. Duplicated *raf1* paralogs (*raf1a* and *raf1b*) are only retained in zebrafish and cavefish.

exception being Atlantic salmon, which retained *pparg* at locus 2 (**Figure 2**). Strikingly, in all euteleostei species examined, the region downstream of *pparg* contained multiple new genes not found in other species (*iffo2b*, *akr7a3*, *mrto4*, *megf6b*), suggesting an euteleostei-specific recombination event that completely changed the sequence downstream of *pparg* (**Figure 2**).

SEQUENCE HOMOLOGY OF TELEOST *pparg* GENES

Extensive synteny at teleost and mammalian *PPARG* suggest the locus is under considerable selective constraint; therefore, we next assessed whether the PPARG amino acid sequence

was equally conserved. We aligned PPARG sequences from representative tetrapods, a basal sarcopterygian, a holosteian, an ostariophysan, and euteleostians (**Figure 1C**). As expected, not all elements of the mammalian PPARG sequence were conserved from mammals to fish (**Figure 1C**). Strikingly, the DBD, hinge region, and LDB exhibited high levels of conservation from mammals to fish (**Figure 1C**). However, we found a large insertion into the hinge region specific to the euteleosts, fugu, and Nile tilapia (**Figure 1C**). We further found euteleost-specific and zebrafish-specific insertions into the AF-1 domain (**Figure 1C**). Aside from these three regions inserted into teleost Pparg, conservation was extensive (**Figure 1C**). Previous studies have identified that the LDB of fish Pparg (red sea bream and Nile tilapia) often contains additional amino acids compared to human PPARG (80, 81). However, the DBD of PPARG is well conserved between fish and mammals (81). These distinct patterns of conservation have been suggested to reflect the fact that PPARG target genes are well conserved, while there may be greater diversity in ligands, which activate PPARG (81) and may explain why some human PPARG agonists are unable to stimulate *pparg* expression across teleost species (81, 82).

CONSERVATION OF Pparg AMINO ACIDS AFFECTED BY DISEASE-ASSOCIATED GENETIC VARIATION IN HUMANS

The considerable sequence conservation between mammalian and teleost *PPARG* suggest that residues affected by naturally occurring, disease-associated, mutations in human *PPARG* may also be conserved in teleosts. To address this, we (i) collected all known human *PPARG* single nucleotide polymorphisms (SNPs) from dbSNP (347 SNPs), (ii) filtered these SNPs to identify 73 SNPs predicted to have a highly deleterious effect on PPARG function, (iii) identified amino acids altered by the deleterious SNPs, which were conserved to teleosts (39 SNPs/amino acids), (iv) filtered the conserved deleterious SNPs to ones that had been experimentally verified to have an effect on adipogenesis and PPARG function in humans (4 SNPs) (9). The resulting collection of SNPs (**Figure 1C**) represent ideal initial targets for modeling Pparg function during teleost adipogenesis and highlight the highly conserved nature of PPARG from mammals to fish.

EXPRESSION DYNAMICS OF *pparg* IN FARMED FISH SPECIES

Although little is known regarding the expression of *pparg* in zebrafish, extensive experiments have been undertaken in farmed fish species to determine the dynamics of *pparg* during adipogenesis. In grass carp (ostariophysi), gilthead sea bream, large yellow croaker, and Atlantic salmon (all euteleostei), Pparg/*pparg* appeared coincident with early stages of adipocyte differentiation and increased gradually throughout adipogenesis (69, 83–87). These dynamics mirror those observed in mammalian 3T3-L1 cells, where *Pparg* mRNA is present at low levels in adipocyte progenitors, and increases upon stimulation of adipogenesis (10, 88). Similar to 3T3-L1 cells, *cebpb* mRNA was also induced prior to *pparg* during differentiation of adipocyte progenitors in the euteleostei, cobia (*Rachycentron canadum*), and in Atlantic Salmon (89). By contrast, in red sea bream (*Pagrus major*) (another euteleostei), *pparg* mRNA appeared to remain stable during a 10-day preadipocyte culture; however, isolated cells were maintained for 4 days prior to induction of adipogenesis; therefore, it remains possible that fluctuations in *pparg* mRNA expression occurred prior to analysis (80). However, by this method, accumulation of LDs appeared late and was not robust (80). Furthermore, in gilthead sea bream (euteleostei), *pparg* mRNA decreased in preadipocytes upon the addition of an adipogenic cocktail (83). The experimental reasons for differences in teleost pparg expression dynamics is unclear; however, in most fish species, the induction and maintenance of *pparg* mRNA during adipogenesis appears largely conserved to mammals. Furthermore, multiple *pparg* isoforms have been found in Nile tilapia and Atlantic salmon (both long and short isoforms) (81, 85, 90, 91), suggesting that teleost *pparg* is alternatively spliced similar to mammalian *PPARG*.

THE FUNCTIONAL ROLE OF Pparg-MEDIATED ADIPOGENESIS IN FARMED FISH SPECIES

In addition to expression dynamics, extensive experiments on Pparg-mediated adipogenesis have been conducted in aquaculturally relevant fish species. Much of the evidence for Pparg-mediated adipogenesis in fish species derive from primary adipocyte progenitor, or "preadipocyte," cell culture systems. Primary preadipocyte cultures have been established in multiple species, including; Atlantic salmon (84), red sea bream (92), rainbow trout (93), grass carp (94), large yellow croaker (69), gilthead sea bream (74), and cobia (89). In all of these systems, primary stromal-vascular cells were isolated from visceral adipose tissue (VAT) [the VAT source most likely equates to the pancreatic VAT and abdominal VAT deposits described in zebrafish (59)]. The preadipocyte culture methods closely follow established methods for the growth and differentiation of mammalian 3T3-L1 cells (95) and enable the incubation of preadipocytes, and differentiated adipocytes, with a range of pharmacological and biological agents to study potential roles during Pparg-mediated adipogenesis.

To our knowledge, no functional genetic data on the role of Pparg in farmed fish species is currently published. However, extensive data exist on pharmacological manipulation of Pparg and adipogenesis. Troglitazone, an insulin sensitizing TZD, potently stimulates preadipocyte differentiation in porcine and human preadipocytes (96, 97); and co-incubation with insulin induced preadipocyte differentiation in rainbow trout (98). A second TZD tested in teleosts, ciglitazone, induced *pparg* expression in preadipocytes of red sea bream (80), suggesting that TZDs induce both *pparg* and adipogenesis in teleosts. The role of several pro- and anti-adipogenic factors have also been studied in fish. Insulin has potent stimulatory effects on *Pparg* mRNA levels, and the proliferation and differentiation of mammalian preadipocytes, acting through IRS1 and the MAPK pathway (99, 100). In large yellow croaker (Percomorpha),

insulin increased *pparg* mRNA, along with stimulating preadipocyte proliferation and differentiation (69). In accordance with mammalian data, Insulin inhibited lipolysis in differentiated adipocytes of rainbow trout (69, 101). Similarly, insulin also stimulated the differentiation of adipocyte progenitors and lipid accumulation in red sea bream (92). These findings suggest that insulin has a conserved role in stimulating *pparg* expression and promoting adipogenesis. Insulin-mediated induction of *pparg* and adipogenesis is also potentially conserved to other fish species, as the insulin-IRS1-MAPK signaling axis is also functional in rainbow trout adipocytes (102). However, unlike in rainbow trout, insulin had no effect on adipocyte lipolysis in gilthead sea bream (103). Tumor necrosis factor alpha (TNFA) is secreted from mammalian adipocytes and inhibits adipogenesis (104). Treating large yellow croaker preadipocytes with human TNFA reduced *pparg* mRNA levels, suppressed proliferation and differentiation, and stimulated lipolysis in differentiated adipocytes (69). An anti-adipogenic role for TNFA was also found in rainbow trout adipocytes at both RNA and protein levels (93, 105). PUFAs inhibit the proliferation and differentiation of mammalian preadipocytes (106, 107). DHA, an omega-3 fatty acid, was used in the treatment of large yellow croaker preadipocytes and led to decreased *pparg* mRNA levels and reductions in cell proliferation (69). It has further been shown that DHA stimulates lipolysis in 3T3-L1 preadipocytes (108); however, DHA did not exert a positive effect on lipolysis within large yellow croaker adipocytes and was actually observed to have an anti-lipolytic effect (69). Interestingly, DHA reduced lipid accumulation in Atlantic salmon adipocytes, although a mechanisms by which this occurred was not identified (85). Conversely, an analog of the saturated fatty acid palmitate, 2-bromopalmitate, increased *pparg* mRNA (red sea bream) (80). *pparg* cooperates with *rxra* to transcribe *fabp4* suggesting that fish Pparg also functions as an obligate heterodimer with Rxr proteins to guide adipogenic gene expression (Nile tilapia) (81). In Atlantic salmon, *pparg* mRNA was induced after addition of liver X receptor (lxr) agonists (109), suggesting Pparg:Lxr coordinate gene expression in teleosts as they do in mammals (110).

CONCLUSION AND FUTURE DIRECTIONS

Peroxisome proliferator-activated receptor gamma is a master regulator of adipogenesis in mammals, and mutations deleterious to PPARG function lead to increased susceptibility to diabetes and cardiovascular disease. In this review, we assessed the literature on Pparg-mediated adipogenesis in teleost fish species, including the biomedical model system, zebrafish, and multiple aquaculturally relevant farmed fish species. We found a high degree of synteny and conservation at/in *pparg* in teleost fish, along with evidence of conserved expression, regulation, and function derived from primary preadipocyte culture studies. Altogether, information on the role of Pparg gleaned from aquaculturally relevant species is likely to be highly informative for future zebrafish and mammalian biomedical studies on adipogenesis.

AUTHOR CONTRIBUTIONS

Background literature research, writing, and review were conducted by RW, PT, and JM. Sequence and locus analysis was conducted by JM.

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Modeling Pancreatic Endocrine Cell Adaptation and Diabetes in the Zebrafish

*Lisette A. Maddison and Wenbiao Chen**

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, USA

Glucose homeostasis is an important element of energy balance and is conserved in organisms from fruit fly to mammals. Central to the control of circulating glucose levels in vertebrates are the endocrine cells of the pancreas, particularly the insulin-producing β-cells and the glucagon producing α-cells. A feature of α- and β-cells is their plasticity, an ability to adapt, in function and number as a response to physiological and pathophysiological conditions of increased hormone demand. The molecular mechanisms underlying these adaptive responses that maintain glucose homeostasis are incompletely defined. The zebrafish is an attractive model due to the low cost, high fecundity, and amenability to genetic and compound screens, and mechanisms governing the development of the pancreatic endocrine cells are conserved between zebrafish and mammals. Post development, both β- and α-cells of zebrafish display plasticity as in mammals. Here, we summarize the studies of pancreatic endocrine cell adaptation in zebrafish. We further explore the utility of the zebrafish as a model for diabetes, a relevant topic considering the increase in diabetes in the human population.

Keywords: zebrafish, glucose homeostasis, β-cell, α-cell, plasticity, regeneration

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***Correspondence:**
Wenbiao Chen
wenbiao.chen@vanderbilt.edu

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INTRODUCTION

Glucose homeostasis is a central physiological mechanism important to maintain proper energy balance and cellular function. While the bulk of the information concerning glucose homeostasis comes from studies in mammals, homeostatic mechanisms are active in many organisms including teleost fish [reviewed in Ref. (1)], which underscores the importance of maintaining tight control of circulating glucose. Maintenance of glucose homeostasis is a coordinated effort between multiple organ systems including the brain, skeletal muscle, liver, and the pancreatic endocrine cells. By appropriately secreting glucagon and insulin into the circulation to regulate the production and uptake of blood glucose, respectively, the pancreatic α- and β-cells play a central role in glucose homeostasis. Interestingly, in conditions where demand for insulin or glucagon exceeds the current secretory capacity, glucose homeostasis is still maintained through increased function and number of α- or β-cells. The regulatory mechanisms of this adaptation or compensation, particularly the compensatory increase of cell number, are not fully defined.

The zebrafish has been firmly established as an attractive animal model to explore questions in developmental biology. This has been aided by the low cost, high fecundity traits of the model (2) as well as the optical transparency (3–6), ease of genome manipulation (7–10), and the amenability toward small molecule screens (11–13). These attractive traits of zebrafish have also inspired investigators to study other biological questions including glucose homeostasis.

GLUCOSE HOMEOSTASIS IN ZEBRAFISH

The systems regulating glucose homeostasis in zebrafish are similar to those of mammals in composition, ontogeny, and function. As in mammals, glucose homeostasis in zebrafish involves brain, skeletal muscle, liver, and the pancreatic endocrine cells. For example, the glucose transporter Glut2 has been found to be critical for proper brain development (14) supporting the importance of appropriate glucose control in the brain. In skeletal muscle, glucose transporters are present (15) and glucose uptake has been found to be insulin sensitive (16), suggesting that like in mammals, skeletal muscle is a major site of glucose disposal. The liver has a critical function in glucose homeostasis as it both uses and produces glucose. Gluconeogenesis is dynamically regulated in the zebrafish liver (17), and glucose-regulated *pck* promoter activity has been leveraged to screen for compounds that impact glucose production (18). Furthermore, risk alleles for altered fasting blood glucose in humans have been found to increase gluconeogenesis in the liver (19). This again supports the conservation of regulatory pathways of glucose homeostasis. Coordinating many aspects of glucose homeostasis are the pancreatic endocrine cells. Of primary focus has been the insulin-producing β-cells and the glucagon-producing α-cells. In the zebrafish, these cells are present as early as 1 day post fertilization and their development is regulated by pathways similar to those for mammals (20–22). The conservation of glucose homeostasis system between zebrafish and mammals supports that zebrafish is a relevant model to study mechanisms of glucose homeostasis, including aspects of pancreatic endocrine cell biology.

PROMOTING β-CELL PROLIFERATION AND DIFFERENTIATION IN LARVAL STAGES WITH SMALL MOLECULES: LINEAGES AND PATHWAYS

Replenishing the β-cell mass has been an active area of investigation for many years as approaches to treat both type 1 and type 2 diabetes. To increase β-cells in adults, often the approach is to manipulate pathways active during development. But there is also a need to understand the mechanisms that promote an increase of β-cells post development, either through neogenesis or through increased proliferation as different mechanisms may be active for adaption to changes in physiology. In embryonic and early larval stages, the pancreatic endocrine cells are primarily coalesced in a single large islet referred to as the principal islet (23). At later larval stages, additional secondary islets are present (24). These secondary islets arise from centroacinar cells in the pancreatic duct (24–28). These cells are Notch sensitive (24, 25, 28) and express markers of endocrine precursors including Nkx6.1 (26) and Nkx2.2 (25). Inducing formation of secondary islets as a way to uncover pathways important in stages beyond early development was the basis of a compound screen (29, 30) and a component of another large-scale screen (30). These screens took advantage of the optical transparency of the zebrafish and transgenic lines that mark the pancreatic endocrine cells. The first screen revealed an important role for retinoic acid signaling in the differentiation of endocrine progenitors (29). Follow-up studies have shown this pathway is functionally conserved in humans (31) and that retinoic acid signaling regulated Sox9b (32), an important transcription factor in endocrine cell differentiation (33). The high-throughput screen was based on increasing endocrine cells in both the principal and secondary islets and yielded several candidate pathways controlling endocrine cell differentiation including NFκB signaling and serotonin signaling (30). Both screens captured changes in both proliferation of endocrine cells and differentiation of precursors. Another compound screen aimed solely to increase β-cell proliferation (34) and relied on expression of markers indicative of the different phases of the cell cycle (35). This screen also identified retinoic acid and serotonin signaling, as well as glucocorticoids, as regulators of proliferation (34). These compound-screening approaches identified both molecules with functions in development, such as Sox9, and also pathways such as serotonin and NFκB which likely also function in post-developmental stages. Ultimately, these compound screening approaches using zebrafish may provide molecules that can be targeted to increase β-cell mass as a treatment for adults with diabetes.

PANCREATIC ENDOCRINE CELL PLASTICITY IN RESPONSE TO INSUFFICIENT HORMONE ACTION

Proper development of the pancreatic endocrine cells is unquestionably crucial to establish homeostatic control. But equally important is understanding the underlying mechanisms that allow adaptation to different physiological stresses, in other words, plasticity. For example, in mammals, the β-cell mass increases during pregnancy (36, 37), with high-fat diet in mice (38–40), and in non-diabetic obese humans (41, 42). With obesity the increase in β-cell mass is an adaptive mechanism to compensate for insulin resistance (43). In type 2 diabetic obese patients, the β-cell mass is decreased compared to non-diabetic counterparts (41, 44), which has been attributed to β-cell death or dedifferentiation, in other words, the loss of β-cell identity (45–47). Zebrafish have been shown to also have β-cell compensatory responses. For example, in states of overnutrition, through culturing in glucose solution or in chicken egg yolk emulsion, the number of β-cells increases (48–53). This treatment also causes β-cell increase in older larvae (52). In juvenile fish, a high calorie diet can promote β-cell proliferation and secondary islet formation (54), indicating that the overnutrition-induced β-cell expansion is not limited only to early larval stages. The compensatory increase in β-cells did not occur with intermittent exposure to the same diets, as would be found in meal-type feeding (52). Consistent with overnutrition as the trigger for the compensatory response, the expansion of the β-cells has been found to be dependent on the nutrient-secretion coupling apparatus in preexisting β-cells (49). Stimulating β-cell secretion through pharmacologic or genetic means increased the number of β-cells in the absence of overnutrition (49). Conversely, reducing β-cell activity inhibited the β-cell expansion in the presence of overnutrition (49). The rapid expansion of β-cells was not through stimulation of β-cell proliferation (49, 52)

based on incorporation of EdU, which suggested differentiation of resident precursors. Lineage tracing experiments indicated that these new cells did not arise from the centroacinar cells in the pancreatic duct (50) but arise from cells with *mnx1* and *nkx2.2* promoter activity (50, 52) likely residing within the principal islet. The non-canonically secreted FGF1 has been proposed to be a candidate molecule stimulating differentiation of these resident endocrine precursors (50). Mutation of *fgf1* abolished the overnutrition-induced β-cell expansion but did not alter the baseline β-cell number, and this could be rescued through transgenic expression of human FGF1 (50). Furthermore, when FGF1 was altered to allow for secretion through the canonical secretion pathway, the basal number of β-cells was increased without overnutrition stimulation (50). Intact leptin signaling is important for these responses (53) as leptin receptor mutant larvae had a higher number of β-cells developmentally but did not increase number of β-cells with high-fat diet feeding. In addition, blocking insulin expression through morpholino injection or through expression of a dominant-negative IRS2 protein increased the number of β-cells during embryonic stages (55). Furthermore, in adult fish with skeletal muscle insulin resistance, there was an initial increase in the number of β-cells (16). These studies suggest that with an increased need for insulin function, either due to elevated nutrient intake or through inhibition of insulin signaling, zebrafish increase the number of β-cells as an adaptive mechanism, similar to what has been observed in mammals. These conserved responses indicate that zebrafish are a useful model to study β-cell adaptive mechanisms, and with the utility of zebrafish in genetic and pharmacological approaches, the role of candidate molecules, such as FGF1, can be rapidly assessed.

Although β-cells are often the focus in glucose homeostasis, the glucagon-producing α-cells also have an important role in modulating glucose production. Glucagon acts as a counterregulatory hormone to insulin, and modulating glucagon signaling is becoming an increasingly attractive approach for diabetic treatments (56). It has been found in mice that the number of α-cells increases with blockade of glucagon signaling either by knocking out glucagon, the glucagon receptor, or Gsα, or by impairing glucagon receptor function with antagonists or monoclonal antibody treatment (57–61). With the β-cells, this suggests an adaptive response to the decreased effectiveness of glucagon. This is also true in zebrafish, where mutation of the two glucagon receptors resulted in an increased number of α-cells (62). The adaptive responses to nutritional or hormonal status in both β-cells and α-cells reflect conservation of metabolic responses between mammals and zebrafish. This further indicates that pathways and molecules identified in zebrafish may indeed be relevant to mammals.

ROBUST β-CELL REGENERATION FOLLOWING ABLATION

Another aspect of the plasticity of pancreatic endocrine cells is regeneration following ablation. Zebrafish has tremendous regenerative capacities, including the β-cells. Similar to compensatory increase of β-cell mass, ablation-induced regeneration is also a response to unmet insulin demand, inferred by the high free glucose levels following ablation (25, 34, 63, 64) and underscores the conservation of β-cell function in zebrafish. Most commonly in zebrafish, β-cells are ablated through β-cell-specific expression of bacterial nitroreductase that converts the prodrug metronidazole to a genotoxic metabolite, resulting in death of the cells (65). There have been other approaches however, including inducible expression of a truncated Bid protein, tBid (49), and mosaic expression of diphtheria toxin (DTA) (66) in larval zebrafish as well as streptozotocin (STZ) treatment in adult fish (67, 68). β-cell regeneration occurs quickly following ablation (25, 65, 69, 70) and has been used as an approach to identify sources of new β-cells. Using lineage tracing in adult fish where β-cells were ablated through nitroreductase/metronidazole, it was determined that the centroacinar cells residing in the pancreatic duct are the primary source of new β-cells based on promoter activity of *nkx6.1* (26) or through Notch responsiveness (25). Ablation of β-cells in larval zebrafish also identified that transdifferentiation of α-cells to β-cells contributes to regeneration (55, 63, 64). Using a combination of pharmacological and morpholino approaches, the α-cell transdifferentiation was found to be dependent on glucagon but not through the modulation of gluconeogenesis (64). This seems to differ from mouse where α- to β-cell transdifferentiation is independent of glucagon signaling (71). The secreted factor IGFBP1 has been found to also enhance α- to β-transdifferentiation following ablation (63). Regeneration following ablation has also been used to identify compounds that increase regeneration (70). This study identified a compound that activates adensosine GPCR to increase proliferation. Interestingly, this compound had a limited capacity to induce β-cell proliferation during development, which may reflect the difference between embryonic immature β-cells and mature β-cells. These studies provide important insights into the origins of and specific pathways leading to new β-cells and exemplify the plasticity of pancreatic endocrine cells. The ablation and recovery studies also exemplify the robust regenerative capacity of zebrafish that is not fully recapitulated in mammalian models. β-cell ablation in mouse using STZ, pancreatic ligation, and partial pancreatectomy causes less robust regeneration (72–77). Understanding the keys that confer the regenerative capacity of zebrafish may provide avenues to boost the regenerative potential in mammals.

MODELING DIABETES IN THE ZEBRAFISH

It is always of interest to produce an animal model that accurately reflects a human disease. While studies in zebrafish have been extremely useful to identify molecules, pathways, and cell types that contribute to the plasticity of the pancreatic endocrine cells, to date there have been no models that accurately reflect the life history of a human with diabetes. This is exclusive of models reflecting the maturity-onset forms of diabetes including targeting NeuroD that models MODY6 (78), Pdx1 that models MODY4 (48), and Hnf1ba that models MODY5 (79). However, these forms of diabetes are quite rare in the overall patient population (80). The approaches to mimic type 1 diabetes by ablating β-cells have highlighted the regenerative nature of zebrafish, and

hyperglycemia is quickly reversed. Although stable expression of DTA can eliminate all β-cells, these fish have growth retardation and fail to thrive (54). For modeling type 2 diabetes, genetically induced muscle insulin resistance using dominant-negative IGF1 receptor (dnIGFR) expression only resulted in glucose intolerance in aged fish but no elevation in fasting blood glucose (16). Likewise, mutation of insulin receptors specifically in the liver resulted in postprandial alterations in glucose (10) but fasting blood glucose was reduced, similar to the liver insulin receptor knockout mice (81). Overfeeding adult fish quickly results in increased fasting glucose (16, 82) but hyperglycemia was reversed by returning to normal feeding. Although zebrafish are glucose sensitive (16, 83), insulin resistance or overfeeding in and of itself may be insufficient to lead to gross dysfunction of glucose homeostasis. A better understanding of the physiology of glucose control in zebrafish is likely necessary for the development of a truly diabetic zebrafish.

Despite the current lack of a robust model for diabetes, the zebrafish stands to contribute to the understanding of the influence of T2D-associated genetic loci on β-cell mass. Genome-wide association studies have identified loci associated with diabetes risk. The challenge is to determine the relevance of these different loci to phenotypes including β-cell mass and β-cell function and further determine the genes that may be influenced by these loci. Given the genetic tractability, the ease of producing mutations via CRISPR/Cas9, and the proven islet cell plasticity, zebrafish are an extremely attractive model to investigate the role of these candidate loci. Recently, O'Hare et al. examined 67 candidate genes from GWAS studies using morpholino and CRISPR-based approaches (84). The impact on β-cell number and regeneration was assayed, and 25 genes that reduced β-cell number when mutated were found. This included genes previously known to influence β-cell number such as *pdx1* and *pax4* as well as some new genes such as *camk1d*. This study, as well as those using genes underlying monogenic forms of diabetes, supports the utility of zebrafish as a model to study the genetic basis of the disease.

To date, all of the screening modalities have relied on measuring changes in the physical number of the β-cells. While secondary measures have also examined free glucose (30, 34, 84), no primary screen has been done to assay for β-cell function either in parallel or instead of changes in cell number. Approaches to achieve this end are currently lacking. Examining calcium signaling through expression of genetically encoded sensors of calcium activity is one approach that may be useful (85), although difficult to employ in a high-throughput screen. To fully understand the physiology of glucose control in the zebrafish, other assays should be developed, beyond those that rely on cell numbers and free glucose assays.

Given all these measures, studies using zebrafish clearly have contributed to the study of glucose homeostasis. From endocrine cell development, plasticity under different conditions, genetic susceptibility, to modeling diabetes, the zebrafish has and will continue to have utility. With the ever increasing number of patients with diabetes, applying as many resources and approaches can only serve to increase knowledge and provide new avenues for therapies.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Nutrient Sensing Systems in Fish: Impact on Food Intake Regulation and Energy Homeostasis

*Marta Conde-Sieira and José L. Soengas **

Laboratorio de Fisioloxía Animal, Departamento de Bioloxía Funcional e Ciencias da Saúde, Facultade de Bioloxía, Universidade de Vigo, Vigo, Spain

Evidence obtained in recent years in a few species, especially rainbow trout, supports the presence in fish of nutrient sensing mechanisms. Glucosensing capacity is present in central (hypothalamus and hindbrain) and peripheral [liver, Brockmann bodies (BB, main accumulation of pancreatic endocrine cells in several fish species), and intestine] locations whereas fatty acid sensors seem to be present in hypothalamus, liver and BB. Glucose and fatty acid sensing capacities relate to food intake regulation and metabolism in fish. Hypothalamus is as a signaling integratory center in a way that detection of increased levels of nutrients result in food intake inhibition through changes in the expression of anorexigenic and orexigenic neuropeptides. Moreover, central nutrient sensing modulates functions in the periphery since they elicit changes in hepatic metabolism as well as in hormone secretion to counter-regulate changes in nutrient levels detected in the CNS. At peripheral level, the direct nutrient detection in liver has a crucial role in homeostatic control of glucose and fatty acid whereas in BB and intestine nutrient sensing is probably involved in regulation of hormone secretion from endocrine cells.

Keywords: nutrient sensors, fish, hypothalamus, liver, Brockmann bodies, intestine, food intake, homeostasis

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****Correspondence:***
José L. Soengas
jsoengas@uvigo.es

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NUTRIENT SENSING MECHANISMS IN FISH

Since sensing and responding to fluctuations in environmental nutrient levels is a requisite for life, is not surprising that different organisms are able to detect extracellular and intracellular levels of sugars, amino acids, and lipids. The sensing of a specific nutrient may occur directly through binding of the sensed molecule to the sensor, or indirectly through detection of a related molecule that reflect nutrient abundance (Ogunnowo-Bada et al., 2014; Efeyan et al., 2015). We provide in the next sections a summary of the findings obtained in fish about glucose and fatty acid sensors.

As for the other main nutrient, amino acid, the increase in mammals in the levels of specific branched-chain amino acids (BCAA) such as leucine inhibits food intake. This process occurs through activation of amino acid sensing systems mediated by activation of target of rapamycin (mTOR) and/or inhibition of AMP-activated protein kinase (AMPK) signaling, or via activation of BCAA metabolism (Heeley and Blouet, 2016; Morrison et al., 2016). Furthermore, the deficiency in essential amino acids (including BCAA) elicits an increase in food intake through amino acid sensing systems mediated by general control nondepressable 2 and eukaryotic initiation factor 2α (Fromentin et al., 2012; Maurin et al., 2014). In fish, no studies have attempted yet to evaluate the possible presence and functioning of comparable amino acid sensing mechanisms and their relationship with food intake control. Their presence in central areas regulating food intake is

however reasonable considering that most fish are carnivorous, and therefore they are strongly dependent (certainly much more than omnivorous mammals in which most studies have been carried out to date) on dietary protein/amino acid levels for functioning. The only studies available in fish demonstrated in peripheral tissues like muscle and liver the effect of changes in amino acid levels in mRNA abundance of mTOR (Seiliez et al., 2008; Wacyk et al., 2012; Tu et al., 2015; Liang et al., 2016; Xu et al., 2016).

The hypothetical mechanisms involved in sensing of glucose, fatty acid, and amino acid in fish are summarized in **Figure 1**.

Glucosensors

Glucosensing is the ability of specialized cells to detect changes in the levels of glucose. This ability relates to food intake control and counter-regulatory responses to changes in levels of plasma metabolites in brain areas like hypothalamus and hindbrain. In pancreatic endocrine cells and intestine it relates to hormone release whereas in liver relates to the metabolic switch between glucose utilization and production in liver. There are several glucosensing mechanisms characterized in mammals. The best known is that mediated by glucokinase (GK), as demonstrated in brain neurons, pancreatic β-cells and hepatocytes (Blouet and Schwartz, 2010; Ogunnowo-Bada et al., 2014; Efeyan et al., 2015). In this mechanism (Marty et al., 2007; Polakof et al., 2011d), glucose is taken up by glucose facilitative carrier type 2 (GLUT2), phosphorylated to glucose 6-phosphate by GK, and then metabolized through glycolysis increasing intracellular ATP/ADP ratio (**Figure 1**). The increased ratio induces closure of ATP-dependent inward rectified potassium channel (K^+_{ATP}) inducing the depolarization of membrane and the entry of calcium into the cell through L-type voltage-dependent calcium channel. This entry of calcium finally results in changes in neuronal activity (brain), modulation of hormone release (endocrine cells) or changes in metabolism (liver). There is also evidence in mammals for GK-independent glucosensing mechanisms as also displayed in **Figure 1** (Fioramonti et al., 2004; Marty et al., 2007; González et al., 2009; Thorens, 2012; Donovan and Watts, 2014). The expression of liver X receptor (LXR) (Mitro et al., 2007) responds to increased glucose levels eliciting a decrease in gluconeogenic capacity (Anthonisen et al., 2010; Archer et al., 2014). The sweet taste receptors (formed by

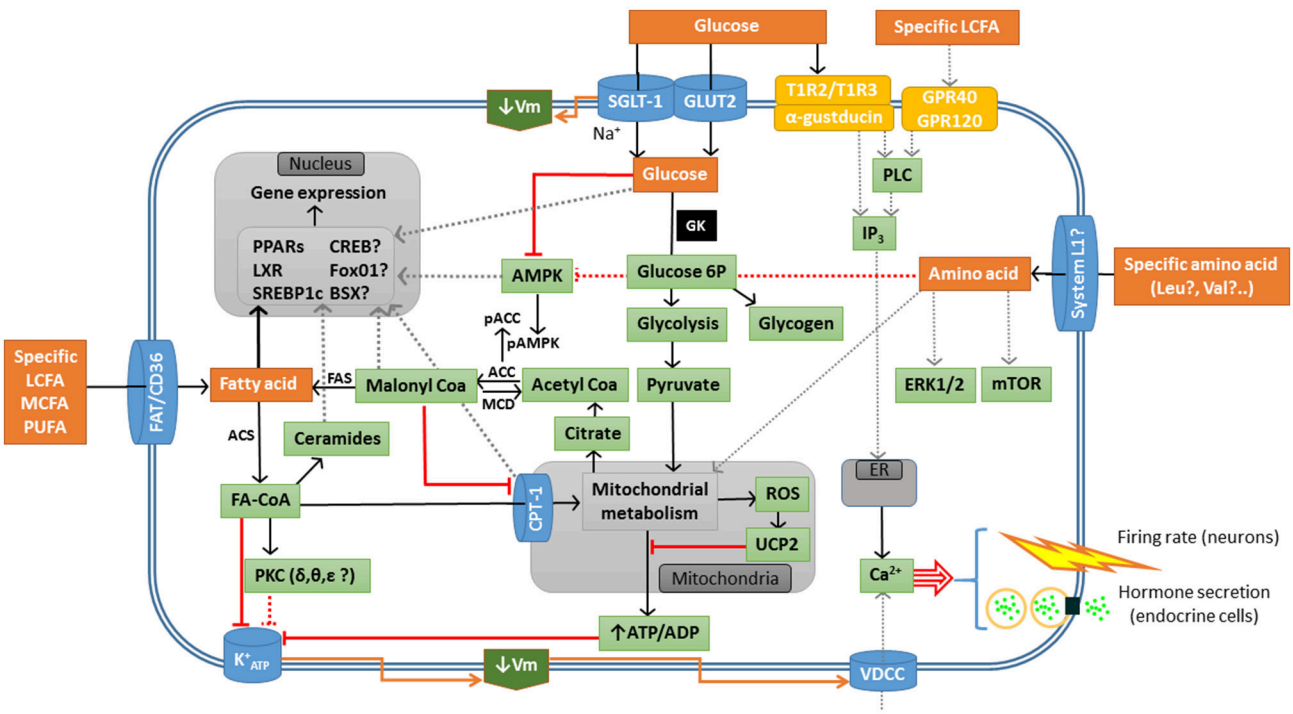

FIGURE 1 | Schematic drawing with a model of different sensing systems for glucose, fatty acid, and amino acid in sensor cells in fish. Black line, activation; gray dotted line, hypothetical activation; red line, inhibition; red dotted line, hypothetical inhibition; ACC, Acetyl-CoA carboxylase; ACS, Acetyl-CoA synthetase; AMPK, AMP-activated protein kinase; BSX, brain homeobox transcription factor; CPT-1, carnitine palmitoyl transferase type 1; CREB, cAMP response-element binding protein; ER, endoplasmic reticulum; ERK, extracellular signal–regulated kinase; FA, fatty acid; FAS, fatty acid synthase; FAT/CD36, fatty acid translocase; FoxO1, forkhead box protein O1; K^+_{ATP}, inward rectifier ATP-dependent K^+ channel; GK, glucokinase (hexokinase IV); GLUT2, facilitative glucose carrier type 2; IP_3, inositol 1,4,5-triphosphate; GPR40. G-protein-coupled receptor 40; GPR120, G-protein-coupled receptor 120; LCFA, long-chain fatty acid; LXR, liver X receptor; MCD, malonyl-CoA decarboxylase; MCFA, medium-chain fatty acid; mTOR, target of rapamycin; PLC, phospholipase C; PPARs, peroxisome proliferator-activated receptors; SGLT-1, sodium/glucose co-transporter 1; SREBP1c, sterol regulatory element-binding protein type 1c; PKC, protein kinase C; PUFA, poly-unsaturated fatty acid; ROS, reactive oxygen species; T1R2, type 1 taste receptor subunit 2; T1R3, type 1 taste receptor subunit 3; UCP2, uncoupling protein 2; VDCC, L-type voltage-dependent calcium channel; Vm, membrane potential.

type 1 taste receptor subunits (T1Rs) 2 and 3, and α-gustducin) respond to changes in glucose levels activating an intracellular signaling cascade (Ren et al., 2009; Kyriazis et al., 2014; Murovets et al., 2015; Herrera Moro Chao et al., 2016). Enhanced glucose levels induce increased expression of sodium/glucose co-transporter 1 (SGLT-1) (Díez-Sampedro et al., 2003; González et al., 2009; Thorens, 2012). The mitochondrial production of reactive oxygen species (ROS) leads to increased expression of uncoupling protein 2 (UCP2) in response to increased glucose levels (Beall et al., 2010; Diano and Horvath, 2012). These different systems might relate since, for instance, T1R3 and α-gustducin are necessary for SGLT-1 response to increased carbohydrate levels in the diet (Wauson et al., 2013).

In fish, evidence obtained in recent years support the presence of a GK-dependent glucosensing mechanism in central and peripheral areas of rainbow trout (Polakof et al., 2011d; Soengas, 2014). Indeed, in rainbow trout changes in the levels of glucose induced dietary (Polakof et al., 2008b,c), intraperitoneal (IP) (Polakof et al., 2007a, 2008a; Conde-Sieira et al., 2010a,b, 2012b; Otero-Rodiño et al., 2015), *in vitro* (Polakof et al., 2007b; Aguilar et al., 2011; Conde-Sieira et al., 2011, 2012a), or intracerebroventricular (ICV) (Polakof and Soengas, 2008) treatments resulted in changes in glucosensing mechanisms in hypothalamus and hindbrain. These include changes in GK mRNA abundance and activity, glucose and glycogen levels, GLUT2 mRNA abundance, glycolytic and glycogenic potentials, and in the activity of K^+_{ATP}. Besides the studies carried out in rainbow trout, a recent study provided evidence of glucose sensing properties in several hypothalamic nuclei in medaka (Hasebe et al., 2016). In peripheral tissues of rainbow trout, the presence and functioning of GK-dependent glucosensing mechanisms is supported by findings in liver (Soengas et al., 2006; Conde-Sieira et al., 2012b), Brockmann bodies (BB, main accumulation of endocrine pancreatic cells) (Polakof et al., 2007a,b, 2008b,c), and intestine (Polakof et al., 2010a; Polakof and Soengas, 2013). Interestingly, the response of glucosensing systems to glucose is more important during the day than during the night in liver but not in hypothalamus, hindbrain, and BB whose responses to hyperglycemic treatment were similar at night and day (Conde-Sieira et al., 2012a).

The presence of GK-independent glucosensing mechanisms and their response to changes in glucose levels has recently been assessed in different central and peripheral areas of rainbow trout (Polakof and Soengas, 2013; Otero-Rodiño et al., 2015, 2016a,b,c). These include hypothalamus (mitochondrial activity, sweet taste receptor, and LXR), hindbrain (SGLT-1), liver (sweet taste receptor), BB (sweet taste receptor, LXR, and mitochondrial activity), and intestine (sweet taste receptor, SGLT-1, and LXR). Furthermore, a recent study (Balasubramanian et al., 2016) also demonstrated increased mRNA abundance of T1R2 and LXR in brain of rainbow trout nutritionally programmed to cope with enhanced carbohydrate levels in the diet.

Figure 2 summarizes the integrative responses of glucosensing systems in different fish tissues to an increase or decrease in glucose levels.

Fatty Acid Sensors

In mammals fatty acid sensing systems are involved in hypothalamus and hindbrain in the detection of changes in the levels of long-chain fatty acid (LCFA) thus contributing to energy homeostasis control (Migrenne et al., 2007; Gao et al., 2013; Duca and Yue, 2014; Efeyan et al., 2015). The best known mechanism is of metabolic nature (**Figure 1**) in a way that a rise in LCFA levels results in increased levels of malonyl-CoA, which inhibits carnitine palmitoyl transferase-1 (CPT-1) then resulting in the inability of mitochondria to import fatty acid-CoA for oxidation (López et al., 2005, 2007). There is also evidence for the presence of alternative mechanisms in mammals (**Figure 1**). These include the increased binding capacity of fatty acid translocase (FAT/CD36) in response to elevated LCFA levels resulting in changes in the expression of several transcription factors (Le Foll et al., 2009). The activation of specific isoforms of protein kinase C in response to increase levels of LCFA results in the inhibition of K^+_{ATP} activity (Benoit et al., 2009; Blouet and Schwartz, 2010). The activity of K^+_{ATP} inhibited by increased capacity of mitochondria to produce ROS in response to increased LCFA levels (Blouet and Schwartz, 2010). Finally, the activity of lipoprotein lipase increases in response to enhanced availability of triglycerides resulting in increased levels of LCFA stimulating G-protein-coupled receptors 40 and 120 (Picard et al., 2013; Ekberg et al., 2016). These systems apparently respond to specific LCFA, such as the monounsaturated fatty acid oleate (C18:1 n-9) (López et al., 2007; Blouet and Schwartz, 2010; Duca and Yue, 2014). The ability of other classes of LCFA differing in the length of their acyl chain and/or in their degree of unsaturation to elicit the activation of these systems has been scarcely assessed to date. The available studies in mammals indicate that neither saturated fatty acids like palmitate (C16:0) nor the presence of two (such as in linoleate, C18:2 n-6) or three (such as in docosahexanoate, C22:6 n-3) double bonds activate fatty acid sensing systems (Gomez-Pinilla and Ying, 2010; Ross et al., 2010; Schwinkendorf et al., 2011; Greco et al., 2014).

Lipids are major nutrients in fish where they metabolically support many different processes (Sheridan, 1994; Tocher, 2003; Polakof et al., 2010b). Therefore, not surprisingly, many studies evaluated the effects of different dietary lipids in fish metabolism (Morash et al., 2009; Torstensen et al., 2009; Sánchez-Gurmaches et al., 2010; Figueiredo-Silva et al., 2012a,b,c; Martinez-Rubio et al., 2013). However, only recent studies provide evidence for the presence of fatty acid sensing systems in central areas of rainbow trout (Librán-Pérez et al., 2012, 2013a, 2014a,b, 2015a,b) and Senegalese sole (Conde-Sieira et al., 2015a) as well as in peripheral areas of rainbow trout (Librán-Pérez et al., 2012, 2013a,b,c, 2015c).

The treatment of rainbow trout with oleate induced responses compatible with fatty acid sensing in hypothalamus (Librán-Pérez et al., 2012, 2013a, 2014a), BB (Librán-Pérez et al., 2012, 2013a, 2015c), and liver (Librán-Pérez et al., 2013b,c, 2015c). These responses include decreased lipogenic and fatty acid oxidation capacities, reduced activity of K^+_{ATP}, and changes in the expression of transcription factors resultant of FAT/CD36 modulation. This response is comparable in general with that

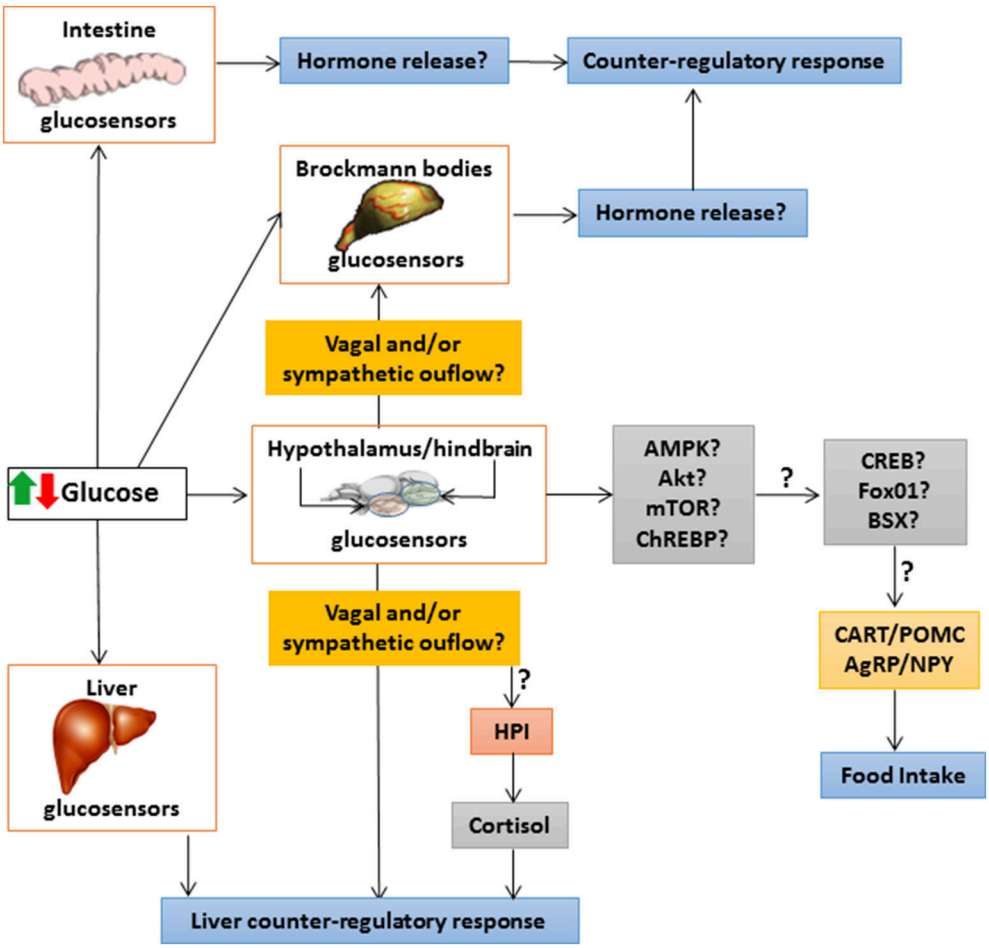

FIGURE 2 | Schematic drawing with a hypothetical model of integrative responses to an increase or decrease in glucose levels of glucosensing systems in different fish tissues. ↑, increase; ↓, decrease; ?, unknown; AgRP, agouti-related peptide; Akt, protein kinase B; AMPK, AMP-activated protein kinase; BSX, hypothalamic homeobox transcription factor; CART, cocaine- and amphetamine-related transcript; ChREBP, carbohydrate-responsive element-binding protein; CREB, cAMP response-element binding protein; FoxO1, forkhead box protein O1; HPI, hypothalamus-pituitary-interrenal axis; mTOR, target of rapamycin; NPY, neuropeptide Y; POMC, pro-opio melanocortin.

reported in mammals. Furthermore, in rainbow trout, similar responses occurred after treatment with the medium-chain fatty acid (MCFA) octanoate, and this is in contrast to mammals (Hu et al., 2011). This different behavior between fish and mammals might relate to the findings that body lipids in teleosts contain considerable amounts of MCFA (Davis et al., 1999; Trushenski, 2009) and/or MCFA oxidation in fish, at least in rainbow trout, is equally preferred compared with that of LCFA (Figueiredo-Silva et al., 2012a), in contrast with mammals (Ooyama et al., 2009). The response of fatty acid sensing systems in rainbow trout hypothalamus to increased levels of oleate or octanoate is also supported by the response of these tissues to specific inhibitors *in vitro* (Librán-Pérez et al., 2013a). Another peculiarity of fatty acid sensing systems in fish is their apparent capacity to respond to changes in the levels of polyunsaturated fatty acid (PUFA) of the n-6 and particularly n-3 series. These PUFA are very relevant for fish since their diets are particularly rich in long chain PUFA (Sargent et al., 2002) and PUFA are therefore abundant in their tissues (Mourente and Tocher, 1992; Tocher, 2003). Furthermore, the brain of marine fish is particularly rich in n-3 PUFA, mainly in α-linolenate (C18:3 n-3), eicosapentanoate (C20:5 n-3), and docosahexanoate (C22:6 n-3) (Tocher et al., 1992; Betancor et al., 2014). Conde-Sieira et al. (2015a) demonstrated that not only oleate but also α-linolenate activated fatty acid sensing systems present in the hypothalamus of Senegalese sole. This is completely different to that described in mammals (see above) and may relate to the importance of n-3 PUFA in fish. However, the capacity of PUFA to activate fatty acid sensing systems appears to be specific of certain PUFA since eicosapentanoate did not induce any significant change in fatty acid sensing systems (Conde-Sieira et al., 2015a).

Although levels of a particular fatty acid cannot be decreased, lipolysis inhibitors have been used to decrease circulating levels of all fatty acids, and this resulted in decreased activity of fatty

acid sensing systems in mammals (Oh et al., 2012, 2014). A similar experimental approach in rainbow trout also resulted in the inhibition of fatty acid sensing systems in hypothalamus, BB, and liver, and these changes apparently relate to the activation of hypothalamus-pituitary-interrenal (HPI) axis (Librán-Pérez et al., 2014b, 2015d).

Figure 3 summarizes the integrative responses to changes in levels of specific fatty acids of fatty acid sensing systems in different fish tissues.

In summary, the evidence obtained in recent years support the presence in fish central and peripheral areas of several of the sensing mechanisms for glucose and fatty acid already characterized in mammalian models. However, these mechanisms are not exactly the same since besides responding to glucose or LCFA they also respond to other molecules such as MCFA or PUFA. Clearly, the assessment of amino acid sensing mechanism is lacking in fish, and this is of crucial importance considering that most fish species are carnivorous. Finally, there is also no information available regarding cellular mechanisms integrating information of main nutrient sensing systems into shared regulatory pathways.

IMPACT OF NUTRIENT SENSING ON FOOD INTAKE REGULATION

In mammals nutrient detection activates directly or indirectly hypothalamic neurocircuits involved in the regulation of food intake, energy expenditure, and homeostasis (Berthoud, 2002; Morton et al., 2006, 2014; Berthoud and Morrison, 2008; Blouet and Schwartz, 2010). These circuits include two clearly defined populations of neurons mostly present in several hypothalamic nuclei including arcuate, as well as in other brain regions like hindbrain (Schwartz et al., 2000; Mobbs et al., 2005; Blouet and Schwartz, 2010; Efeyan et al., 2015). The first population responds to rises in circulating levels of glucose, fatty acid, or amino acid with the enhancement of AgRP and NPY expression. The second population responds to rises in levels of the same nutrients with enhanced co-expression of CART and POMC. Accordingly, in response to a rise in the levels of nutrients CART/POMC neurons depolarize while AgRP/NPY neurons hyperpolarize (Levin et al., 2004; Fioramonti et al., 2007). These populations also inhibit each other producing signals to higher-order neurons (Marty et al., 2007). Hypothalamic projections terminating in the hindbrain also causes a flow of efferent information to tissues involved in energy balance including liver, adipose tissue, and endocrine pancreas (Zheng and Berthoud, 2008).

In fish, NPY/AgRP and POMC/CART neurons are present in brain areas analogous to those in mammals (Cerdá-Reverter and Canosa, 2009). In addition, the expression of these neuropeptides relates to food intake control since feeding conditions change mRNA abundance of neuropeptides (Volkoff et al., 2005, 2009; Volkoff, 2006; Hoskins and Volkoff, 2012). Indeed, food deprivation decreased mRNA abundance of CART in goldfish (Volkoff and Peter, 2001), cod (Kehoe and Volkoff, 2007), and Atlantic salmon (Murashita et al., 2009) while values increased with re-feeding in channel catfish (Kobayashi et al., 2008), and

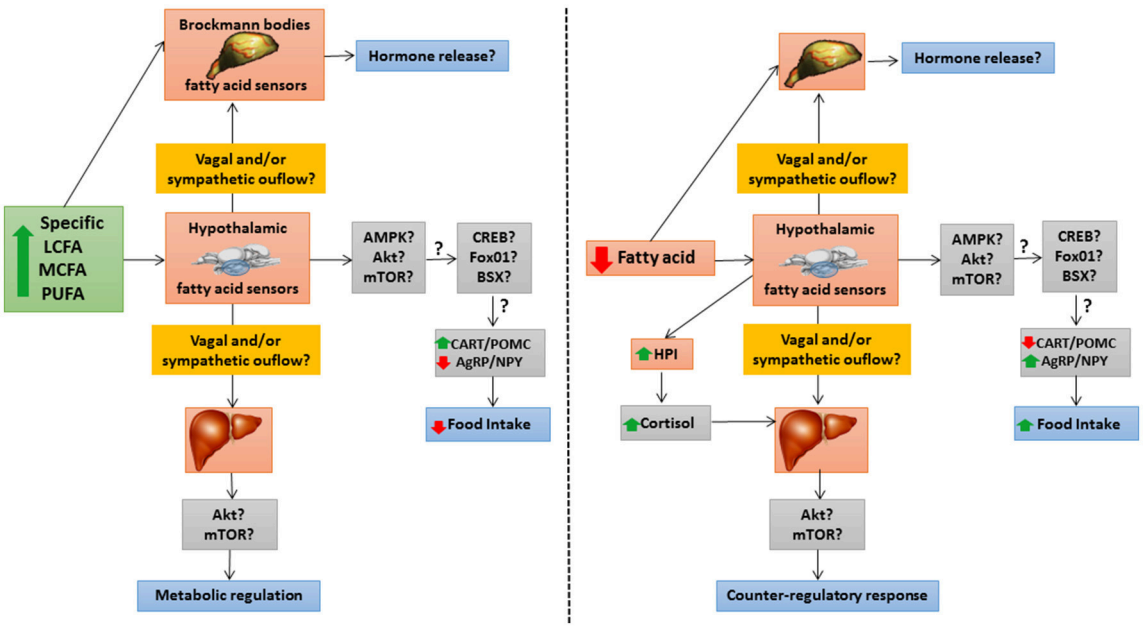

FIGURE 3 | Schematic drawing with a hypothetical model of integrative responses to an increase (left panel) or decrease **(right panel)** in levels of specific fatty acids of fatty acid sensing systems in different fish tissues. ↑, increase; ↓, decrease; ?, unknown; AgRP, agouti-related peptide; Akt, protein kinase B; AMPK, AMP-activated protein kinase; BSX, hypothalamic homeobox transcription factor; CART, cocaine- and amphetamine-related transcript; CREB, cAMP response-element binding protein; FoxO1, forkhead box protein O1; HPI, hypothalamus-pituitary-interrenal axis; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; mTOR, target of rapamycin; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PUFA, poly-unsaturated fatty acid.

post-prandial changes occurred in goldfish (Volkoff and Peter, 2001) and channel catfish (Peterson et al., 2012). As for POMC, its mRNA abundance increased post-prandially in rainbow trout (Gong and Björnsson, 2014), medaka (Chisada et al., 2014), and Atlantic halibut (Gomes et al., 2015). The mRNA levels of AgRP increased with food deprivation in hypothalamus of goldfish (Cerdá-Reverter and Peter, 2003), zebrafish (Song et al., 2003), carp (Zhong et al., 2013), and sea bass (Agulleiro et al., 2013), though not in Atlantic salmon (Murashita et al., 2009) whereas no post-feeding changes occurred in medaka (Chisada et al., 2014). AgRP mRNA levels also increased in hypothalamus of GH-transgenic carp that also displayed increased food intake (Zhong et al., 2013). The mRNA abundance of NPY decreased post-feeding in grass carp (Zhou et al., 2013), goldfish (Kehoe and Volkoff, 2007), and zebrafish (Tian et al., 2015) but responses were contradictory in orange-spotted grouper (Tang et al., 2013), rainbow trout (Gong and Björnsson, 2014), and zebrafish (Chen et al., 2016). Finally, decreased mRNA abundance of NPY occurred in food-deprived rainbow trout (Gong et al., 2016b).

Glucosensors and Regulation of Food Intake

In mammals, the detection of changes in glucose levels by glucosensing mechanisms results in regulatory responses, including food intake, allowing the animal to control blood glucose levels (Marty et al., 2007). Accordingly, reduced glycaemia increases food intake whereas enhanced glycaemia decreases food intake (Morton et al., 2014; Ogunnowo-Bada et al., 2014; Rogers et al., 2016).

Similar changes in food intake in response to altered glucose levels occur in fish (Polakof et al., 2011d, 2012a). Decreased food intake occurred in rainbow trout fed with a diet enriched in carbohydrates (Kaushik et al., 1989; Suárez et al., 2002; Krogdahl et al., 2004; Polakof et al., 2008b,c; Figueiredo-Silva et al., 2013). A similar response was observed after ICV or IP hyperglycaemic treatments in the same species (Ruibal et al., 2002; Polakof et al., 2007a, 2008a; Conde-Sieira et al., 2010a,b, 2012b). In contrast, increased food intake occurred in rainbow trout fed a diet with a reduced amount of carbohydrates (Sánchez-Muros et al., 1998; Capilla et al., 2003; Polakof et al., 2008b,c) or after IP or ICV hypoglycaemic treatments (Polakof et al., 2007a, 2008a; Conde-Sieira et al., 2010a,b). Comparable responses of food intake to changes in glucose levels also occurred in other fish species including goldfish (Narnaware and Peter, 2002), tilapia (Saravanan et al., 2012; Figueiredo-Silva et al., 2013), Siberian sturgeon (Gong et al., 2014) or sea bass (Castro et al., 2015).

In brain areas producing AgRP/NPY and POMC/CART histochemical studies in rainbow trout support the presence of GK (Polakof et al., 2009) suggesting a functional relationship between glucosensors and neuropeptides. However, few studies in fish described changes in the mRNA abundance of those neuropeptides in response to changes in glucose levels. The mRNA abundance of hypothalamic NPY decreased in hyperglycaemic-treated rainbow trout (Conde-Sieira et al., 2010b, 2012b; Aguilar et al., 2011; Otero-Rodiño et al., 2016a). A similar decline occurred in fish fed with a carbohydrate-enriched diet, such as in rainbow trout (Figueiredo-Silva et al., 2012c) and goldfish (Narnaware and Peter, 2002) whereas in the whole brain of gilthead sea bream no changes occurred (Babaei et al., 2017). CART mRNA levels in hypothalamus increased in response to elevated glucose levels in catfish (Subhedar et al., 2011) and rainbow trout (Conde-Sieira et al., 2010b, 2012b; Otero-Rodiño et al., 2015) or after rainbow trout were fed with a carbohydrate-enriched diet (Figueiredo-Silva et al., 2012c). Hypothalamic POMC mRNA levels increased in hyperglycaemic rainbow trout (Conde-Sieira et al., 2010b; Otero-Rodiño et al., 2015). Finally, AgRP mRNA abundance did not display changes in hypothalamus of rainbow trout after hyperglycaemic treatment (Otero-Rodiño et al., 2015, 2016a). Therefore, the mRNA abundance in glucosensing central areas (hypothalamus and hindbrain) of the four neuropeptides involved in the food intake regulation is affected by changes in glycaemia, and this is compatible with the changes observed in food intake (Polakof et al., 2008a,b).

Fatty Acid Sensors and Regulation of Food Intake

In fish fed with a lipid-enriched diet, a decrease in food intake usually takes place. This occurred for instance in rainbow trout (Peragón et al., 2000; Rasmussen et al., 2000; Gélineau et al., 2001; Forsman and Ruohonen, 2009; Figueiredo-Silva et al., 2012c; Saravanan et al., 2013), chinook salmon (Silverstein et al., 1999), polka-dot grouper (Williams et al., 2006), Senegalese sole (Bonacic et al., 2016) or grass carp (Li et al., 2016). Moreover, enhanced lipid storage is also usually associated with a reduced food intake (Shearer et al., 1997; Silverstein et al., 1999; Johansen et al., 2002, 2003). Therefore, lipid metabolism is clearly influencing food intake control in fish. Considering the relative high importance of fatty acids within the lipid pool, both in fish diets and in tissue composition, is not surprising that the available studies in fish focussed on fatty acids.

In recent studies in rainbow trout a decrease in food intake was observed after IP (Librán-Pérez et al., 2012) or ICV (Librán-Pérez et al., 2014a; Velasco et al., 2016a,b) administration of oleate or octanoate, with the effect being more important for octanoate. The effect of octanoate is specific of fish (at least rainbow trout) since in mammals treatment with this fatty acid did not affect food intake (López et al., 2007; Hu et al., 2011). Moreover, when rainbow trout fed diets containing different lipid composition, the lower food intake occurred in fish with the highest levels of fatty acid in plasma (Luo et al., 2014). This finding supports that central fatty acid sensing mechanisms mediated the lipid-induced decrease in food intake. Further support come from results obtained in rainbow trout where the decrease in food intake induced by treatment with a fatty acid synthase (FAS) inhibitor is counteracted by the simultaneous presence of an acetyl-CoA carboxylase inhibitor (Librán-Pérez et al., 2012), i.e., a response similar to that of mammals (Loftus et al., 2000; Gao and Lane, 2003; Hu et al., 2011). In Senegalese sole IP treatment with oleate, α-linolenate, or eicosapentanoate also resulted in a decrease in food intake (Conde-Sieira et al.,

2015a). Furthermore, when levels of circulating fatty acid decreased through pharmacological treatment a clear increase in food intake occurred in rainbow trout (Librán-Pérez et al., 2014b).

In mammals, the activation of fatty acid sensing systems results in food intake inhibition through changes in the expression of anorexigenic and orexigenic neuropeptides (López et al., 2005; Oh et al., 2016). Accordingly, the increase in LCFA levels results in a decrease in the mRNA abundance of AgRP and NPY as well as in an increase in mRNA abundance of CART and POMC. Therefore, not surprisingly, several studies have described changes in mRNA abundance of neuropeptides in fish fed lipid-enriched diets. Feeding fish with these diets resulted in increased mRNA abundance of POMC in rainbow trout (Librán-Pérez et al., 2015b), and increased mRNA abundance of CART in rainbow trout (Figueiredo-Silva et al., 2012c; Librán-Pérez et al., 2015b) and Atlantic salmon (Hevrøy et al., 2012). Feeding diets enriched in lipids also induced a decrease in NPY mRNA abundance in grass carp (Li et al., 2010) but not in rainbow trout (Figueiredo-Silva et al., 2012c; Librán-Pérez et al., 2015b) or orange-spotted grouper (Tang et al., 2013). Finally, AgRP mRNA abundance did not change in Atlantic salmon (Hevrøy et al., 2012) but decreased in rainbow trout (Librán-Pérez et al., 2015b) fed with lipid-enriched diets.

Other available studies described the impact of treatments with specific fatty acids on mRNA abundance of orexigenic and anorexigenic neuropeptides. In rainbow trout oleate either through IP (Librán-Pérez et al., 2012), *in vitro* (Librán-Pérez et al., 2013c) or ICV (Librán-Pérez et al., 2014a; Velasco et al., 2016a,b) treatments resulted in hypothalamus in a decrease in mRNA abundance of NPY and an increase in mRNA abundance of CART and POMC. Changes observed in NPY mRNA levels after oleate treatment are comparable to those of mammals (Blouet and Schwartz, 2010). The changes displayed by neuropeptides point to an enhancement of the anorexigenic potential, which is in agreement with the effects in food intake after treatment with the same fatty acid. The treatment of rainbow trout with octanoate also resulted in decreased NPY mRNA abundance after ICV treatment (Librán-Pérez et al., 2014a), and increased mRNA abundance of CART and POMC after ICV and *in vitro* treatments (Librán-Pérez et al., 2013c, 2014a). These changes also suggest an enhancement of the anorexigenic potential in hypothalamus in response to octanoate treatment supporting the reduced food intake observed after treating the same species with octanoate (Librán-Pérez et al., 2012, 2014a). This effect of octanoate is exclusive to fish, at least rainbow trout, since in mammals octanoate does not induce any change in mRNA abundance of neuropeptides (Hu et al., 2011). In Senegalese sole, the treatment with oleate also induced a decrease in the mRNA abundance of AgRP while that of CART increased, i.e., a balance favoring an anorexigenic response (Conde-Sieira et al., 2015a). In the same species, the IP treatment with the PUFAs α-linolenate or eicosapentanoate (Conde-Sieira et al., 2015a) decreased mRNA abundance of AgRP (α-linolenate) and increased mRNA abundance of CART (α-linolenate and eicosapentanoate) thus favoring enhanced anorexigenic potential, in a way similar to the effects elicited by oleate. This was the first time in any vertebrate species in which any PUFA induced changes in hypothalamic mRNA abundance of neuropeptides involved in food intake control. Interestingly, parameters involved in fatty acid sensing changed only in the case of α-linolenate (Conde-Sieira et al., 2015a) suggesting a complex relationship between changes in fatty acid sensing and neuropeptide mRNA abundance.

In a way similar to that described above for fatty acid sensing systems and food intake responses, the decrease in rainbow trout of circulating levels of fatty acid resulted in decreased mRNA abundance of POMC and CART. This change favors enhanced orexigenic potential (Librán-Pérez et al., 2014b), i.e., the opposed response of that elicited by increased levels of fatty acids.

Linking Nutrient Sensing and Neuropeptide Control of Food Intake

The mechanisms linking the function of nutrient sensing systems with changes in the expression of neuropeptides, which ultimately regulate food intake, are mostly unknown in mammals. Changes in the expression of neuropeptides might relate to modulation of forkhead box01, phosphorylated cAMP response-element binding protein, and/or brain homeobox transcription factor (Diéguez et al., 2011). The actions of these factors would result in the enhancement of CART and POMC expression and the inhibition of AgRP and NPY expression resulting in decreased food intake (López et al., 2007; Diéguez et al., 2011). However, it is not clear how these transcription factors relate to the activity of the different nutrient sensing systems. Several possibilities have been suggested in mammals (López et al., 2007; Diéguez et al., 2011; Gao et al., 2013; Morton et al., 2014) including direct action of malonyl CoA or CPT-1, indirect action through CPT-1 inhibition, modulation by AMPK, mTOR, protein kinase B (Akt), or carbohydrate-responsive element-binding protein and/or involvement of ceramides.

In fish, several recent studies carried out in rainbow trout provided evidence for several of these hypothetical mechanisms. Indeed, Librán-Pérez et al. (2015b) demonstrated that protein levels of AMPK, Akt, and mTOR increased in hypothalamus of fish fed a lipid-enriched diet. Furthermore, Gong et al. (2016a) demonstrated increased Akt protein levels in isolated hypothalamic cells incubated with leptin. Finally, Velasco et al. (2016b) also suggested the possible involvement of ceramides in the connection between activation of hypothalamic fatty acid sensing systems, neuropeptide mRNA abundance, and control of food intake. Besides these preliminary studies, there is no other evidence in fish about hypothalamic pathways related to integration of metabolic information coming from different nutrient sensor systems (glucose, fatty acids, amino acids) into a shared pathway controlling food intake via neuropeptide expression.

Food intake regulation is a complex process in which nutrient sensing systems are apparently involved in fish, in a way again comparable to that of mammals with notable differences including the capacity of several nutrients like MCFA or PUFA to modify food intake control in fish. Again, there is no information regarding the involvement of amino acid sensing systems in fish

on food intake regulation, and this clearly needs assessment in the near future. Once characterized such a putative effect, the next step would be the assessment of how and why changes in those sensing systems translate into expression of anorexigenic and orexigenic neuropeptides ultimately regulating food intake. The possible mechanisms are mostly unknown, even in mamamals, and therefore it is quite probable that important differences between fish and mammals arise considering their different gastrointestinal morphology and physiology, and dietary habits.

IMPACT OF NUTRIENT SENSING ON ENERGY HOMEOSTASIS

Nutrient sensing mechanisms in mammals are also implicated in the regulation of energy homeostasis through processes other than food intake (Levin, 2006; Blouet and Schwartz, 2010; Morton et al., 2014), such as hormone secretion and energy expenditure (Morgan et al., 2004; Pocai et al., 2005; Le Foll et al., 2009; Roh et al., 2016). The homeostatic control carried out by nutrient sensing systems occurs at both central and peripheral levels. At the central level, the brain integrates multiple metabolic inputs from the periphery as nutrients, gut-derived satiety signals and adiposity-related hormones eliciting a counter-regulatory response in peripheral tissues modulating various aspects of metabolism (Morton et al., 2014; Rogers et al., 2016). At peripheral level, nutrient sensing systems modulate energy metabolism either directly or indirectly through endocrine effectors (Marty et al., 2007; Morton et al., 2014).

Central Nutrient Sensing and Counter-Regulation

Central Nutrient Sensing and Regulation of Hepatic Metabolism

ICV administration of glucose or a LCFA like oleate in mammals results in a decrease of hepatic glucose production and lipogenesis (Obici et al., 2002; Morgan et al., 2004; Migrenne et al., 2011). The downstream mechanism(s) involved are presumably based on sympathetic and parasympathetic systems that provide direct innervations to liver and endocrine pancreas via the splanchnic nerve and vagus nerve, respectively (Morgan et al., 2004; Migrenne et al., 2006; Blouet and Schwartz, 2012; Roh et al., 2016).

In fish, central glucose administration affects liver metabolism. In rainbow trout ICV administration of glucose resulted in liver in decreased levels of glucose and glucose 6-phosphate, increased capacity for glycolysis and glycogenesis, and decreased capacity of glucose export into plasma (Polakof and Soengas, 2008). The presence of glucose in the brain appears to be a signal of energy abundance indicative that no production and release of glucose from liver is necessary to sustain plasma glucose levels (Polakof and Soengas, 2008). Central treatment with oleate or octanoate in rainbow trout also induced changes in several parameters related to fatty acid and glucose metabolism in liver directed to counter-regulate the elevated fatty acid levels detected in the brain (Librán-Pérez et al., 2015c). These changes in liver include increased levels of glucose and glycogen, decreased levels of fatty acids and total lipids, decreased mRNA abundance of GK and fructose 1,6-bisphosphatase as well as FAS and CPT-1 activities. The changes in glucose metabolism observed in liver are similar to those reported in mammals where ICV administration of oleate (but not octanoate) resulted in a marked decrease of hepatic glucose production via decreased glycogenolysis and glucose release (Obici et al., 2002; Morgan et al., 2004). Furthermore, the results obtained in liver metabolism were similar when comparing central (Librán-Pérez et al., 2015c) and IP (Librán-Pérez et al., 2013b) administration of fatty acid, which would indicate that sensing capacity in liver is indirect and therefore dependent on the previous sensing in brain. These changes in hepatic metabolism after central administration of glucose or fatty acid are indicative of a functional connection between central nutrient sensing and production/release of fuels from liver (Marty et al., 2007). The mechanisms involved are also likely based on sympathetic and parasympathetic systems (Morgan et al., 2004; Migrenne et al., 2006) since, at least in rainbow trout, vagus and splanchnic nerves are present in the gastrointestinal tract though it is not clear whether or not branches of those nerves arrive to the liver (Burnstock, 1959; Seth and Axelsson, 2010).

Interestingly, in rainbow the HPI axis is also likely involved in the counter-regulatory response of liver metabolism to a fall of circulating FA levels, in order to restore the normal values (Librán-Pérez et al., 2014b, 2015d), in a way comparable to that described in mammals (Oh et al., 2012, 2014).

Central Nutrient Sensing and the Pancreatic Counter-Regulatory Response

Central glucose detection is involved in mammals in the pancreatic counter-regulatory response to hypoglycaemia in order to restore normal blood glucose levels (Blouet and Schwartz, 2010). The brain, especially the hypothalamus and brain stem, receives and integrates this information to control the counter-regulatory response by modulating pancreatic insulin and glucagon secretion via the parasympathetic and sympathetic efferent nerves that innervate pancreatic α- and β-cells (Ogunnowo-Bada et al., 2014; Roh et al., 2016). This response involves suppression of insulin secretion, activation of glucagon secretion, activation of catecholamine secretion from the adrenal glands, and the activation of hepatic glucose production by the autonomic nervous system (Marty et al., 2007). Conversely, central glucose administration suppresses the counter-regulatory hormonal responses to hypoglycaemia (Roh et al., 2016). In mammals, several studies demonstrate the involvement of central glucosensors and their components in the counter-regulatory response (Miki et al., 2001; Evans et al., 2004; Sanders et al., 2004; Marty et al., 2005; McCrimmon et al., 2005). These central glucosensors can modulate not only the counter-regulatory response to hypoglycaemia in the pancreatic cells by modulating the glucagon secretion, but also the glucose-stimulated insulin secretion in the β-cells, through activation and inhibition of the sympathetic or parasympathetic branches, respectively (Thorens, 2011; Chan and Sherwin, 2012; Osundiji et al., 2012).

In fish, central administration of glucose in rainbow trout resulted in increased GK activity and expression in BB (Polakof and Soengas, 2008). This may suggest an activation of the glucosensor system in BB that could result in increased insulin levels in plasma as part of the system trying to counter-regulate the increase in plasma glucose levels elicited by ICV treatment (Polakof and Soengas, 2008). Other studies carried out in rainbow trout also support the connection between glucose levels and pancreatic function (Polakof et al., 2012a,b). Indeed, plasma insulin levels decrease and plasma glucagon levels increase in fish subjected to natural or experimental deprivation of food (Navarro and Gutiérrez, 1995). Moreover, in zebrafish exposed to high glucose levels, insulin expression was also apparently enhanced (Jurczyk et al., 2011).

Several studies in mammals suggest that not only glucose, but also fatty acid detection in central nutrient sensing areas can alter the pancreatic function through alterations of sympathetic nervous activity (Migrenne et al., 2006; Blouet and Schwartz, 2010). Central administration of lipids that do not change plasma fatty acid concentrations, induce increased glucose-induced insulin secretion counteracted by the inhibition of β-oxidation (Cruciani-Guglielmacci et al., 2004). Furthermore, oleate injection leads to increments in plasma insulin levels without altering glycaemia, suggesting that fatty acids *per se* can regulate neural control of insulin secretion (Migrenne et al., 2011).

In fish, ICV treatment with oleate or octanoate elicited several changes in BB lipid metabolism (Librán-Pérez et al., 2015c), which, in general, are different than those obtained after IP administration using the same fatty acid (Librán-Pérez et al., 2012) or to those described in mammals after ICV administration of oleate (MacDonald et al., 2008). Therefore, contrary to that observed in mammals, fatty acid sensing in BB of rainbow trout appears to be mainly direct and probably not dependent on previous central sensing. Furthermore, the action of peripheral hormones is probably influencing sensing capacity since results obtained after IP administration of fatty acid *in vivo* differed from those obtained with the same tissue *in vitro* (Librán-Pérez et al., 2013a).

Peripheral Nutrient Sensing and Energy Homeostasis

Metabolic Response of Liver to Changes in Nutrient Abundance

In fish, as in mammals, the regulation of glucose levels in blood depends on the balance between glucose utilization via glycolysis or glycogenesis, and glucose production via gluconeogenesis or glycogenolysis in liver. An imbalance in this regulation could be responsible of glucose intolerance in some fish species (Enes et al., 2009; Polakof et al., 2012a). This regulation relies on the differential response to variations in glycaemia of enzymes involved in hepatic metabolism. GK has been shown to be essential in fish liver for induction by glucose of key glycolytic and lipogenic enzymes and repression of genes involved in gluconeogenesis (Vaulont et al., 2000) thus acting as a glucosensor (Magnuson and Matschinsky, 2004; Polakof et al., 2011d). Thus, many fish species increased GK activity and/or expression as well as glycolytic potential in liver under hyperglycaemic conditions induced by glucose administration or by feeding diets with high contents of carbohydrates (Tranulis et al., 1996; Panserat et al., 2000; Enes et al., 2006, 2009; Conde-Sieira et al., 2010a, 2015b, 2016; Castro et al., 2016).

Changes in circulating levels of glucose also modulated other components of the GK-dependent glucosensing machinery in liver of different fish species (Hemre et al., 2002; Polakof et al., 2008b, 2011d; Enes et al., 2009). These include variations in glucose and glycogen levels, GLUT2 mRNA abundance, glycolytic and glycogenic potentials, and in the activity of K^+_{ATP} occurred in the liver of hyperglycaemic rainbow trout (Conde-Sieira et al., 2010a, 2012a). Moreover, in rainbow trout fed a carbohydrate-enriched diet an up-regulation occurred in these parameters while feeding a carbohydrate-free diet resulted in a down-regulation (Polakof et al., 2008b). As for GK-independent mechanisms, experimental results obtained in fish liver indicate enhanced mitochondrial activity in response to increased levels of glucose in rainbow trout (Craig et al., 2013; Otero-Rodiño et al., 2016b). However, these responses were not reflected in other fish species such as zebrafish (Seiliez et al., 2013), red sea bream (Liang et al., 2003) or grass carp (Li et al., 2010). Furthermore, experiments *in vitro* carried out in rainbow trout did not confirm the presence of a glucosensing mechanism in liver mediated by the mitochondrial activity (Otero-Rodiño et al., 2016c). The mechanism based on sweet taste receptor appears to be operative in liver of rainbow trout since the responses obtained with this tissue *in vitro* (Otero-Rodiño et al., 2016c) are compatible with the responses described in mammalian liver (Treesukosol et al., 2011) although with some differences to those presented *in vivo* (Otero-Rodiño et al., 2016b). A glucosensor based on the hepatic LXR seems to work differentially in fish liver compared with mammals since gluconeogenesis is not inhibited by hyperglycaemia either induced by glucose administration or by feeding fish with carbohydrate-enriched diets (Panserat et al., 2001; Kirchner et al., 2008; Polakof et al., 2011d; Otero-Rodiño et al., 2016b,c). However, in other fish species such as Senegalese sole, gilthead sea bream or common carp a clear inhibition of gluconeogenesis occurred under hyperglycaemic conditions (Panserat et al., 2002b; Kamalam et al., 2013; Conde-Sieira et al., 2015b, 2016) although no studies regarding glucosensing mechanisms based on LXR are available in these species.

Other metabolic sensors regulate intermediary metabolism in mammals through control of intracellular glucose use (Polakof et al., 2012a), including AMPK (activated when the energy level in the cell is low) or mTOR (activated when the levels of nutrients increase). In fish, AMPK phosphorylation decreased in liver and mTOR phosphorylation increased in liver and muscle of rainbow trout under post-prandial conditions (Seiliez et al., 2008; Lansard et al., 2010; Polakof et al., 2011e). Furthermore, the pharmacological activation of hepatic AMPK and the inhibition of mTOR pathway induce glucose catabolism and increased gluconeogenesis besides decreased glycolysis in trout liver, respectively (Lansard et al., 2010; Polakof et al., 2011e). These findings suggest the existence in fish of a system induced by feeding carbohydrates with similar consequences

on glucose metabolism as those observed in mammals (Seiliez et al., 2008; Lansard et al., 2010; Polakof et al., 2011e). Moreover, under hyperglycaemic conditions a decrease in the mRNA abundance of sirtuin-1 (another integrative nutrient sensor) is observed in liver and BB of rainbow trout (Otero-Rodiño et al., 2016b), similar to that described in mammals (Ruderman et al., 2010; Velásquez et al., 2011). Therefore, evidences exist in fish regarding the functioning of integrative energy and nutrient sensors in response to changes in the levels of a nutrient like glucose (Otero-Rodiño et al., 2016b).

Increased circulating fatty acid levels also induce metabolic changes in liver of fish as in mammals, in order to restore normal conditions. High content of lipids in the diet reduce lipogenic potential and increases β-oxidation in the liver of many fish species (Dias et al., 2004; Figueiredo-Silva et al., 2010; Borges et al., 2013; He et al., 2015; Librán-Pérez et al., 2015b; Li et al., 2016). Furthermore, dietary lipid level affects glucose metabolism inducing hyperglycaemia, and reducing glycolytic capacity and increasing gluconeogenic potential in liver, as described in several fish species like rainbow trout (Gélineau et al., 2001; Panserat et al., 2002a; Figueiredo-Silva et al., 2012a,b), other salmonids (Mazur et al., 1992; Hemre and Sandnes, 1999), grouper (Cheng et al., 2006), sunshine bass (Hutchins et al., 1998), and Senegalese sole (Borges et al., 2014). The long-term use of lipid-enriched diets in fish can compromise glucose homeostasis due to an impairment on insulin signaling and a down regulation of the Akt and mTOR pathways, as observed in rainbow trout or Senegalese sole (Panserat et al., 2002a; Figueiredo-Silva et al., 2012b; Borges et al., 2014).

Several of the putative components of fatty acid sensing mechanisms are present in fish liver (Kolditz et al., 2008; Plagnes-Juan et al., 2008; Lansard et al., 2009; Skiba-Cassy et al., 2009; Polakof et al., 2010b). Moreover, the peripheral administration of oleate or octanoate induces in rainbow trout enhanced fatty acid catabolism as well as reduced lipogenic and glycolytic potentials, suggesting a direct action of fatty acid administration on hepatic glucose and lipid metabolism (Librán-Pérez et al., 2013b). However, under *in vitro* conditions (Librán-Pérez et al., 2013c), administration of oleate or octanoate induces changes opposed of those observed *in vivo*, which indicates that fatty acid sensing capacity in liver is indirect and probably be the result of previous hypothalamic sensing. The finding that ICV treatment in rainbow trout with the same fatty acid induced changes in fatty acid sensing systems (Librán-Pérez et al., 2015c) similar to those obtained after IP treatment supports this hypothesis.

Nutrient Sensing in BB and the Modulation of Hormone Release

In mammals, the glucosensing mechanism based on GK present in pancreatic β-cells is involved in modulation of insulin release in response to changes in blood glucose levels (Rutter et al., 2015), which therefore constitutes an essential mechanism for the maintenance of glucose homeostasis (MacDonald et al., 2005; Polakof et al., 2011d).

Experimental evidences suggest that a glucosensor system linked to insulin secretion is present in pancreatic endocrine cells in fish. Indeed, insulin release is stimulated by glucose (Epple et al., 1987; Mommsen and Plisetskaya, 1991; Hrytsenko et al., 2008; Jurczyk et al., 2011) as well as by 2-deoxyglucose, mannose and K^+ (Ronner and Scarpa, 1987; Ronner, 1991) and inhibited under hypoglycaemia induced by food deprivation (Navarro and Gutiérrez, 1995). These changes may relate to those observed in the pancreatic glucosensor system in fish under altered conditions of glycaemia. In rainbow trout BB these include increased GK activity and expression, GLUT2 expression, glycolytic capacity as well as glucose and glycogen levels in hyperglycaemic fish (Polakof et al., 2007a,b). In the same species, feeding fish with diets enriched in carbohydrates upregulates glucosensing response in BB whereas feeding fish with diets poor in carbohydrates resulted in a down-regulation of glucosensing response in the same tissue (Polakof et al., 2008b,c). Some GK-independent mechanisms also present in BB of rainbow trout respond to increased levels of glucose with changes in parameters related to mitochondrial activity, LXR, and sweet taste receptor both *in vivo* (Otero-Rodiño et al., 2016b) and *in vitro* (Otero-Rodiño et al., 2016c).

In mammals, lipid metabolism in the β-cell is also critical for the normal regulation of insulin secretion (MacDonald et al., 2008) and fatty acids directly regulate insulin release from pancreatic β-cells (Nolan et al., 2006). In fish, the available experimental results also demonstrate enhanced insulin release in response to increase levels of fatty acid (Barma et al., 2006). Moreover, insulin treatment in rainbow trout enhances the potential of lipogenesis and decreases the potential of fatty acid oxidation in several tissues (Plagnes-Juan et al., 2008; Lansard et al., 2010; Polakof et al., 2010b, 2011d; Caruso and Sheridan, 2011). In rainbow trout, the decreased mRNA levels of FAS and CPT1c in BB after treatment with oleate or octanoate (Librán-Pérez et al., 2012) suggest that components of putative fatty acid sensing systems respond in BB to increased fatty acid levels. This response could modulate insulin secretion from this tissue, as reported in mammals (Keane and Newsholme, 2014), with the main difference that in fish fatty acid sensing systems are also responsive to a MCFA like octanoate. This mechanism appear to be mainly the result of a direct action of fatty acid in β-cells (Librán-Pérez et al., 2013a) though an indirect action by previous hypothalamic sensing mediated by vagal and/or splanchnic outflow cannot be discarded (Librán-Pérez et al., 2015c).

Glucosensing Capacity in Gut

The gastrointestinal tract in mammals has an important role in the complex signaling network that controls food intake, metabolism and energy homeostasis since it releases several energy-related gastrointestinal hormones that send nutritional information to the control areas in the brain through afferent nerves (Schwartz et al., 2000; Roh et al., 2016). Accordingly, the presence of nutrient sensing mechanisms have been proposed in mammalian enteroendocrine cells (Miguel-Aliaga, 2012) and enterocytes (Pfannkuche and Gäbel, 2009). Glucose can be sensed in the gastrointestinal tract by mechanisms dependent on sweet taste receptors and gustducin, which are activated by glucose leading to the release of glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (Kokrashvili et al.,

2009; Miguel-Aliaga, 2012). Other glucosensing mechanisms controlling hormonal release in mammalian gastrointestinal tract involve electrogenic or metabolic processes mediated by SGLT-1 and GLUT2/GK (Miguel-Aliaga, 2012).

In fish intestine, histochemical studies evidence the presence of components of different glucosensing systems (SGLT-1 and GK) in enterocytes and enteroendocrine cells of rainbow trout (Polakof et al., 2010a). Furthermore, molecular evidence also pointed to the presence in fish intestine of glucosensing mechanisms involving components of metabolic (GK/GLUT2), electrogenic (SGLT-1), nuclear (LXR) and sweet taste receptor systems (Ishimaru et al., 2005; Geurden et al., 2007; Hashiguchi et al., 2007; Kirchner et al., 2008; Cruz-García et al., 2009; Polakof et al., 2010a). However, only few studies characterized the response of these systems in intestine to increased levels of glucose. In black bullhead enterocytes enhanced glucose uptake through SGLT-1 occurred in fish fed a diet rich in carbohydrates (Soengas and Moon, 1998) whereas in zebrafish GLUT2 mRNA abundance in intestine changed in parallel with changes in glucose levels (Castillo et al., 2009). In rainbow trout, increased glycogen levels, GK activity, glycolytic capacity, and transcript levels of GK, SGLT-1, and LXR, as well as decreased transcript levels of T1R and gustducin occurred in intestine of hyperglycemic trout (Polakof et al., 2010a; Polakof and Soengas, 2013). These systems seem to operate in fish in a different way compared with other vertebrate species (Polakof and Soengas, 2013) but certainly appear to be functional, and thus presumably involved in fish gastrointestinal physiology, especially through production and release of gastrointestinal hormones.

Possible Glucosensing Capacity in Head Kidney and Its Role on Cortisol Release

One study using head kidney perifused cultures in rainbow trout demonstrated that in the presence of ACTH, cortisol release increased in parallel with the increase of glucose in the medium (Conde-Sieira et al., 2013). These changes could relate to the presence of a glucosensing system in putative interrenal cells in head kidney that would respond to glucose levels in a way similar to that of pancreatic β-cells for insulin release. Accordingly, immunohistochemical studies indicate the presence of GK protein in interrenal cells and SGLT-1 protein in both interrenal and chromaffin cells of rainbow trout (Conde-Sieira et al., 2013). However, metabolite levels and enzymes activities involved in glucosensing mechanisms did not show a clear response to changes in circulating glucose levels in head kidney of rainbow trout, probably due to the high cellular heterogeneity of the tissue assessed (Conde-Sieira et al., 2013). A further study in rainbow trout (Gesto et al., 2014) supports that cortisol release under stress conditions in rainbow trout might relate to hyperglycemia previously elicited by catecholamine action.

As a whole, the nutrient sensing systems characterized in fish are involved in the regulation of energy homeostasis through mechanisms other than regulation of food intake. The evidence obtained in recent years pointed to a role of these systems in counter-regulatory mechanisms as well as in the regulation of hormone release, though the evidence is preliminary in some cases.

ENDOCRINE MODULATION OF NUTRIENT SENSING

Several hormones modulate the response of nutrient sensing systems in mammals to changes in the levels of nutrients. These hormones provide information about homeostasis, status of energy stores, and the presence of food and its composition in the gastrointestinal tract. These include ghrelin, insulin, leptin, cholecystokinin (CCK), GLP-1, adiponectins, cannabinoids, and glucocorticoids (Diéguez et al., 2009; Blouet and Schwartz, 2010; Morton et al., 2014).

Results obtained in recent years in fish provide evidence for the modulatory role of several of these hormones in the activity of nutrient sensing systems as well as in the mRNA abundance of neuropeptides related to the control of food intake. Moreover, several of these hormones modulate peripheral nutrient sensing systems.

As in other vertebrates, insulin administration modifies glucose and lipid metabolism in fish, by enhancing the glucose uptake in liver and muscle, increasing hepatic glycolytic and lipogenic potentials, and depressing gluconeogenesis and fatty acid oxidation (Mommsen and Plisetskaya, 1991; Plagnes-Juan et al., 2008; Jin et al., 2014). The effects on lipid metabolism depend on the dose of insulin administered as well as the feeding status of fish (Polakof et al., 2010b, 2011f). Insulin is present and synthesized in fish brain (Caruso et al., 2008) where insulin receptors are also present (Gutiérrez and Plisetskaya, 1994; Leibush et al., 1996). Insulin treatment resulted in contradictory effects in food intake in fish. In rainbow trout IP administration of insulin inhibited (Librán-Pérez et al., 2015a) or activated (Polakof et al., 2008a; Conde-Sieira et al., 2010b) food intake whereas ICV treatment with insulin inhibited food intake in rainbow trout (Soengas and Aldegunde, 2004) but not in catfish (Silverstein and Plisetskaya, 2000). The putative anorectic effects of insulin would be in agreement with the increased anorexigenic potential elicited by insulin treatment as demonstrated increased mRNA abundance of CART in rainbow trout (Librán-Pérez et al., 2015a) and catfish (Subhedar et al., 2011) as well as decreased NPY mRNA abundance in rainbow trout (Librán-Pérez et al., 2015a). As for insulin capacity to modulate the activity of nutrient sensing systems, its administration in rainbow trout inhibits glucosensing response in hypothalamus, hindbrain, BB, and intestine (Polakof et al., 2007a, 2008a, 2010b; Conde-Sieira et al., 2010b). As for fatty acid sensing systems, no clear effects of insulin treatment were observed in rainbow trout hypothalamus (Librán-Pérez et al., 2015a), in contrast to mammals (Duca and Yue, 2014). However, in liver and BB insulin treatment potentiates the effect of oleate and octanoate on fatty acid sensing systems (Librán-Pérez et al., 2015a).

Leptin treatment is usually anorectic in fish as demonstrated studies in rainbow trout (Murashita et al., 2008; Kling et al., 2009; Aguilar et al., 2010; Gong et al., 2016a), goldfish (Volkoff et al., 2003; de Pedro et al., 2006; Vivas et al., 2011) and striped bass (Won et al., 2012). This anorectic effect occurred in parallel with changes in the expression of neuropeptides generally indicating an enhanced anorexigenic potential. Thus,

leptin treatment induced a decrease in NPY mRNA levels in hypothalamus of rainbow trout (Murashita et al., 2008; Aguilar et al., 2011), hypothalamus and telencephalon of goldfish (Volkoff et al., 2003), and in whole brain in grass carp (Li et al., 2010). POMC mRNA abundance increased in response to leptin treatment in rainbow trout (Murashita et al., 2008; Aguilar et al., 2011; Gong et al., 2016a). Leptin treatment also increased CART mRNA levels in hypothalamus of goldfish (Volkoff and Peter, 2001), catfish (Subhedar et al., 2011), and rainbow trout (Murashita et al., 2008; Aguilar et al., 2011; Gong et al., 2016a). Furthermore, leptin receptor knockout for medaka displayed (compared with the wild type) a higher food intake, as well as decreased POMC mRNA abundance, and increased NPY and AgRP mRNA abundance (Chisada et al., 2014) whereas zebrafish knockout for leptin displayed changes in mRNA abundance of genes related to glucose but not to lipid metabolism (Michel et al., 2016). The anorectic effects of leptin could relate, at least in part, to the activation of nutrient sensing systems. In fact, leptin treatment clearly activates central glucosensing systems in rainbow trout (Aguilar et al., 2010, 2011). There is little evidence for the action of leptin on nutrient sensing systems in peripheral tissues of fish. The only available study showed that ICV leptin treatment in rainbow trout did not affect liver glucosensing capacity, although an increased glycogenolytic potential possibly mediated by the activation of the sympathetic nervous system occurred in rainbow trout liver (Aguilar et al., 2010).

Few studies have assessed the effects of GLP-1 on food intake in fish to date. GLP-1 treatment resulted in an inhibition of food intake in catfish (Silverstein et al., 2001) and coho salmon (White et al., 2016) but not in channel catfish (Schroeter et al., 2015). In rainbow trout GLP-1 treatment (Polakof et al., 2011b) elicited in hypothalamus and hindbrain the activation of glucosensing systems with increased mRNA abundance of CART and POMC, and decreased mRNA abundance of NPY, i.e., changes clearly indicative of enhanced anorexigenic potential. In the same species, GLP-1 IP treatment also resulted in the activation of GK-mediated glucosensing mechanism in liver (Polakof et al., 2011b).

Treatments with CCK produce anorectic responses in fish as demonstrated in rainbow trout (Gélineau and Boujard, 2001; Jönsson et al., 2006), coho salmon (White et al., 2016), goldfish (Himick and Peter, 1994; Kang et al., 2010), catfish (Silverstein and Plisetskaya, 2000), sea bass (Rubio et al., 2008), and winter flounder (MacDonald and Volkoff, 2009). Furthermore, CCK treatment in rainbow trout activated glucosensing capacity in hypothalamus and hindbrain (Polakof et al., 2011a), and this is accompanied by decreased NPY mRNA levels in hindbrain and hypothalamus, thus supporting increased anorexigenic potential. In liver of rainbow trout IP administration of CCK also activated glucosensing capacity (Polakof et al., 2011a).

The effects of ghrelin treatment on food intake in fish are controversial. Increases were noted in goldfish (Miura et al., 2006), brown trout (Tinoco et al., 2014), rainbow trout (Velasco et al., 2016a,b), striped sea bass (Picha et al., 2009) or cavefish (Penney and Volkoff, 2014) whereas decreases occurred in rainbow trout (Jönsson et al., 2010), channel catfish (Schroeter et al., 2015), and tilapia (Peddu et al., 2009). In rainbow trout ghrelin treatment activates central glucosensing systems (Polakof et al., 2011c), an effect opposed of that in mammals (Wang et al., 2008). In contrast ghrelin treatment induces an inhibition of fatty acid sensing systems in rainbow trout hypothalamus and hindbrain (Velasco et al., 2016a,b) in a way similar to that described in mammals, and these changes agree with those of mRNA abundance of neuropeptides that decreased for POMC/CART and increased for AgRP/NPY. Increased mRNA abundance of NPY occurred in hypothalamus of ghrelin-treated goldfish (Miura et al., 2006). Central ghrelin treatment also modulates indirectly hepatic liver metabolism resulting in increased potential for lipogenesis and decreased potential for fatty acid oxidation, as indicative of inhibition of fatty acid sensing (Velasco et al., 2016c).

A reduction in food intake is a typical response to stress in fish, and at least part of this response might depend on changes in the ability of stress to alter nutrient sensing systems regulating food intake. A readjustment in the activity of hypothalamic glucosensing mechanisms occurred in stressed rainbow trout (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). This effect might relate to any of the components of the HPI axis such as corticotropin releasing factor (CRF), which is involved in the effects of stress on food intake in mammals (Evans et al., 2004; McCrimmon et al., 2006). Accordingly, the treatment of rainbow trout hypothalamus with CRF altered functioning of glucosensing mechanisms (Conde-Sieira et al., 2011) in a way similar to that observed under stress conditions (Conde-Sieira et al., 2010a).

Finally, melatonin is mainly involved in fish in the timing of rhythmic events, but also in growth, endocrine function, and metabolism (Falcón et al., 2010). In rainbow trout, melatonin *in vitro* treatment in hypothalamic tissue activated glucosensing mechanisms and elicited a response in the expression of neuropeptides compatible with an enhancement of orexigenic potential (Conde-Sieira et al., 2012a). In contrast, in liver a clear down-regulation of glucosensing potential occurred in response to melatonin treatment (Conde-Sieira et al., 2012b). This differential tissue response to melatonin treatment might relate to the day-night differences in glucosensing capacity observed in liver of rainbow trout (Conde-Sieira et al., 2012b).

In summary, several hormones involved in the regulation of energy homeostasis are involved in the modulation of glucose and fatty acid sensing systems in fish. Despite most studies were carried out with glucosensing systems, few with fatty acid sensing systems and none with putative amino acid sensing systems, a preliminary conclusion can be obtained in a way that anorexigenic/anabolic hormones demonstrated to activate nutrient sensing systems whereas orexigenic/catabolic hormones inhibit them. There are differences in the direction and magnitude of the responses compared with the mammalian model, which among other reasons might relate to the high degree of hormone variants present in fish (as a result of their additional genome duplication), and/or to the clear difference in dietary habits between both models.

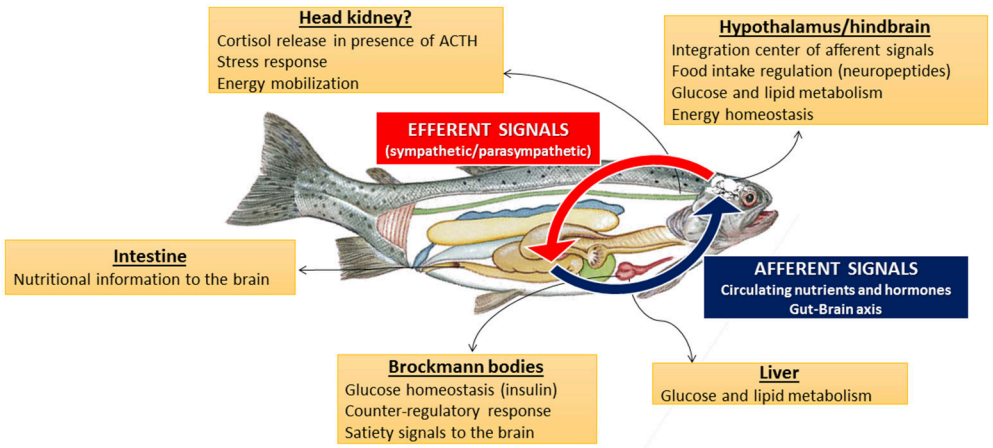

FIGURE 4 | Schematic drawing summarizing functions of nutrient sensing systems in central and peripheral tissues of fish.

CONCLUSIONS

Research carried out in recent years provided information for the presence and functioning of putative nutrient sensing systems either in peripheral or central areas of the few fish species assessed to date regarding this issue, mainly rainbow trout, as summarized in **Figure 4**.

The main role of these systems is to participate in the control of homeostasis through modulation of feeding behavior or other processes such as energy expenditure or hormone secretion. The known mechanisms are comparable to those of mammals in several aspects but clear differences arise in others, such as the fish capacity of detecting changes in circulating levels of MCFA or PUFA. These differences between fish and mammals might relate to at least three different reasons, among others. A first reason might relate to the large importance of amino acids for metabolic purposes in fish, not only in carnivorous but also in herbivorous and omnivorous species. A second reason may be due to the high variety of dietary fish habits resulting in large differences in gastrointestinal morphology and function. A third reason may rely on the existence in fish of multiple gene variants in neuropeptides, hormones, and metabolic effectors resulting from the additional gene duplication of actinopterygians. The assessment of these topics, together with the possible presence and functioning of amino acid sensing systems in fish, as well as the elucidation of signaling pathways linking activity of sensors with the effectors controlling homeostasis, such as expression of neuropeptides controlling food intake, hormone secretion or metabolic changes, are open questions demanding further research in the near future.

AUTHOR CONTRIBUTIONS

Both authors designed the paper, wrote and approved the final version of the manuscript.

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REVIEW
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Regulation of Agouti-Related Protein and Pro-Opiomelanocortin Gene Expression in the Avian Arcuate Nucleus

Timothy Boswell[1]* *and Ian C. Dunn*[2]

[1] School of Biology, Newcastle University, Newcastle upon Tyne, UK, [2] Royal (Dick) School of Veterinary Studies, Roslin Institute, University of Edinburgh, Easter Bush, UK

The arcuate nucleus is generally conserved across vertebrate taxa in its neuroanatomy and neuropeptide expression. Gene expression of agouti-related protein (AGRP), neuropeptide Y (NPY), pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) has been established in the arcuate nucleus of several bird species and co-localization demonstrated for AGRP and NPY. The proteins encoded by these genes exert comparable effects on food intake in birds after central administration to those seen in other vertebrates, with AGRP and NPY being orexigenic and CART and α-melanocyte-stimulating hormone anorexigenic. We have focused on the measurement of arcuate nucleus AGRP and POMC expression in several avian models in relation to the regulation of energy balance, incubation, stress, and growth. AGRP mRNA and POMC mRNA are, respectively, up- and downregulated after energy deprivation and restriction. This suggests that coordinated changes in the activity of AGRP and POMC neurons help to drive the homeostatic response to replace depleted energy stores in birds as in other vertebrates. While AGRP and POMC expression are generally positively and negatively correlated with food intake, respectively, we review here situations in some avian models in which AGRP gene expression is dissociated from the level of food intake and may have an influence on growth independent of changes in appetite. This suggests the possibility that the central melanocortin system exerts more pleiotropic functions in birds. While the neuroanatomical arrangement of AGRP and POMC neurons and the sensitivity of their activity to nutritional state appear generally conserved with other vertebrates, detailed knowledge is lacking of the key nutritional feedback signals acting on the avian arcuate nucleus and there appear to be significant differences between birds and mammals. In particular, recently identified avian leptin genes show differences between bird species in their tissue expression patterns and appear less closely linked in their expression to nutritional state. It is presently uncertain how the regulation of the central melanocortin system in birds is brought about in the situation of the apparently reduced importance of leptin and ghrelin compared to mammals.

Keywords: melanocortin, AGRP, pro-opiomelanocortin, melanocortin 4 receptor, hypothalamus, birds, leptin

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***Correspondence:**
Timothy Boswell
timothy.boswell@ncl.ac.uk

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INTRODUCTION

Neural circuitry in the arcuate nucleus of the hypothalamus is well established in mammals as being particularly important for the regulation of energy balance (1, 2). Two neuronal cell types are involved. One synthesizes both agouti-related protein (AGRP) and neuropeptide Y (NPY), and the other produces α-melanocyte-stimulating hormone (α-MSH) and other peptides from the pro-opiomelanocortin (POMC) precursor together with cocaine- and amphetamine-regulated transcript (CART). AGRP and α-MSH peptides secreted from arcuate nucleus neurons bind to melanocortin receptors in the brain and collectively comprise the components of the central melanocortin system. AGRP/NPY neurons exert anabolic effects on food intake and body mass while POMC/CART neurons are catabolic. Evidence from non-mammalian vertebrate taxa suggests that the neuronal circuitry has been evolutionarily conserved both in its neuroanatomical location and in the genes the cells express. For example, AGRP, NPY, POMC, and CART mRNAs have all been localized in several teleost fish species in the lateral tuberal nucleus (NLT), the equivalent of the mammalian arcuate nucleus (3–6). Comparable observations have been made in birds, where the arcuate nucleus has historically been named the infundibular nucleus. Immunoreactive cell bodies and mRNA have been localized in the arcuate nucleus in several bird species for NPY, AGRP, and POMC (7–12). Furthermore, the co-expression of AGRP and NPY mRNA characteristic of laboratory rodents has been demonstrated in individual arcuate nucleus neurons in the Japanese quail (*Coturnix coturnix japonica*) (13). Less is known about CART in birds and its co-expression with POMC has yet to be formally demonstrated. However, immunoreactive CART neuronal cell bodies have been identified in the arcuate nucleus of the zebra finch (*Taeniopygia guttata*) (14).

We consider in this review the extent to which the evolutionary neuroanatomical conservation of the arcuate nucleus neurons implicated in energy balance in birds is conserved at the functional level, with an emphasis on our recent studies on the regulation of AGRP and POMC expression in several avian models.

AGRP, POMC, AND ENERGY HOMEOSTASIS

Nutritional Sensitivity of AGRP and POMC Gene Expression

It is well established in laboratory rodents that neuropeptide gene expression in the arcuate nucleus AGRP/NPY and POMC/CART neurons is sensitive to nutritional state (physiologically reported in mammals by variation in plasma leptin concentrations) as part of a counter-regulatory response to loss of body energy stores during situations of negative energy balance such as fasting or food restriction (2). Comparable findings have been obtained in birds in response to both experimental food deprivation and chronic restriction. For example, AGRP mRNA was increased by food deprivation for 24 h in adult Japanese quail, a response also consistently observed after food deprivation for 24–48 h in domestic broiler chicks (11, 15–18). AGRP expression returned to baseline levels after 24 h refeeding (15–18). We have extended these observations to investigate the effects of chronic food restriction in growing broiler breeder hens. Food restriction of the parent hens of broiler chickens during the growth phase is a common practice in the poultry industry in order to mitigate poor reproductive performance when birds are fed *ad libitum* (19). We observed that AGRP mRNA was strongly increased in 12-week-old hens that had been maintained on an industrial food restriction regime for 6 weeks and gene expression returned to baseline after 2 days' refeeding (20). A further comparison within the same experiment indicated that the level of AGRP mRNA was sensitive to feeding history: in two groups of hens sampled at the same body mass, AGRP gene expression was significantly higher in a group that had been maintained at an intermediate level of food restriction for 6 weeks compared to a group that had been maintained on commercial food restriction for 4 weeks before being released onto 2 weeks of *ad libitum* feeding. These results suggested that AGRP gene expression in broiler breeder hens is at least as sensitive to chronic food restriction as in mammals such as sheep, Siberian hamsters (*Phodopus sungorus*), rats, and golden spiny mice (*Acomys russatus*) (21–24). Furthermore, its expression is more closely linked to feeding state than reported in some rat studies of chronic food restriction where either no change was observed in AGRP mRNA, or where arcuate nucleus neuropeptide mRNAs had not returned to baseline levels after 4 weeks' refeeding (25, 26).

It would be predicted from mammalian studies that food deprivation and restriction would exert an opposite, inhibitory, effect on POMC mRNA levels compared to AGRP mRNA because POMC gene expression induces a catabolic effect on energy balance (2). However, in birds, some studies have reported no change in POMC mRNA level after 24–48 h food deprivation in Japanese quail and broiler chicks (11, 17) while significantly decreased POMC expression has been observed in other broiler chick studies (15, 16, 18). For chronic food restriction, we detected no difference in POMC expression after 6 weeks' restriction in our study of 12-week-old broiler breeder hens mentioned above, but another investigation reported a significant decrease after 7 days' restriction in both 3-week-old broiler and layer chicks (20, 27). The greater variability in detecting altered POMC mRNA and the reduced magnitude of the change in POMC expression compared to that for AGRP in response to food deprivation or restriction may reflect a greater relative importance of increased AGRP expression in the counter-regulatory response to lost energy stores or, alternatively, a possibly greater role for regulatory control at the level of POMC-derived peptide secretion as has been reported in mammals (28).

Nutritional Regulation of AGRP- and POMC-Derived Peptide Synthesis and Secretion

The significance of altered AGRP and POMC gene expression in response to manipulation of energy status is beginning to be understood in laboratory rodents. For example, the respective increased and decreased AGRP and POMC mRNA following

a fast appears to be matched by parallel changes at the level of increased release of AGRP peptide and decreased release of α- and γ-MSH peptides (29–31) and by, respectively, increased and decreased firing of AGRP and POMC neurons (32, 33). Furthermore, optogenetic approaches have demonstrated that feeding behavior can be directly induced or inhibited by light activation of AGRP and POMC neurons, respectively (34). In contrast, most studies of the central melanocortin system in birds have focused on mRNA measurements as an indicator of the activity of the neurons. However, a few investigations have demonstrated the presence of central melanocortin system peptides in the avian arcuate nucleus. For example, AGRP immunoreactivity was identified in the Khaki Campbell duck (*Anas platyrhynchos*) arcuate nucleus (12), and evidence for altered synthesis of AGRP peptide is available for the ring dove (*Streptopelia risoria*), where increased numbers of AGRP-immunoreactive cell bodies were observed in the medio-basal hypothalamus following a 48-h fast and also during the post-hatching phase of the reproductive cycle when the parent birds are in negative energy balance as a result of the demands of feeding offspring (10, 35). Relatively little is known in birds about the processing of POMC-derived peptides in the hypothalamus and their relative importance for energy balance regulation. However, immunoreactive neuronal cell bodies for α-MSH, β-endorphin, and N-terminal POMC (pro-γ-MSH) were detected in the arcuate nucleus of broiler chickens (7), and co-localization of POMC mRNA and α-MSH peptide was observed in individual arcuate nucleus neurons in Japanese quail (11).

Given the general lack of information in birds about AGRP and POMC signaling above the mRNA level, the significance of changes in gene expression in relation to energy status is generally inferred from the mammalian literature and from the behavioral effects of the encoded peptides. There is behavioral evidence that domestic pigeons and Japanese quail eat more in a refeeding period after food deprivation compared to control birds that had been allowed to feed freely over the experimental fasting phase (13, 36). This increased food intake combined with knowledge about the effects of energy deprivation on AGRP and POMC gene expression is consistent with the idea that fasting stimulates food intake by increased secretion of AGRP peptide and, in some situations, reduced secretion of POMC-derived peptides such as α-MSH.

Central Melanocortin Receptors

The avian central melanocortin system peptides appear to exert their effects by acting on melanocortin receptors in the hypothalamus as in mammals. Characterization of the pharmacological properties of the five chicken melanocortin receptor subtypes *in vitro* revealed a relatively greater affinity for ACTH-derived peptides than for α-MSH compared to their mammalian orthologs (37). This might suggest a more significant role in birds for ACTH as a central melanocortin receptor ligand. Studies have not been performed to localize or quantify ACTH peptide in the avian hypothalamus, but its synthesis and secretion have been reported in the hypothalamus of laboratory rats (28), and an inhibitory effect on feeding of the centrally administered peptide has been observed in rats, domestic pigeons (*Columba livia*), and broiler chicks (38–40). Another POMC peptide, β-endorphin, has been localized in the chicken arcuate nucleus, as noted above (7). It stimulates food intake after central injection of the ostrich and mammalian peptide, respectively, in the domestic pigeon and the chicken (41, 42). Further investigation is needed to explore the context and significance of β-endorphin secretion from POMC/CART neurons.

Pharmacological studies have been performed in birds on the melanocortin 4 receptor (MC4R), the melanocortin receptor most strongly associated with the regulation of food intake in mammals. The synthetic compounds HS014 and HS024 were identified *in vitro* as selective antagonists and melanotan II (MTII) as a potent agonist in the chicken (37). The predicted stimulatory effects on food intake for the antagonist, and inhibitory effects for the agonist, have been confirmed for HS014 and MTII in ring doves and in broiler and layer chickens following central or peripheral injection (27, 35, 43). Several studies have investigated the effect of central injection of MSH peptides on food intake in domestic chicks. Inhibitory effects of α-, β-, and γ2-MSH have been observed (44–47) with reports of differential sensitivity between broiler and layer and high and low-growth lines (48, 49). However, while the amino acid sequence of α-MSH is conserved across vertebrates, the chicken sequences for β- and γ2-MSH differ between birds and mammals and appear to have a less potent effect on food intake in chicks when administered centrally compared to mammalian versions (43). This suggests a more prominent role for α-MSH among the avian POMC-derived peptides. As in mammals, there is evidence for competition between the α-MSH agonist and the AGRP antagonist at central melanocortin receptors because central injection of AGRP dose-dependently attenuated the inhibitory effect on feeding of α-MSH in domestic chicks (45). Central injections of AGRP alone stimulated food intake, as expected, in the ring dove but with an apparently lower potency than observed in laboratory rodents, which may reflect the use of heterologous human AGRP fragments in the ring dove experiment (35). Layer chicks were observed to be more sensitive in their feeding response to central AGRP injection than broiler chicks (45).

While pharmacological and behavioral studies suggest that the actions of AGRP- and POMC-derived peptides, particularly α-MSH, are conserved between birds and mammals, knowledge is lacking in birds about the site of action of the peptides and the central melanocortin receptor subtypes that naturally mediate their signaling effects: the projections of AGRP/NPY and POMC/CART neurons have not been studied, and the central melanocortin receptors involved have not been precisely localized. Both MC4R and melanocortin 3 receptor (MC3R) mRNA have been quantified in the chicken hypothalamus in real-time PCR studies (15, 17, 50, 51) and the melanocortin 5 receptor more generally in the brain (50, 52). Increased MC4R gene expression has been observed after 48 h food deprivation in broiler chicks (15, 17), which may be in response to decreased agonistic drive. Little is known about the possible role of the MC3R in the regulation of energy balance in birds, but its hypothalamic expression was higher in chickens lines selected for low compared to high body weight (50, 51).

REGULATION OF AGRP AND POMC EXPRESSION BY INCUBATION, STRESS, AND PREPARATION FOR MIGRATION

Effects of Incubation and Stress

While the dynamic coordinated changes in arcuate nucleus AGRP and POMC gene expression in response to energy shortage are well established in mammals and conserved in birds, less is known about the occurrence and influence of altered basal expression of these genes. Uniquely avian models are available to test the hypothesis that variation in the levels of AGRP and POMC mRNA measured in individuals with free access to food promotes the expression of natural seasonal changes in feeding behavior and metabolism. We have recently investigated this in a chicken strain that exhibits natural incubation behavior. Hens incubate their eggs over a 3-week period that is associated with a suite of behavioral and physiological changes known as "broodiness" (53). Hens spend an increasing amount of time sitting on their eggs and cease laying. Linked to this is the expression of natural anorexia when birds reduce their food intake and reduce body mass over the incubation period (54). Experimental food deprivation and refeeding in junglefowl hens suggested that the level around which body mass is defended (or set point) is reduced during incubation, and the concept was extended to other vertebrate species of natural "animal anorexias" that are expressed in phases of life history such as hibernation or territorial defense where the time available to feed is limited (55, 56).

We drew on these studies to investigate how AGRP and POMC gene expression changes during the incubation phase in hens (57). One possible outcome was that there would be no change in gene expression because in birds with free access to food, body mass is at its appropriate defended level despite the fact that body mass is lower: altered AGRP and POMC expression would only be expected in response to perturbation such as food deprivation or restriction. Alternatively, it was possible that changes in basal gene expression play a role in promoting the loss of body mass. In this case, increased POMC expression would be expected, combined with unaltered, or reduced, AGRP expression. We controlled for possible confounding effects of, respectively, enlarged and regressed ovaries in laying and incubating hens by pair-feeding two groups of laying hens to the amount of food eaten voluntarily by incubating birds (57). This resulted in ovarian regression in those groups that matched that shown in the incubating hens. One of the pair-fed groups was allowed to refeed for 5 days so that food intake and body mass stabilized to reveal the natural *ad libitum* food intake level in birds with regressed ovaries. In birds sampled 21 days after the onset of incubation, POMC gene expression was increased to a level on the border of statistical significance in the incubating hens compared to the two control groups, consistent with the idea that this is linked to an increased anorexic drive. However, unexpectedly, AGRP mRNA was higher in both incubating and pair-fed birds, compared to the re-fed control group. This finding is interesting in suggesting that increased AGRP gene expression (and the assumed associated increase in AGRP peptide signaling) does not necessarily result in increased food intake. This situation was not unique to incubation. A related experiment arose from our observation that hens transferred from single housing in a cage to housing in a pen showed reduced food intake in their new environment presumably because they perceived the transfer as stressful (57). Measurements of AGRP and POMC gene expression 6 days after the housing transfer showed results comparable to the incubation experiment, this time with significantly increased POMC mRNA combined with increased AGRP expression. This result is again consistent with increased POMC expression contributing to anorexic drive that leads to the reduced food intake. The fact that AGRP gene expression is increased during incubation and after housing transfer suggests that its sensitivity to reduced energy availability is maintained despite an apparent change in the defended set point for body mass during incubation. The normal stimulatory effects of AGRP expression on food intake may be overridden by a relatively greater inhibitory influence of the increased POMC expression and, in the case of incubation, a possible inhibitory influence on feeding of hypothalamic vasoactive intestinal polypeptide, the expression of which is causatively linked to incubation behavior in birds (53). The fact that AGRP gene expression is increased in these situations may be of adaptive significance in promoting more rapid restoration of energy stores when incubation ends and as part of the recovery from the effects of a stressor. For incubation, it is also possible that increased AGRP mRNA is linked to altered daily patterns of behavior during incubation when expression of ingestive behavior is confined to two daily recesses from nest sitting but during which feeding may be relatively intense (53).

Photoperiodic Effects and Migratory Physiology

Another opportunity provided by avian models to investigate the possibility of seasonal regulation of AGRP and POMC gene expression is represented by species that show increased appetite and fat deposition as preparation for migratory flight (58). Laboratory studies in captive birds have revealed that the increased appetite (hyperphagia) and fat deposition that occur before migration are stimulated by changes in daylength and appear to involve changes in the level around which body mass is regulated as has been suggested for incubation (59). Seasonal changes in reproductive physiology mediated by photoperiod in birds and seasonal mammals have been linked to release of thyroid-stimulating hormone from the pars tuberalis of the pituitary gland. This results in conversion within the mediobasal hypothalamus of thyroxine into triiodothyronine (T3) that promotes release of gonadotropin-releasing hormone from neuron terminals in the median eminence (60). It is possible that locally increased tissue concentrations of T3 mediate seasonal cycles in appetite and fat deposition in addition to reproduction. This is suggested by a mammalian study of Siberian hamsters that received hypothalamic implants of T3. This procedure on short day animals induced changes in body mass characteristic of exposure to long photoperiods (61). Furthermore, there is evidence in domestic chicks that experimentally increased T3 stimulates hypothalamic AGRP gene expression both *in vivo* and *in vitro* (62). However, there is limited evidence in hamsters for an important role for the arcuate nucleus and its neuropeptides in

driving seasonal cycles in food intake and body mass (63). We are currently investigating whether the situation is similar in birds by quantifying AGRP gene expression in Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) after photostimulation. This will test whether increased AGRP gene expression is associated with seasonally increased food intake in this migratory species.

INFLUENCE OF GROWTH AND SEX ON AGRP AND POMC EXPRESSION

In addition to a possible role of basal changes in gene expression in regulating reproductive and seasonal changes in food intake and body weight, our recent studies suggest that AGRP and POMC expression may be related to growth. In our chronic food restriction experiment on broiler breeder hens (20) reviewed in relation to energy homeostasis above, we noted that AGRP gene expression was inversely related to body mass in the groups maintained on *ad libitum* feeding at the time of sampling (**Figure 1**). Birds were the same age at the time they were killed and had all been maintained on commercial restricted feeding for the first 6 weeks. The highest body mass was attained in the birds fed *ad libitum* over the next 6 weeks and the lowest in birds that were only allowed to refeed for 2 days. AGRP mRNA remained significantly elevated in birds fed *ad libitum* for 2 weeks compared to 6 weeks. Thus, the level of AGRP expression could be regarded as a measure of the growth potential of the different experimental groups, with the highest expression in birds that were furthest away from the natural growth trajectory. We have further evidence for this at the genetic level. When we compared AGRP gene expression between males and females in 12-week-old broiler breeder chickens that had been re-fed for 2 days after food restriction, expression was significantly higher in males and this difference was replicated in *ad libitum*-fed fully mature chickens of another genetic strain (64). The higher AGRP expression in males is consistent with the fact that they grow faster and attain a higher mature body mass. It would therefore be predicted that AGRP mRNA would be higher in fully fed birds in chicken strains that grow more rapidly. We obtained evidence for this from trait linkage analysis of birds from a broiler-layer cross that differed in growth rate (65). We identified a genotype that explained 19% of the difference in body mass between the lines that was associated with lower global tissue expression of the cholecystokinin A receptor (CCKAR) in the high-growth haplotype. We demonstrated that the high-growth birds were less sensitive to the inhibitory effects on food intake of intraperitoneal cholecystokinin (CCK) injection and that they showed significantly higher expression of AGRP (but no difference in POMC mRNA). This suggests that the tone of CCK signaling influences AGRP expression. It could be predicted that increased AGRP expression is causative in generating higher growth by stimulating food intake. However, we have been unable to find a consistent difference in daily food intake between the lines. This demonstrates again, as we observed for incubation, that high AGRP gene expression can be dissociated from increased feeding. Any effects of AGRP on growth rate must therefore be independent of food intake. The mechanisms involved are currently unclear and point to a need in birds to investigate the effects of central melanocortin system signaling on metabolic aspects of energy balance regulation distinct from feeding behavior.

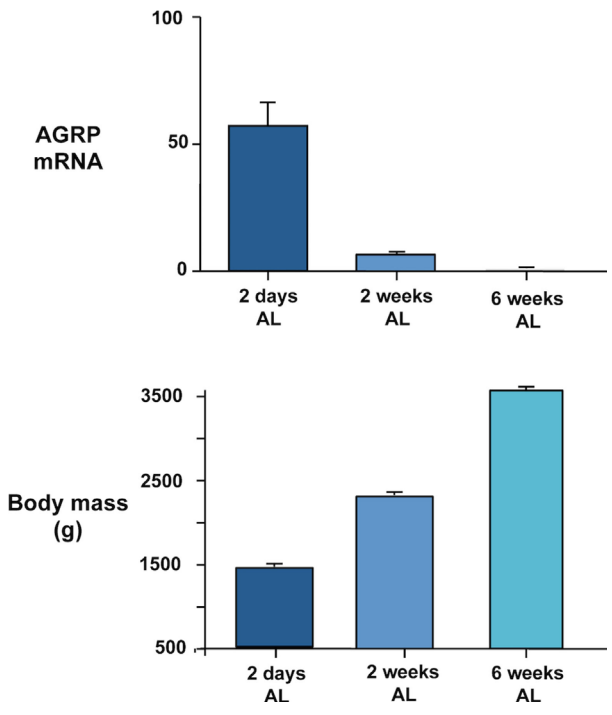

FIGURE 1 | Inverse relationship between AGRP gene expression quantified in dissected basal hypothalamus by real-time PCR (upper panel) and body mass (lower panel) in female broiler breeder chickens (Ross 308 line). Birds were maintained on a commercial food restriction program from hatch before being transferred to *ad libitum* (AL) feeding at 2 days, 2 weeks, and 6 weeks before the birds were killed at 12 weeks of age. Further experimental details are provided in Ref. (20).

While our observations linking AGRP expression to growth in birds are preliminary and require further investigation, they serve to highlight a possible involvement of the central melanocortin system in growth regulation that has received limited attention in vertebrates compared to its more general effects on energy homeostasis. Experimental inactivation or natural mutation of the MC4R in laboratory mice and humans is associated with increased linear growth (66, 67). The mechanisms underlying this effect are not fully understood but appear to be independent of growth hormone secretion and involve hyperinsulinemia (68, 69). More direct evidence for a link between the central melanocortin system and growth has been obtained from teleost fish for which it has been reported that transgenic overexpression of the natural melanocortin receptor antagonist agouti-signaling protein reversed the pattern of sexually dimorphic growth in zebrafish (*Danio rerio*) (70) and that AGRP and POMC neurons project directly to the pituitary gland (71). Transgenic overexpression of AGRP itself in zebrafish led to both obesity and increased linear growth, while suppression of AGRP expression reduced larval growth rate and was mediated through the MC4R (71, 72). Thus it seems that AGRP exerts more pleiotropic

effects on hormonal axes through the pituitary gland in teleosts compared to mammals and more investigation is needed to determine whether this applies to non-mammalian vertebrates more generally including birds.

REGULATION OF AGRP AND POMC EXPRESSION BY METABOLIC HORMONES, HYPOTHALAMIC ENERGY SENSING, AND GUT FILL

Leptin

Less is known in birds than mammals about the regulatory feedback signals to the arcuate nucleus that mediate the physiological changes in AGRP and POMC gene expression reviewed above. This is due in part to uncertainty over the status of leptin in birds, with leptin being a key regulator of the central melanocortin system in mammals (2). Leptin genes have recently been identified in several avian species after a 20-year search (73–77). However, they have low (about 30%) amino acid sequence identity with mammalian leptins, with variable tissue expression patterns between species and limited expression in adipose tissue (78). Mammalian leptins inhibit food intake in birds after central or peripheral injection but peripheral infusion of "chicken leptin" (later shown to be mouse leptin) had no effect on AGRP and POMC gene expression in young broilers (79). Thus, unlike the situation in mammals, there is currently no direct evidence to support an action of leptin on AGRP and POMC gene expression, but further investigation is needed that takes into account the new information on avian leptin.

Ghrelin

A pronounced difference between birds and mammals is also apparent for the effects of ghrelin on food intake. There is commonality in that, as in mammals, ghrelin is expressed in the avian stomach (proventriculus) and its gene expression and circulating protein are increased by fasting and decreased by refeeding in layer chickens and Japanese quail (80). Also, in free-living garden warblers (*Sylvia borin*) sampled at a stopover site during spring migration, plasma ghrelin concentrations were positively associated with higher fat scores (81). However, unlike the situation in mammals, central and peripheral injections of mammalian and chicken ghrelin decrease, rather than stimulate, feeding (80, 81). There is evidence in mammals for a regulatory influence of ghrelin on AGRP/NPY neurons (82). Expression of the ghrelin receptor (GHSR) has been detected in the domestic chick hypothalamus (83), but its localization there, including in the arcuate nucleus, is unknown. The inhibitory effect of centrally administered ghrelin on food intake appeared to be mediated *via* corticotropin-releasing factor rather than through AGRP/NPY neurons because ghrelin injection did not influence NPY gene expression (84).

Hypothalamic Energy Sensing

At the local tissue level, there is suggestive evidence for a link in birds between arcuate nucleus neuropeptide gene expression and hypothalamic energy sensing as there is in mammals. Immunoreactivity for the energy sensor AMP-activated protein kinase (AMPK) was observed in the chicken arcuate nucleus and food deprivation for 48 h increased phosphorylated AMPKα in parallel with AGRP mRNA (17, 85). In broiler chicks, 24 h food deprivation led to increased expression of the AMPK subunit mRNAs AMPKα2, AMPKβ1, AMPKβ2, and AMPKγ1 along with, respectively, increased and decreased AGRP and POMC mRNAs (16). When domestic broiler chicks were fed with the AMPK inhibitor α-lipoic acid (α-LPA), hypothalamic AMPKα1 mRNA was decreased along with food intake, confirming α-LPA's inhibitory effect (86). However, the pattern of expression of AGRP and POMC was opposite to that predicted for a regulatory effect of AMPK. The same study (86) also confirmed the expression in the chick hypothalamus of hypoxia-inducible factor-1α, a nuclear transcription factor that influences POMC gene expression in mammals in response to local hypoxia (87). More generally, microarray and real-time PCR analysis of gene expression in the hypothalamus of broiler chicks food-deprived for 48 h suggested that fasting induces metabolic switching (16, 18). Genes associated with fatty acid oxidation and inhibition of glycolysis were upregulated, and those linked to fatty acid synthesis and transport downregulated, in parallel with increased AGRP and reduced POMC gene expression. The suggestion of metabolic switching was supported by central injection of compounds influencing glycolysis and fatty acid oxidation (18). Injection of α-LPA decreased food intake, which is consistent with the finding of the dietary administration study above and with α-LPA stimulating glycolysis through inhibition of pyruvate dehydrogenase kinase isoform 4 (18). In contrast, injection of the glycolytic inhibitor 2-deoxyglucose stimulated food intake. The possible importance of the metabolic sensor sirtuin 1 in mediating the metabolic switching was suggested by the fact that administration of its inhibitor NADH and activator NAD+, respectively, decreased and increased food intake (18). However, the effect of these manipulations on neuropeptide gene expression was not reported so that it is not clear to what extent the changes in food intake observed are directly attributable to regulatory effects on AGRP/NPY and POMC/CART neurons. Thus, overall, although suggestive, evidence for a direct effect of metabolic sensing on AGRP and POMC gene expression is lacking and further investigation is needed.

Local Hypothalamic Signaling

Signals that may influence central melanocortin system gene expression within the hypothalamus have been explored by microarray and pathway analysis in newly hatched broiler chicks that were food deprived for up to 48 h and re-fed (15). Functional interactions, supported by hypothalamic cell culture experiments, were identified within a network of six genes encoding the neuropeptide relaxin-3, the neuropeptide receptors NPY5R and somatostatin receptor 5, and the β2 adrenergic and metabotropic glutamate receptor 8 neurotransmitter receptors together with POMC, which appeared to play a central role. POMC expression was downregulated by fasting while the other genes were upregulated.

Insulin

For metabolic hormones, evidence is needed for co-expression of hormone receptors in individual AGRP/NPY and POMC/CART neurons. So far, this has been established only for insulin. Before the discovery of leptin, insulin was favored in mammals as a long-term regulator of energy balance because fasting, or basal, concentrations report body fat content, the hormone is transported into the brain, and insulin receptors are present on AGRP/NPY neurons (2). It is less clear whether it plays a similar role in the long-term regulation of energy balance of birds: although circulating insulin concentrations are correlated with food intake in chickens, they did not differ under fasting conditions between selected lines of fat and lean birds (88). However, central injection of insulin decreased food intake in layer chicks and the effect was blocked by coadministration of the MC4R antagonist HS014 (89, 90). A direct effect of insulin on arcuate nucleus neurons was suggested by the demonstration of co-localization of insulin receptor immunoreactivity with that of NPY, and with α-MSH in individual neurons (91). Insulin stimulates POMC gene expression and inhibits that of AGRP and NPY in the brain of laboratory rodents (92), and this regulatory influence appears to have been conserved to some extent in birds. Thus, in the chick studies, central insulin injections consistently stimulated POMC expression, but a decrease in NPY mRNA was observed in one study and not another, and no decrease in AGRP mRNA was detected (89, 90). Overall, however, the results suggest an involvement of insulin in the response of POMC/CART and possibly AGRP/NPY neurons to fasting.

Corticosterone and Thyroid Hormones

Other metabolic hormones that have a regulatory influence on AGRP/NPY and POMC/CART neurons in mammals are corticosterone and thyroid hormones. Circulating corticosterone is increased by fasting in birds and mammals and, respectively, increases and decreases AGRP and POMC gene expression in laboratory rodents (93, 94). There is evidence in domestic chicks for a suppressive effect of corticosterone on POMC expression (62, 95). However, there is more variability in the response of AGRP gene expression to centrally or peripherally administered corticosterone or to the glucocorticoid receptor agonist dexamethasone (62, 95–97). Thyroid hormones have already been mentioned in the context of preparation for migration above. There is evidence in both birds and mammals for a stimulatory effect of T3 on AGRP gene expression (63, 98). Thus, overall, there is some commonality in the regulatory influences of corticosterone and T3 on AGRP/NPY and POMC/CART neurons between birds and mammals.

Cholecystokinin

We identified a possible link between signaling by the gut peptide CCK and AGRP expression in the context of growth as reviewed above (65). It is unclear whether the increased AGRP expression we observed in high-growth haplotype chickens is a secondary consequence of reduced CCKAR expression and experimental manipulations of CCK signaling in other chicken strains are needed. In mammals, there is evidence for an involvement of the central melanocortin system, both in the hindbrain and the hypothalamus, in the inhibitory effects of CCK on feeding because MC4R knockout mice show a reduced sensitivity (99, 100).

Gut Fullness Effects

In addition to feedback effects by metabolic hormones, we have recently investigated the possibility for a sensitivity of AGRP gene expression to signals arising from gut fullness. This has been in the applied context of attempting to improve the welfare of broiler breeder chickens that experience prolonged hunger during the industrial practice of food restriction when birds receive a limited ration of food per day. As an alternative, it is possible to use alternative diets containing a high proportion of food that is normally high in fiber and of low energy density (101). This could potentially mitigate some of the undesirable effects of constant hunger by providing more total food and therefore more opportunity for the expression of natural foraging and ingestive behaviors. However, it is uncertain whether birds fed on such diets still experience a "metabolic hunger." To address this, we provided restricted-fed 12-week-old broiler breeder birds with *ad libitum* access to food for 2 days compared to birds re-fed a diet over the same time period that was diluted with the non-nutritive bulking agent ispaghula husk, and to birds that remained on a restricted diet (64). We measured significantly increased AGRP expression and decreased POMC expression compared to fully fed controls in both food-restricted birds and in those receiving the dietary bulking agent. This suggests that AGRP and POMC gene expression is insensitive to mechanosensory signals relating to gut fullness. We are performing further experiments to confirm this using other more industrially relevant diet formulations.

CONCLUSION

The striking neuroanatomical conservation among vertebrates of the arcuate nucleus AGRP/NPY and POMC/CART neurons appears to be accompanied in birds by functional conservation in these cells of a coordinated signaling response to energy deprivation whereby AGRP/NPY neurons are stimulated and POMC/CART neurons are inhibited, which promotes replacement of lost energy stores when food becomes available. However, the picture is somewhat incomplete in birds compared to mammals with investigations tending to be skewed toward the measurement of gene expression rather than being focused on secretion of the peptides. Information is lacking on the connections of the neurons, both within and outside the hypothalamus, and little is known about the metabolic and energetic effects of the arcuate nucleus peptides distinct from their effects on food intake. Knowledge in birds has also been limited by a lack of availability of transgenic methods to assess the effects on energy balance of experimental genetic activation and inactivation, although such methods now appear to be close to routine application (102).

The use of unique avian models to explore aspects of the regulation of the central melanocortin system has highlighted possible differences from mammals and emphasized adaptations that may be representative of those in other non-mammalian vertebrates. These include changes in basal expression of AGRP and POMC in relation to seasonal changes in food intake, energy balance,

and reproduction that tend not to be apparent in mammalian models (63). There are also situations in birds in which AGRP gene expression is dissociated from the pattern of food intake, particularly in relation to sexually dimorphic growth, which widens the perspective of investigations into the functions of the melanocortin system in birds and other vertebrates beyond the regulation of appetite.

The apparent functional conservation of changes in central melanocortin system gene expression in response to energy shortage is somewhat puzzling from the evidence available on how the system is regulated by feedback signals. Circulating leptin is acknowledged to play a prominent regulatory role on the system in mammals whereas in birds its sites of synthesis are variable and less focused on adipose tissue, and it appears to act more as an autocrine/paracrine signaling factor than as a circulating hormone (78). The recent discovery of avian leptin genes together with the knowledge that the pattern of expression is more representative of other non-mammals than mammals offers the opportunity for the regulation of the avian melanocortin system to be viewed from a new perspective. More commonality between birds and mammals is evident in the regulatory effects of other metabolic hormones such as insulin, corticosterone, and T3. However, it is presently unclear whether these hormones exert the main regulatory effects on the system with diminished input from leptin and ghrelin, or whether other regulatory mechanisms are present that are either ancestral and representative of other non-mammalian vertebrates rather than mammals or are unique and linked to the general adaptations that have evolved in birds to support flight.

AUTHOR CONTRIBUTIONS

TB wrote the article with substantial intellectual and editorial input from ID on its content and structure. Both authors approved the work for publication.

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REVIEW
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Appetite-Controlling Endocrine Systems in Teleosts

Ivar Rønnestad[1], Ana S. Gomes[1], Koji Murashita[1,2], Rita Angotzi[1], Elisabeth Jönsson[3] and Hélène Volkoff[4]*

[1] Department of Biology, University of Bergen, Bergen, Norway, [2] Research Center for Aquaculture Systems, National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, Tamaki, Mie, Japan, [3] Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden, [4] Departments of Biology and Biochemistry, Memorial University of Newfoundland, St John's, NL, Canada

Mammalian studies have shaped our understanding of the endocrine control of appetite and body weight in vertebrates and provided the basic vertebrate model that involves central (brain) and peripheral signaling pathways as well as environmental cues. The hypothalamus has a crucial function in the control of food intake, but other parts of the brain are also involved. The description of a range of key neuropeptides and hormones as well as more details of their specific roles in appetite control continues to be in progress. Endocrine signals are based on hormones that can be divided into two groups: those that induce (orexigenic), and those that inhibit (anorexigenic) appetite and food consumption. Peripheral signals originate in the gastrointestinal tract, liver, adipose tissue, and other tissues and reach the hypothalamus through both endocrine and neuroendocrine actions. While many mammalian-like endocrine appetite-controlling networks and mechanisms have been described for some key model teleosts, mainly zebrafish and goldfish, very little knowledge exists on these systems in fishes as a group. Fishes represent over 30,000 species, and there is a large variability in their ecological niches and habitats as well as life history adaptations, transitions between life stages and feeding behaviors. In the context of food intake and appetite control, common adaptations to extended periods of starvation or periods of abundant food availability are of particular interest. This review summarizes the recent findings on endocrine appetite-controlling systems in fish, highlights their impact on growth and survival, and discusses the perspectives in this research field to shed light on the intriguing adaptations that exist in fish and their underlying mechanisms.

Keywords: appetite control, feed intake, hormones, neuropeptides, teleosts, adaptations, fasting, voracious feeding

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****Correspondence:***
Ivar Rønnestad
ivar.ronnestad@bio.uib.no

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INTRODUCTION

Control of food intake and energy metabolism is vital for the development and survival of an organism. These processes ensure optimal allocation of energy resources to cover the basic maintenance of metabolism and immune system, the cost of foraging and other daily activities, somatic growth, reproductive investment, and sufficient energy stores to survive periods of low food availability (1).

Terminology for gene names: GENE (All capitals), Mammalian protein; Gene (First letter capital), Fish Protein; *Gene* (First letter capital, italic), Mammalian gene; *gene* (small letters only and italic), Fish gene.

Food intake is affected by external factors, such as temperature and photoperiod, stress, predators, and food availability, as well as by internal factors, such as genetics, life stage, gut filling, and stored energy. The hypothalamus is the hub that controls appetite and energy balance and integrates peripheral signals related to food intake and digestion, metabolism, and energy storage (**Figure 1**). These include not only endocrine signals (gut peptides, the focus of this review) but also other signals such as nutrient levels through central nutrient sensing systems and the presence/absence of food in the gastrointestinal (GI) tract through vagal afferents projecting to the brain.

Fishes represent over 30,000 species with an enormous variation in their ecological niches and habitats as well as life history adaptations, transitions between life stages and feeding behaviors. In the context of food intake and appetite control, common adaptations to extended periods of starvation or periods of abundant food availability are of particular interest. Also, the large variations in appetite between species and within a species (individual variation) are intriguing. A large fraction of fish species has indeterminate growth, i.e., these species continue to grow during their whole life span. This contrasts with growth in mammals and other model animals including zebrafish (*Danio rerio*), which reach a maximum length size as adults. Thus, while control of appetite and food intake is often viewed as a behavioral component of maintaining an energy balance (2), the general concept of energy homeostasis needs to be used with caution.

This review summarizes the recent findings on appetite-controlling systems in fish with a focus on peptide hormones. A major goal is to discuss perspectives in this research field that can reveal how fish adapt to their specific ecological requirements.

CENTRAL CONTROL

The physiological mechanisms that control appetite are relatively well conserved among vertebrates, and many of the neuropeptides and hormones involved in the central appetite regulation in mammals are also found in fish (3–7). However, differences in appetite-controlling systems can be found as a response to the large diversity in feeding habits of teleost species (8), yet the mechanisms for many of these adaptations remain unexplored.

Central signals arising in the hypothalamus are crucial for the control of food intake, and this brain area produces both orexigenic (appetite-stimulating) and anorexigenic (appetite-inhibiting) factors. The main hormones and neuropeptides so far described in teleosts and their possible involvement in the central control of appetite are presented in **Figure 1** and described below.

NPY

Neuropeptide Y (NPY) is one of the strongest orexigenic signals in mammals, and the NPY/agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) are the principal inducer of feeding (9, 10). The relative importance of NPY in feeding regulation seems to vary among teleosts. In goldfish (*Carassius auratus*) (11, 12), carp (*Cyprinus carpio*) (13), channel catfish (*Ictalurus punctatus*) (14), zebrafish (15), rainbow trout (*Oncorhynchus mykiss*) (16), and Nile (17) and red (18) tilapias (*Oreochromis* sp.), NPY injections increase feeding. Food deprivation increases brain *npy* expression in several species, including goldfish (19), chinook and Coho salmon (*Oncorhynchus tshawytscha*; *Oncorhynchus kisutch*) (20), zebrafish (15, 21), winter skate (*Leucoraja ocellata*) (22), tiger puffer (*Takifugu rubripes*) (23), and winter (24) and Brazilian (25) flounder (*Pseudopleuronectes americanus*;

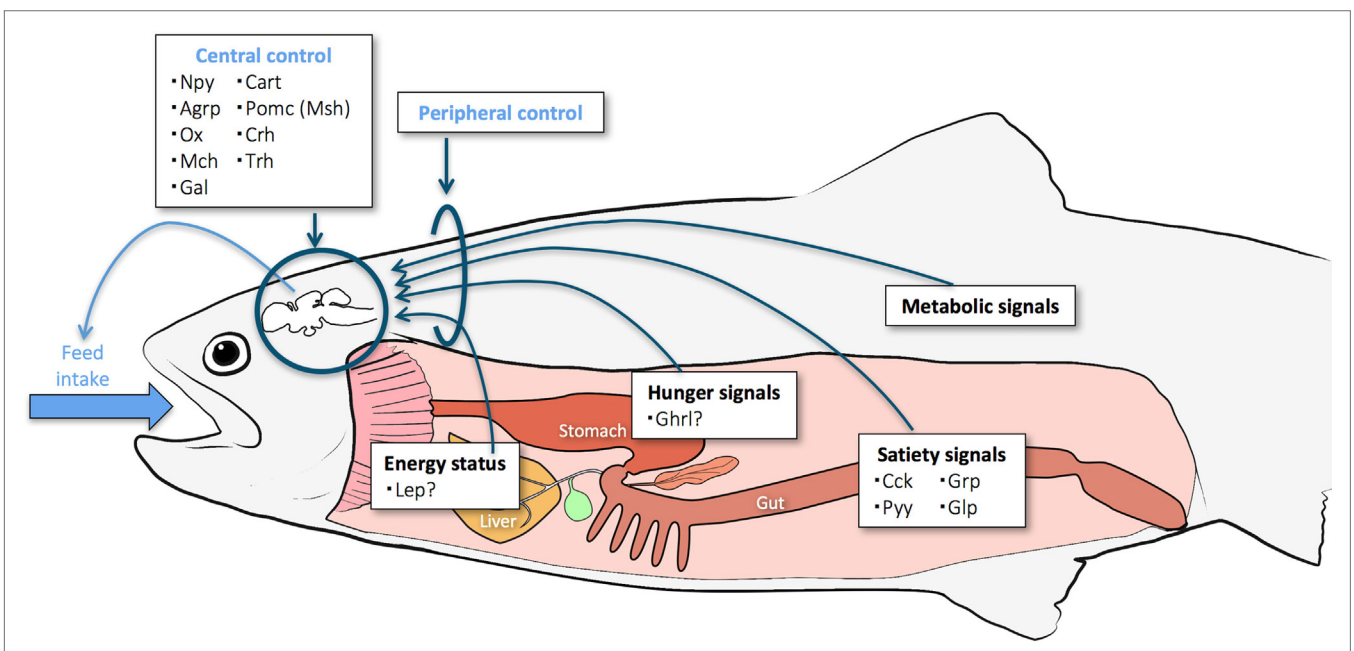

FIGURE 1 | Key organs and signaling pathways believed to be involved in control of appetite in fish. Some of the central and peripheral endocrine factors explored so far are listed.

Paralichthys brasiliensis), suggesting an orexigenic role. In some species, such as Atlantic cod (*Gadus morhua*) (26), tiger puffer (23), snakeskin gourami (*Trichogaster pectoralis*) (27), Brazilian flounder (25), channel catfish (28), and cobia (*Rachycentron canadum*) (29), *npy* brain expression levels are high around feeding time and decrease post-feeding, further suggesting a role of Npy as a short-term appetite stimulator in fish. Npy treatments have also been shown to stimulate fish growth/growth hormone (GH) secretion both *in vitro* [goldfish (30)] and *in vivo* [tilapia (*Oreochromis mossambicus*) (17, 31); orange-spotted grouper (*Epinephelus coioides*) (32)].

However, in Atlantic cod, fasting does not affect *npy* brain expression (26), in cunner (*Tautogolabrus adspersus*), short-term fasting decreases *npy* brain expression (33), and in both Atlantic salmon (*Salmo salar*) (34) and larval Atlantic halibut (*Hippoglossus hippoglossus*) (35), *npy* expression increases after feeding, suggesting that Npy might have a minor role as a feeding stimulator in these species. GH transgenesis, which results in increased feeding rates, does not affect brain *npy* levels in Coho salmon (36) and carp (37) but decreases *npy* levels in zebrafish (38).

In goldfish (39), Senegalese sole (*Solea senegalensis*) larvae (40), rainbow trout (41), and both Atlantic cod larvae (42) and adults (43), *npy* brain expression is modulated by diet, which is consistent with the role of NPY containing neurons in sensing the metabolic status (e.g., glucose levels) as reported for mammals (10) and fish [e.g., tilapia (44)]. However, in cobia (29), *npy* expression does not appear to correlate with diet-induced changes in food intake.

CART

The peptide cocaine-amphetamine-related transcript (CART) was originally isolated from rat brain as a transcript regulated by acute administration of cocaine or amphetamine (45, 46). In goldfish, *cart* brain expression also increases following treatment with amphetamine (47). CART is a potent anorexigenic peptide in mammals (48–50) and birds (51), and CART injections inhibit food intake in goldfish (52).

Several *cart* genes have been identified in some fish species [e.g., two in goldfish (53), four in zebrafish (54), six in medaka (*Oryzias latipes*) (55), and seven in Senegalese sole (56)] whereas only one *cart* has been reported for others [e.g., Atlantic salmon (57), Atlantic cod (26), Atlantic halibut (35), and channel catfish (28)]. Post-feeding increases in *cart* brain expression have been reported for several fish species such as catfish (28), Atlantic salmon (34) and goldfish (53) suggesting that Cart acts as a short-term satiety factor in fish. Fasting has been shown to decrease *cart* brain expression in several fish species, and these changes are sometimes gene-specific. In goldfish, although the expression of both *cart* genes decreases after fasting, *cart1* appears more affected than *cart2* (53). In both zebrafish (58) and medaka (55), only one *cart* is affected by fasting, and in Senegalese sole, three out of seven *cart* genes are affected (56). However, fasting does not affect *cart* expression in other species such as winter flounder (24) or Atlantic halibut larvae (35), perhaps since only one gene has been identified in these species to date. Cart is also involved in sensing metabolic status, as hypothalamic *cart* mRNA levels change in response to changes in the levels of glucose in rainbow trout (41) or fatty acids in rainbow trout (59) and Senegalese sole (60).

Orexin

Orexins (OXs) A and B (or hypocretin 1 and 2) are neuropeptide products of a single gene precursor, prepro-orexin (*pOX*), through proteolytic cleavage. Two orexin receptors have been identified, OXR1 and OXR2. OX-A binds to both receptors with similar potencies whereas OX-B binds preferentially to OXR2 receptors (61). In mammals, orexins have been implicated in the regulation of many physiological functions, including feeding, sleep–wake cycles, reproduction, and cardiovascular function (62–65). Orexins and/or orexin receptors have been identified in several fish species, including goldfish (66), cavefish (*Astyanax fasciatus mexicanus*) (67), zebrafish (68), barfin flounder (*Verasper moseri*) (69), orange-spotted grouper (70), Atlantic cod (71), winter flounder (72), and dourado (*Salminus brasiliensis*) (73). Orexins have been shown to increase feeding and locomotor behavior in both mammals (74) and fish (75–81). Consistent with its role as an orexigenic peptide, *ox* brain mRNA expression increases following fasting [e.g., cavefish (67), goldfish (77, 82), zebrafish (68), winter flounder (72), Atlantic cod (71), and dourado (73)] and around feeding time [e.g., cavefish (67), orange-spotted grouper (70), and goldfish (83)].

Galanin

Galanin (GAL) is a 29–30 amino acid peptide first identified in mammals (84) and shown to have actions in brain and peripheral tissues to increase appetite and regulate metabolism (85, 86). Gal has been isolated in several fish species [reviewed in Ref. (87)] and appears to act as an orexigenic peptide. Injections of GAL stimulate food intake in goldfish (88) and tench (*Tinca tinca*) (89). Although long-term food deprivation does not affect brain *gal* mRNA expression in goldfish, the expression levels of *gal* decrease after the scheduled feeding time in fed fish, suggesting that Gal is a short-term regulator of appetite (90). Related to its role in metabolism, high *gal* mRNA expression has been linked to increased locomotion in zebrafish (91).

MCH

Melanin-concentrating hormone (Mch) was first isolated from the salmon pituitary as a skin-paling factor (92, 93) and later isolated and identified as an orexigenic factor in mammals (94). In fish, the role of Mch in food intake regulation is still unclear. In goldfish, central injections of MCH inhibit appetite, and fasting induce a decrease in brain Mch-immunoreactive (ir) cells (95–97), suggesting an anorexigenic role. However, in other teleost species, such as winter flounder (98), barfin flounder (99), zebrafish (100), and Atlantic cod (101), fasting-induced increases *mch* mRNA levels and -ir cells, pointing to an orexigenic role.

CRH

The corticotropin-releasing hormone (CRH) family includes CRH [or corticotropin-releasing factor (CRF)], urocortin (Ucn), urocortin 2, and urocortin 3. Members of the CRF family of neuropeptides have been shown to decrease feed intake in mammals

(102). In goldfish, Crf and urotensin I (UI, the homolog of UCN in mammals) stimulate the hypothalamic–pituitary–interrenal axis (the fish homolog to the hypothalamic–pituitary–adrenal axis) to induce secretion of glucocorticoids (e.g., cortisol) and act as anorexigenic factors (6, 103). Central injections of CRF (104–106) or UI inhibit food intake in goldfish. Similar effects have been shown in rainbow trout (106). In Ya fish (*Schizothorax prenanti*), fasting decreases *crf* brain expression levels (107), consistent with the anorexigenic role of Crf-related peptides in fish.

Melanocortin System

The vertebrate melanocortin system is phylogenetically well conserved, and it has been identified in fish, amphibians, and mammals (108–110). It consists of (1) melanocortin peptides, which includes melanocyte-stimulating hormones (α-, β-, and γ-MSH) and adrenocorticotropic hormone, all derived from the gene pro-opiomelancortin (*Pomc*), (2) five G protein-coupled melanocortin receptors (MCRs), and (3) endogenous melanocortin antagonists, agouti and AgRP (111). In vertebrates, components of the melanocortin system are involved in a diverse range of physiological functions, including regulation of food intake, appetite, and anticipatory behavior (112).

The melanocortins are posttranslational products of the POMC prohormone, which also gives rise to the opiate peptide β-endorphin. Posttranslational processing of the POMC prohormone is tissue-specific, which results in the production of different POMC peptides by different cell types and, therefore, multiple physiological functions. *Pomc* is a single copy gene in mammals and birds, but in most teleosts, there are two to three different *pomc* transcripts [e.g., zebrafish (113), carp (114), barfin flounder (115), gilthead sea bream (*Sparus aurata*) (116), and sockeye salmon (*Oncorhynchus nerka*) (117)], proposed to result from the whole or partial genome duplication (118). In salmonids, Atlantic salmon and rainbow trout, three copies of *pomc* gene and one splice variant have been described, i.e., *pomc* (*-a1*, *-a2*, *-a2s*, and *-b*) (119, 120). However, the functions of the fish *pomc* subtypes remain largely unexplored. In rainbow trout, fasting induces increased expression levels of both hypothalamic *pomca1* and *pomcb* (121), whereas in olive flounder (*Paralichthys olivaceus*), *pomc2* but not *pomc1* and *pomc3* mRNA levels increase with fasting (122), suggesting a form-specific response of *pomc* in some species.

The repertoire of MCRs (MC1R to MC5R) found at the target cells has undergone significant diversification and specialization. Therefore, MCRs differ in their affinity for the different melanocortins, agouti, and AgRP. Of importance to energy homeostasis are MC3R and MC4R that are expressed throughout the central nervous system (CNS). Fish Mcr and ligands are expressed in a highly conserved pattern relative to mammals (123, 124). This conservation is also seen in the melanocortin neural circuits involved in hypothalamic control of energy homeostasis, underlining that the melanocortin functions originated early in evolution (125). The presence of Mc4r in teleosts has been reported in several species [e.g., goldfish (126), zebrafish (127), spotted scat (*Scatophagus argus*) (128), snakeskin gourami (129), fugu (109), common carp (130), and Ya fish (131)]. In Atlantic salmon, several paralogs of Mcr have been described, *mc1r* (*-p1* and *-p2*), *mc2r*, *mc4r* (*-a-p1*, *-a-p2*, *-b-p1*, and *b-p2*), *mc5r*, *mrap2* (*-p1* and *-p2*) (Lars Ebbesson, Uni Environment, Bergen, Norway, personal communication). Mc3r seems to have been lost early in teleost evolution and is not present in salmonids, as observed for pufferfishes, tiger puffer and tetraodon (*Tetraodon nigroviridis*) (132). The only known *mc3r* in teleosts is the zebrafish *mc3r*; however, *mc3r* has also been identified in the spiny dogfish (*Squalus acanthias*) (133). In snakeskin gourami, the *mc4r* mRNA expression varies during daily feeding and fasting period, and its correlation with *npy* expression indicates a role in feed intake control (27, 129). However, in barfin flounder and sea bass (*Dicentrarchus labrax*), progressive fasting did not modify the hypothalamic *mc4r* mRNA expression (134, 135). Intracerebroventricular injections of MCR agonist decrease food intake in juvenile rainbow trout (136) and in goldfish (126, 137) in a dose-dependent manner, whereas the injection of MCR antagonists increases food intake in rainbow trout and in goldfish (137). The importance of Mc4r in the regulation of fish growth is also emphasized by naturally occurring mutations of the Mc4r in swordtails (*Xiphophorus nigrensis* and *Xiphophorus multilineatus*), which dramatically affects growth (138, 139).

An interesting fact is the existence of two endogenous antagonists in the melanocortin system, agouti and AgRP. These proteins are paracrine-signaling molecules and act as subtype-selective endogenous antagonists. AgRP exerts its major physiological function in the hypothalamus, where it acts as a potent orexigenic factor (140) due to its ability to antagonize the MC3R and MC4R (141). *agrp* genes have been identified in several fish species (57, 124, 126, 130, 131, 142–144). Hypothalamic *agrp* expression in goldfish (137), sea bass (*agrp1*, not *agrp2*) (144), and zebrafish (124) dramatically increased during fasting. In addition, GH-transgenic common carp has higher feed intake and higher hypothalamic *agrp1* mRNA expression levels than non-transgenic fish (37). *agrp* mRNA abundance in the hypothalamus of rainbow trout (59) and Senegalese sole (60) also responds to changes in the levels of specific fatty acids. Altogether, it is suggested that the role of AgRP in energy homeostasis and its relation to the melanocortin system is conserved across vertebrates (51, 145).

ANATOMICAL LOCATIONS OF CENTRAL APPETITE CONTROL SYSTEMS

Control of appetite is an evolutionarily conserved process resulting from a close interplay between multiple neuronal and peripheral signals, which are integrated in the hypothalamus and processed in a specific spatial and temporal order to regulate hunger and satiety (4, 146). The mammalian hypothalamus consists of numerous interconnecting nuclei organized into complex neuronal networks where ARC nucleus, ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), and lateral hypothalamus (LH) play crucial roles in food intake control and energy expenditure [reviewed in Ref. (146)]. The ARC contains two distinct neuronal populations referred to as "first order" neurons, releasing appetite stimulators NPY/AgRP and appetite suppressors POMC/CART (1, 147). Neuronal

projections from the first order neurons connect to other hypothalamic nuclei (PVN, DMN, VMN, and LH) (148). These "second order" nuclei express potent orexigenic factors such as orexins and MCH in the LH, and anorexigenic neuropeptides such as CRH and thyrotropin-releasing hormone (TRH) in the PVN. Lesioning studies in these nuclei have long recognized their functional significance in generating satiety and hunger responses [reviewed in Ref. (149)].

The existence of a functional (and to lesser extent anatomical) equivalence of appetite-controlling brain regions in fish has been demonstrated, based on electrical stimulation and brain lesion studies [reviewed in Ref. (4)]. The teleostean hypothalamic neurons are organized in a similar fashion as their mammalian counterparts and are distributed in conserved clusters within the ventral diencephalon (150–153). Yet, very little is known about the fish anatomical homologs to mammalian hypothalamic VMN, DMN, PVN, and LH nuclei, owing to the lack of specific neuronal molecular markers for distinct neuronal classes. In addition, expression domains of fish appetite control genes do not appear anatomically confined to their putative hypothalamic homologous areas.

The lateral tuberal nucleus (NLT; also known as ventral periventricular hypothalamus Hv) might be a feeding center and the teleostean homolog of the mammalian ARC [reviewed in Ref. (153)]. *pomc*, *agrp*, and *leptin* receptor transcripts are found in neurons within the NLT of goldfish (126, 137) and zebrafish (154), and ir and/or gene expression studies have identified Npy in the NLT of several teleosts (155, 156), as well as sturgeon [*Acipenser transmontanus* (157) and elasmobranch fish (158)]. *npy* and *cart* transcripts are also present in the NLT of juvenile Atlantic cod (159). In addition, Msh-α and Agrp-ir-cells are found in discrete populations in the NLT of zebrafish (125).

A recent study shows high homology between the zebrafish neurosecretory preoptic area (POA) and the mammalian PVN (153, 160). This homology is consistent with the presence of fish *trh* and *crh* ortholog genes in the POA, although their expression is not exclusive to the POA (161–164). The mammalian PVN is an important site of NPY synthesis and release (146, 165), and recent evidence indicates that Npy-ir cells and *npy* mRNAs are also present in the POA of fish (159, 166), further supporting functional homology between PVN and POA structures.

Functional and to some extent anatomical homologies could also exist between the mammalian and fish LH. In mammals, LH is an important site of orexins and MCH expression and believed to act as a "feeding center" (146). The LH is the site of transit for neuronal fibers interconnecting hypothalamic nuclei and forebrain to midbrain structures. A similar neuronal pattern has been observed in the LH of zebrafish, where *pOx*-expressing neurons send projections to the midbrain and the spinal cord (167, 168). In addition to the LH, the POA and the rostral NLT are also important sites of *pOx* expression in fish, as recently observed by double-fluorescence *in situ* hybridization in Atlantic cod larvae, in which the caudal domain of *pOx*-expressing neurons in the POA overlaps with the rostral-most *cart* cell population in the NLT (159). *pOx* mRNA expression in the POA has also been reported in zebrafish (169).

Furthermore, the strong expression of *cart* mRNAs and the absence of orexigenic modulators such as *npy* or *pOx* in the diffuse nucleus of the inferior hypothalamic lobe of Atlantic cod has recently led to the hypothesis that this nucleus may be the VMN homolog and that may serve as "satiety center" in fish (159) as in mammals (149, 167).

mRNAs of several appetite signals have been detected in the brain of different fish in extra-hypothalamic areas analogous to those characterized in mammals, suggesting a functional relationship between them (26, 41, 58, 159, 170, 171). It is, however, important to underline that canonical appetite genes (e.g., *Npy* and *Cart*) in mammals are modulated by many factors and their wide brain distribution may reflect various physiological roles and responses to changing environmental conditions (45, 172). All these mechanisms are still largely unknown in fish.

PERIPHERAL SIGNALS

The GI-Tract

The GI-tract is the largest endocrine organ in vertebrates and produces around 30 different neuropeptides and hormones. These peptides act on several tissues, including the GI-tract itself, exocrine glands, and the CNS (173, 174). Most of the GI peptides are sensitive to the gut nutrient content, and some of them are important in the control of appetite and meal size (174, 175). GI peptides may act on the CNS *via* an endocrine action by traveling in the blood, which requires that they pass the blood–brain barrier, and/or by stimulating afferent vagal nerve fibers (174, 176, 177). Studies on rainbow trout show that appetite returns when 80–90% of the stomach content has been emptied (178), indicating that gut filling, feed digestion, and transit rates may affect appetite control with both hunger and satiety signals. Indeed, most of the gut-derived appetite-regulating factors are also involved in digestion, thus coordinating these two processes (179).

GHRL

Ghrelin (GHRL) is mainly produced in the stomach of fish and mammals, or in the intestine of some stomachless species (180). Ghrl has been shown to have an orexigenic function in several fish species, including goldfish (177, 181), tilapia (182), brown trout (*Salmo trutta*) (183), and grass carp (*Ctenopharyngodon idellus*) (184), which is consistent with its role in mammals (185, 186). However, in rainbow trout, opposite effects of Ghrl on feed intake have been reported from two independent studies: one showed that central injection of Ghrl increased feed intake after 24 h (187) whereas the other study showed that short-term (1 h) central and long-term (weeks) peripheral administration of Ghrl suppressed appetite (174). The different time scales may, at least partly, explain the contradictory results. Recently, an anorexigenic response was also reported in channel catfish after Ghrl administration (188). In goldfish, appetite-regulating neuropeptides in the CNS, such as Npy and Ox, seem to mediate Ghrl-induced feeding (181, 189), but interactions between Ghrl and central appetite regulators are inconsistent in other examined fish species. For example, Ghrl increased (in tilapia and rainbow trout) (182, 187), decreased (in rainbow trout) (190), or did not affect

(in brown trout and channel catfish) (183, 188) hypothalamic *npy* expression. Moreover, Ghrl decreased (in rainbow trout) (187) or had no effect (in channel catfish) (188) on *pomc* expression. A CRH receptor antagonist (α-helical CRF 9–41) abolished Ghrl-induced feeding (191) whereas Ghrl administration did not affect central *crh* expression in rainbow trout (187). In goldfish, it appears that peripheral Ghrl may stimulate feeding by acting on gastric vagal afferents that transmit information to brain appetite centers (177). Indirect effects on food intake, through stimulatory actions on digestion, could subsequently affect onset of feeding. For instance, rat GHRL evoked intestinal contraction in zebrafish (192, 193), but homologous Ghrl did not affect GI-tract contractility in goldfish and rainbow trout (194). The presence of GH secretagogue receptor in the fish pituitary and brain (particularly hypothalamus and telencephalon) also suggests a direct action of octanoylated Ghrl in these tissues (195, 196).

CCK

Cholecystokinin (CCK) is secreted by the proximal intestine and mainly acts as a short-term satiety factor at the same time as it promotes digestion through its many actions on the digestive system of vertebrates (174, 197). CCK is characterized by an evolutionary conserved biologically active C-terminal octapeptide (CCK-8) among vertebrates (198, 199), and Cck-ir cells have been observed in the intestine of most fish groups (174). Central or peripheral administration of sulfated CCK-8 suppresses food intake in goldfish (200) and channel catfish (14). Oral CCK administration inhibits feed intake in sea bass (201), while oral treatment with CCK antagonists increases food intake in rainbow trout (202). A single *cck* gene has been cloned in several teleost species, including yellowtail (*Seriola quinqueradiata*) (203), Atlantic herring (*Clupea harengus*) (204), and pirapitinga (*Piaractus brachypomus*) (205). However, two different *cck* sequences were identified in Japanese flounder (*Paralichthys olivaceus*), tetraodon (206), Atlantic salmon (207), and white sea bream (*Diplodus sargus*) (208), and three distinct *cck* genes exists in rainbow trout (209). All the identified *cck* genes in teleosts are predominantly expressed in the GI-tract and brain, including hypothalamus, telencephalon, and optic tectum.

Both circulating levels of Cck and *cck* gene expression are influenced by macronutrients, although these effects appear to be species-specific. For example, rainbow trout fed a high fat diet had higher plasma Cck levels compared with fish fed a high protein diet (210) and oral administration of single bolus of fat (oleic acid) or protein (casein), but not carbohydrate (starch), increased *cck* expression in yellowtail gut (211). In addition, *cck* expression levels increased following a meal in yellowtail pyloric caeca (212) and circulating Cck levels increase postprandially in rainbow trout (213). Fasting decreases gene expression or protein levels of Cck in the gut of yellowtail and white sea bream (203, 208). These results support the anorexigenic function of Cck and the conservation of this function in the teleost lineage. Some studies, however, show opposite effects; in Coho salmon, *cck* gene expression in the gut increased during winter fasting (214). In Atlantic salmon, on the other hand, intestinal *cck* mRNA expression was unchanged after 6 days of fasting (207). Furthermore, there are variations in the distribution pattern of Cck-producing cells within the intestinal segments among species (204, 215, 216) as well as in the fasting response among *cck* isoforms (207–209) suggesting diverging roles among species and *cck* isoforms. The action of CCK is initiated by its binding to two subtypes of cognate receptors (CCK-1R and CCK-2R), which results in satiety (197). Cck receptor genes have been isolated in yellowtail (*cck-1r*) (217), Atlantic salmon (*cck-1r*, *cck-2r1*, and *cck-2r2*) (218), and goldfish (*cck-1r* and *cck-2r*) (219). The primary structure of fish Cck receptors as well as their tissue distribution patterns is highly conserved; *cck-1r* is widely distributed within the GI-tract, while *cck-2r* is mainly expressed in the brain. Furthermore, *cck-1r* expression levels increased after feeding in yellowtail pyloric caeca (217), suggesting that Cck-1r mediates the effects of Cck on appetite, as in mammals (220). Further studies on Cck receptors are required to elucidate the detailed mechanisms underlying the anorexigenic function of Cck in fish.

PYY

Peptide YY (PYY) is a member of the NPY family. But, while NPY is well known to have a strong orexigenic function in the CNS (1), peripheral PYY mainly produced in the distal intestine (221) inhibits food intake in mammals (222). PYY consists of two forms: 36 (PYY1–36) or 34 (PYY3–36) amino acids (223). Two isoforms of the gene *pyy*, *pyya*, and *pyyb* (previously named *py*) (224) have been identified in teleost species, including sea bass (155), Atlantic salmon (207), and piranha (*Pygocentrus nattereri*) (225). To date, the *pyy* gene expression patterns are similar among the studied fish species, being predominantly expressed in the brain and GI-tract (203, 226). On the other hand, controversial results have been reported when analyzing intestinal segments from fed versus fasted fish. Fasting decreased (in piranha) (225), increased (in yellowtail) (203), or did not affect (in Atlantic salmon) (207) *pyy* expression. After feeding, GI-tract *pyy* mRNA expression increased in grass carp (227), while it decreased in yellowtail (212). These observations suggest that *pyy* response to fasting/feeding might be species-specific (225). Central and peripheral Pyy1–36 injection reduced food intake in goldfish (228), while administration of the truncated form Pyy3–36 had no effect on food intake in channel catfish (188) or goldfish (228). These results suggest that Pyy3–36 is not a major endogenous form of Pyy in fish (228, 229). The current mammalian model indicates that PYY suppresses appetite through the inhibition of NPY and subsequent activation of POMC neurons (230); however, the effects of GI-tract-derived Pyy on CNS are still uncertain in fish. PYY inhibits GI motility and pancreatic exocrine activity in mammals (175), and a similar digestive function has also been suggested for Pyy in teleosts (207, 211).

GRP

Gastrin-releasing peptide (GRP) is a homolog of the amphibian bombesin (Bbs) and is released from the GI-tract. In mammals, GRP decreases feed intake (231) and stimulates gastric acid secretion and motility (232). Bbs/Grp also appears to stimulate gastric secretion and motility in teleosts (233–235). In teleost species, Bbs/Grp-like peptides have been detected in the GI-tract of rainbow trout (236) and chub (*Squalius cephalus*) (237), and *bbs*/*grp* cDNA sequences have been published for goldfish (238), zebrafish (239),

and Atlantic cod (240). Restricted feeding decreased *grp* expression in the gut of Atlantic cod (240) and zebrafish, but the *grp* decreasing pattern was reversed in the latter after refeeding (239). Central or peripheral injections of Bbs suppress feed intake in goldfish (200), which might be attributed to Bbs-induced reduction in *ghrl* gut expression (241). In addition, peripheral injections of Bbs/Grp decrease feeding in channel catfish (188) and Coho salmon (242). On the other hand, feeding status or diet composition does not seem to influence plasma Grp levels in rainbow trout (210). These observations indicate that teleost peripheral (gut) Grp may have an anorexigenic function and its signaling pathway is not endocrine but *via* neuronal circuits or local paracrine action, as proposed for the mammalian model (231).

The Evolution of Leptin Teleost Genes

The leptin gene (O*b*) was first identified in double mutant (*Ob*/*Ob*) mice (243) and presented an obese phenotype associated with impaired metabolic functions. Since obesity is linked to several comorbidities in humans, including type II diabetes and cardiovascular disease (244, 245), leptin has been extensively investigated in both humans and murine models. The first fish leptin was identified in 2005 (246). Leptin orthologs and several duplicated paralogs, originating from the whole-genome duplication (WGD) events, have recently been identified in teleost species (247, 248). These include 3R-leptin duplicated paralogs (A and B) in zebrafish (249), medaka (250), orange-spotted grouper (251), tilapia (252), chub mackerel [*Scomber japonicas* (253)], and European and Japanese eel [*Anguilla anguilla* and *Anguilla japonica* (254)], as well as two conserved leptin paralogs [*lepAI*/*lepAII* and *lepA1*/*lepA2* (255, 256)]; in common carp and goldfish, as a result of the ancestral lepA doubling at the basal root of cyprinids (256, 257) about 8 million years ago (258). In salmonids, additional "recent" 4R-leptin duplicates have been identified consistently with the (pseudo) tetraploid state of their genome (259–261).

Leptin functions are mediated *via* class-I helical cytokine receptors (long-form LEPR) through intracellular JAK/STAT signal transduction pathways (262, 263), in an evolutionarily conserved manner as suggested by transfection assay studies for carp (264), rainbow trout (265), and tilapia (252) receptors. In humans, alternative splicing of the LEPR gene leads to expression of long (LEPRb) and short (LEPRa, -Rc, -Rd) isoforms (266).

Single leptin receptors have been identified in most fishes (154, 250, 251, 267, 268), but two 3R-duplicated *lepR* genes are present in the ancestral teleost eel. This suggests that a loss of the second *lepR* (*lepRB*) may have occurred after the clupeocephals/elopomorphs split during teleost radiation (254). At the root of extant salmonids, the *lepRA* was then further duplicated by the 4R-WGD as deduced by the recently cloned *lepRA2* in Atlantic salmon (269). Like mammals, LepR isoforms that arise from alternative splicing of the C-terminal exon have been identified in fish (260, 264, 270, 271). LepR splice variants encode for circulating soluble binding proteins (LepBPs) that may function in leptin modulation, transport, and clearance (265, 271, 272). The characterization of the *leptin-lepR* system in the context of WGD(s) in teleost genomes and overall evaluation of their functional significance are instrumental to understand to which extent leptin duplicates have contributed to species-specific feeding adaptations.

Leptin Signaling—The Liver and Adipose Tissue

In mammals, leptin is an anorexigenic hormone released into the blood stream mainly by adipocytes. It acts as a lipostatic factor in a negative feedback loop between fat tissue and hypothalamic brain regions so that the organism can maintain energy balance and adequate fat mass reservoirs (273–276). Leptin signaling in the CNS is exerted on different hypothalamic neurons to inhibit the expression of the orexigenic NPY and AgRP and stimulate anorexigenic POMC and CART (120, 277–280). In fish, liver is the main secretory source of LepA (249, 250, 260, 270, 281–283), although some studies reported moderate mRNA expression and secretion from the adipose tissue (260, 270, 281, 284, 285). Central and peripheral administration of recombinant leptin, using homologous or heterologous leptin, produces anorectic effects in several fish species, suggesting that the regulatory role of leptin on appetite is well conserved in vertebrates (120, 279, 282, 286–289).

Leptin variations in response to feeding status (postprandial, short- and long-term fasting/food restriction) have been reported at the level of gene expression and protein among fish orthologs as well as among paralogs. For instance, postprandial increases in hepatic *lepA* and *lepB* expression are observed within 9 h in common carp (255), and hepatic *lepA* in orange-spotted grouper (251) and mandarin fish [*Siniperca chuatsi* (289)], suggesting that leptins may act as a satiety signal. In longer-term fasting (after 7 days and after 3 weeks), a significant increase in hepatic *lepA* expression was observed in orange-spotted grouper, but not in carp (289). Prolonged feed restriction induced hepatic upregulation of *lepA* expression in salmonids (290–292) and chub mackerel (253). In contrast, liver *lepA* expression decreases during catabolic states in striped bass (*Morone saxatilis*) (282), and hepatic mRNA expression of *lep1*, *lep2*, *lepRa*, and *lepRb* does not correlate to feeding status in eels (254).

lepB expression is low or absent in the liver of several teleosts and is mostly found in the CNS (253, 261, 289). The brain expression profiling of *lepA-B* paralogs in relation to feeding status shows species-specific variations among orthologs, paralogs, and time exposure to catabolic states. For instance, short-term fasting induces a downregulation of both *lepA* and l*epB* in the brain of mandarin fish (289), whereas it has no effect on *leptin(s)*/*lepR* in orange-spotted grouper (251). Long-term fasting has no effect on either *lepA* or *lepB* in Nile tilapia, *Oreochromis niloticus* (252), and eel (254), while in salmon, it induces upregulation of *lepA1* and *leprA1* expression and downregulation of *lepB1–2* genes in the brain (269). The increases in *lepA1* and *leprA1* mRNA upon fasting are in line with most studies on plasma leptin in salmonids (291–293). Also, in Mozambique tilapia (*Oreochromis mossambicus*), hepatic *lepA* mRNA as well as circulating LepA is higher in fasted than fed fish (294), as is seen with salmonids. Rising leptin plasma levels could be adaptive during catabolic states inducing anorexigenic effects at the level of the CNS, and a consequent reduction of energy-demanding foraging behavior during periods of limited food availability (291, 295). Interestingly, in burbot

(*Lota lota*), plasma leptin levels decrease following fasting at 2°C but not at 10°C, implying that metabolic rate may influence leptin in catabolic conditions (296).

Given the lipostatic role of leptin in mammals, putative similar roles have been investigated in teleosts. The *lepB* gene has been proposed to be involved in lipid metabolism in chub mackerel (253) and mandarin fish (289). However, plasma levels do not correlate with body adiposity in salmonids (293, 297). Leptin patterns in adipose tissue vary widely among species and between duplicates; in salmon, only *lepA1–2* are found with *lepA1* type being higher expressed (260, 261). Low *lepAI–II* expression has been reported in visceral adipose tissue of common carp (298). The differential leptin expression in adipose tissue between fish species and mammals may be a result of the divergent fat allocation patterns observed for the various species but also related to differences between endotherm and ectotherms.

In vivo recombinant LepA treatments suggest anti-adipogenic effects and stimulatory actions on fat metabolism in several teleosts (287, 299, 300). Consistently, LepA treatment *in vitro* stimulates lipolysis in rainbow trout adipocytes (284). In addition, *lepr*-deficient medaka exhibit increased visceral fat depots compared to wild types, which is consistent with the body composition of the leptin receptor-deficient db/db mice and Zucker obese rats (243, 301).

While these findings suggest that leptin is involved in mobilization of lipid stores in fish, emerging literature suggests that rather than a canonic "lipostat" signaling for adipostasis (as in mammals), leptin might be important in other metabolic processes. Recent fish studies suggest roles of leptin in glucose homeostasis (302–304) and in the coordination of energy metabolism and somatic growth (305). Leptin receptor-deficient zebrafish do not exhibit increased appetite or adiposity but display β-cell hyperplasia and increased levels of *insulin* mRNA and alterations in glucose homeostasis, suggesting that leptin might act as a glucostat rather than a lipostat in fish. In both rainbow trout (303) and tilapia (304), either peripheral or central treatment of homologous LepA induces hyperglycemia and glycogenolysis. In tilapia, lipase gene expression was not altered, suggesting the hormone is important in mobilizing glucose. Thus, the contradictory leptin data attained so far on gene expression, *in vivo* and *in vitro* recombinant leptin administrations or leptin plasma levels in response to different feeding status, suggest an independent evolution of leptin functions among teleosts. Species-specific responses among orthologs may reflect defined metabolic adaptations to the widely diverse fish life histories. Similarly, leptin duplicates may be under different selective processes and respond to modulation of nutritional status in a spatiotemporal specific manner.

Other Tissues

In mammalian species, there is a range of other peripheral tissues that produce and release factors (peptides/cytokines) that affect appetite, such as the thyroid and pancreatic hormones.

Thyroid

The thyroid axis consists of hypothalamic TRH, pituitary thyrotropin (TSH), and thyroid hormones [thyroxin (T4) and tri-iodothyronine (T3)]. In mammals, the thyroid axis plays a significant role in energy expenditure, as it increase basal metabolic rate, control appetite, and food intake and regulate body weight (306, 307). The few studies that have targeted the role of the thyroid axis on fish feeding suggest a stimulatory effect. For instance, in goldfish, injections of either TRH or T4 increase feeding and locomotion (82, 308), and treatment with the antifouling agent tributyltin increases weight gain and food intake, as well as serum thyroid hormone levels (309). In Amur sturgeon (*Acipenser schrenckii*), low feeding rates result in low thyroid hormones serum levels (310). In both winter flounder (72) and goldfish (82), fasting induces increases in hypothalamic *trh* mRNA expression, further suggesting an orexigenic role.

Pancreas

The pancreas secretes mainly insulin and glucagon-related peptides, which have been shown to affect metabolism in fish (311). Plasma insulin and glucagon levels increase after feeding in fish; however, their specific role in the food intake regulation is largely unknown.

Complete isletectomy in the goby (*Gillichthys mirabilis*) results in hyperphagia (312), and in rainbow trout, intraperitoneal injections of insulin decrease food intake (313), suggesting an anorexigenic role for insulin in fish.

The vertebrate proglucagon (*Pg*) gene encodes three peptide hormones, namely, glucagon, glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) (314). In mammals, GLP-1 and GLP-2 are satiety signals, mainly produced by the GI-tract (315, 316). In fishes, the pancreas synthesizes glucagon and Glp-1, and the intestine releases glucagon, Glp-1, and Glp-2 (317). To date, the *pg* gene has also been isolated in several teleost species (314), and duplicate *pg* genes have been identified in all teleost species for which the genomic sequencing has been completed (318). Although, to our knowledge, there is no information on glucagon and Glp2, Glp-1 appears to act as an anorexigenic factor in fish. In channel catfish, central administration of GLP-1 has a potent inhibitory effect on feed intake, but peripheral injection showed only a weak or no effect on appetite (188, 319). On the other hand, peripheral GLP-1 injection strongly decreased feed intake in Coho salmon (242), suggesting that the peripheral (GI-tract) anorexigenic Glp-1 effects might be species-specific in fish. In rainbow trout, peripheral injections of Glp-1 increase plasma glucose levels, decrease hindbrain *npy* and *pomc* mRNA levels and increase hindbrain *cart* expression levels, suggesting that Glp-1 regulates not only food intake but also glucose homeostasis (320). Although mammalian GLP-1 inhibits gastric emptying (321), the function of Glp-1 on digestion (speed) is still unclear in fish.

SELECTED FISH ADAPTATIONS IN THE ENDOCRINE REGULATION OF FEEDING

Owing to their large diversity, fishes display a wide range of interesting adaptations in the feeding biology and appetite to different environmental conditions and food availability. Research on these comparative aspects both with regards to evolution

and function is still largely unexplored and only a few species, mainly with commercial interest, have been studied. Below, we provide some examples and discuss other adaptions that could be explored further.

Long-term Seasonal Fasting (The Arctic Charr)

The anadromous (sea-migrating) life-strategy of Arctic charr (*Salvelinus alpinus*) is characterized by substantial seasonal changes in food intake, growth, and adiposity. In the wild, most of the annual growth and energy accumulation occurs because of an intense appetite burst during the short seawater residence in summer, whereas overwintering in freshwater is characterized by anorexia and depletion of energy reserves (322–325). The seasonal cycle in food intake and growth in this species seems to be a strictly genetically programmed process as captive offspring of Arctic charr exhibit pronounced seasonal changes in food intake and growth when held at constant temperature and given food in excess (326, 327). Because of the physiologically regulated seasonal feeding cycles, Arctic charr represent an interesting model for investigation of adaptive mechanisms underlying long-term regulation of appetite and energy homeostasis (328).

It has been suggested that the seasonal feeding cycle is regulated by a lipostatic mechanism (297, 328–330). Leptin, the principal regulator of the lipostatic mechanism in mammals (331), does not appear to be involved in signaling the large variations of adiposity in the Arctic charr (297). However, hepatic leptin production increases at the end of the winter fasting period (297), when fat mobilization and increased plasma glucose occurs (325). It is possible that leptin has a role in depressing metabolism during long-term seasonal fasting, when fat stores are depleted by the suppression of liver lipolytic pathways (292, 297). It is also possible that leptin is more important as a glucostat than an adipostat in Arctic charr, as suggested in zebrafish (302).

The role of Ghrl in controlling the seasonal variation in appetite of charr has also been explored. Stomach *ghrl* mRNA expression seems to be negatively correlated with feed intake and growth (332), supporting that Ghrl acts as an anorexigenic factor, as suggested in one study on rainbow trout (191). The expression levels of a range of putative central appetite-controlling genes in Arctic charr such as *pomc*, *cart*, *mc4r*, *agrp*, and *npy* were not correlated to its annual feeding cycle (333). Further studies are needed to understand how anadromous Arctic charr can maintain an anorexic state when overwintering despite the massive loss of fat reserves.

Long-term Fasting Related with Reproduction (The Mouthbrooder)

Mouthbrooder fish hold their eggs in their mouth until their young are free-swimming. Several fish are classified as mouthbrooders, some being paternal (male holds eggs) and others maternal (most common). Eggs can be fertilized in the environment or in the female's mouth (in the case of maternal brooding). Teleost mouthbrooder fish include cichlids (e.g., mbuna *Astatotilapia burtoni*) and tilapias such as *Oreochromis mossambicus* and *Oreochromis niloticus*, sea catfish (e.g., *Ariopsis felis*), cardinalfish (e.g., *Pterapogon kauderni*), and gouramis (e.g., dwarf gourami *Colisa lalia*). While guarding eggs, most mouthbrooders do not eat or feed less, often resulting in a weight decrease (334–338).

Very little is known about the endocrine mechanisms responsible for brooding-induced fasting. Fed mbuna females with large ovarian eggs (pre-spawning or spawning) have larger gonadotropin-releasing hormone (Gnrh1) neurons (339), which has also been observed in convict cichlid, *Amatitlania nigrofasciatus* (340) and higher mRNA expression levels of whole brain *gnrh1* (major Gnrh form involved in reproduction), than mouthbrooding females carrying eggs, which is reflected by higher gonadosomatic indexes and higher circulating levels of sex steroids (341). However, no significant differences are seen in *gnrh2*, in contrast with fasting-induced changes reported for other fish species [e.g., winter flounder (342) and Ya fish (343)]. Similarly, no differences are seen in *npy*, *pomc* or *mch* whole-brain expression, between mbuna holding eggs in their mouths and pre-spawning females (341). However, *orexin* increases in fasting mbuna females, which is consistent with its stimulatory role on feeding and inhibitory actions on spawning (66). The increase in *cck* is more surprising, as Cck is a satiety factor that is normally secreted when the GI-tract is full. This increase in *cck* might be a response to long-term fasting to attenuate hunger and prevent feeding by counteracting increases in orexigenic peptides such as orexin.

Interestingly, when comparing fed and fasted mouthbrooding females from which eggs/fry have been removed, no differences in brain expressions of appetite regulators (*npy*, *cck*, *orexin*, *pomc*, and *mch*) were seen (341), possibly because of changes in physiology and metabolism. However, as no information is available about the effects of fasting on appetite regulators for pre-spawning females or immature fish, it is difficult to draw definitive conclusions on the changes that lead to brooding-induced fasting.

Long-term Fasting in Aquaculture (Trout and Salmon)

Like the above-mentioned Arctic charr, many other fish species, including rainbow trout and Atlantic salmon, tolerate long fasting periods. Rather than a genetically driven seasonal halt in feed intake as in charr, they adapt to long periods with low food availability in the wild. To better understand the potential role of various peptides in this process, plasma protein and/or gene expression levels of candidate appetite-regulating hormones and neuropeptides have been analyzed during variable periods of food deprivation in salmon and trout.

Leptin

The picture of leptin endocrinology dynamics in fish during fasting is not clear-cut, even within species, e.g., rainbow trout. Recent data on two lines of rainbow trout bred for either high (fat line) or low (lean line) muscle lipid content indicate that leptin response to fasting may be plastic and dependent on selective breeding, environmental factors and/or energy status and body composition (344). The two lines of trout differ in the fat deposition pattern: the fat line has higher total energy reserves, higher muscle adiposity, and lower visceral adiposity than the lean line.

A 4-week fasting period decreased plasma Lep in the lean line while Lep levels and hepatic *lep* expression remained unchanged in the fat line (344). This contrasts previous results in rainbow trout, where leptin levels increase or remain unchanged during fasting, despite a decrease in condition factor (293, 345).

Tissue *lep* gene expression was also unaltered in long-term fasted fish except for an increased expression in fat rich muscle tissue (346). In the same study, the fasted fish displayed hyperphagia when they could refeed, eating as much as up to 8.4% of their body weight (346). Hence, even though the fasted fish were clearly in a catabolic state, hungry and mobilizing energy stores, leptin production and plasma levels remained unchanged.

Unlike the observation mentioned above (346), appetite does not always return immediately when food becomes available for anorectic/food-deprived salmonids (345, 347). During a 72-h refeeding period for long-term fasted rainbow trout, there was a large variability in the time to start feeding between individuals, and some did not feed at all in the beginning. This response may have been caused by high leptin levels in these individuals (345). Leptin generally did not start to decrease until some food had been ingested, raising the question of which mechanism is responsible for triggering the onset of appetite. In fine flounder (*Paralichthys adspersus*), leptin also decreases after, but not before refeeding (291). This fast leptin response indicates that there is a short-term meal-related regulation of leptin release (291, 345).

Available data on the relation between leptin and energy status in Atlantic salmon are still limited to those from food restriction studies or experiments using diets with different energy content (260, 290, 348, 349). Plasma leptin levels were not different between fish that were fed full or restricted (60%) rations for 10 months, although hepatic *lepA2* expression was higher in the fed than in the fasted salmon (260). In a shorter trial (7 weeks), feed-restricted fish had higher plasma leptin levels and elevated hepatic *lep* expression levels than controls fed to satiation (290), which is consistent with some of the previous studies on rainbow trout (293, 345). Restricted feeding during several months (April–September) in Atlantic salmon parr undergoing sexual maturation showed that fish with the highest fat stores had the lowest leptin levels (349). Similarly, fish on a high-energy diet had lower leptin levels than fish on a low energy diet with less adipose stores (348). Taken together, these studies lend further support to the notion that leptin is not a long-term adiposity signal in salmonids. The results obtained from fish species are also interesting in the context of studies on wild mammals with seasonal changes in adiposity and feeding behavior, showing a large variability in the link between plasma leptin levels, fasting, and adiposity (350–353).

Ghrelin

The response of plasma Ghrl and *ghrl* mRNA expression to fasting in fish is highly variable between studies and fasting duration (354). There are few studies investigating the response of Ghrl to long-term fasting in Atlantic salmon and rainbow trout. In rainbow trout, plasma Ghrl levels decreased after 1–3 weeks of fasting (213). In Atlantic salmon, 2 days of fasting led to elevated plasma Ghrl levels, indicating an effect of short-term feeding status on Ghrl release, a response consistent with this "hunger hormone." However, after 14 days of food-deprivation, Ghrl levels were unchanged in fasted salmon compared to fully fed controls (355). Whether these differences are a result of true species differences in Ghrl function (see section above about ghrelin), domestication processes or experimental design remains unclear.

Fasting-Induced Changes in Central Appetite Regulatory Neuropeptides

The recent study by Jørgensen et al. (346) is one of few that have investigated potential changes in the expression of hypothalamic appetite-regulating peptides during fasting in a salmonid species. Rainbow trout was fasted for 4 months, and among the peptides that were measured in the hypothalamus (*lepa1*, *cart*, *agrp*, *pomca1*, *pomca2*, *pomcb*, *npy*, *mc4r*, and c*rf*), few fasting-induced effects were observed. There was an increased gene expression of *pomca1* and *pomcb*, suggesting that increased *pomc* transcript levels may be a potential mechanism for a reduced appetite and foraging activity in catabolic conditions.

Peripherally injected Lep seems to increase the expression of *pomc-a1* and *-a2* with a concurrent transient reduction in *npy* gene expression (279). In rainbow trout, the leptin receptor is localized in mediobasal hypothalamic appetite centers, and it seems that Pomc and Cart mediate leptin's acute anorexigenic effect in this species (295). It may be speculated that during long-term fasting in salmonids, increased circulating leptin levels stimulate hypothalamic Pomc neurons, suppressing appetite. Brain sensitivity (amount of receptor levels) to, e.g., leptin and Ghrl will also influence appetite. At the termination of a 7-week feeding/fasting experiment, fed Atlantic salmon parr showed an increase in *lepr* gene expression in the brain, while the *lepr* gene expression in food-deprived fish was unaltered despite increased plasma Lep levels. This was interpreted by the authors as the possible result of a negative feedback of Lep on its receptor (290).

Life-Stage Transition (First Feeding Larvae to Juveniles)

Most fish species spawn eggs, in which the developing embryo relies on yolk nutrients until it is sufficiently developed to capture, ingest, and digest feed. After onset of exogenous feeding, the larvae continue to grow and develop into juveniles—a transition triggered by environmental cues that induce a coordinated program to remodel the organism. The transition involves a wide range of changes in behavior, habitat, and physiology, and many fish larvae change food sources as they become adults; therefore, it has major consequences for feeding behavior and most likely in the control of appetite (356).

Several studies have aimed to understand the various aspects of the feeding biology and nutritional requirements of developing fish larvae to improve their performance in aquaculture. However, very few have focused on the mechanisms that control appetite and food intake (42, 357). This may be partly explained by biological and technical challenges when working with fish larvae, such as the accurate determination of food intake, the use of individual larva (instead of pools), or the handling of individual variability in growth and development.

There are several described cases where fish larvae continue to eat, despite having an apparently full GI-tract. For instance, Atlantic halibut larvae continue to ingest prey despite a full gut and with gut transit rates so high that the prey is eliminated (defecated) undigested and sometimes even alive (358). Apparently, the feedback systems and satiety signals originating in the GI-tract are not functional in these early stages. It has been argued that fish larvae have adapted to low concentrations and availability of prey in the wild. Consequently, satiety signals may not be required to prevent overfeeding. In aquaculture conditions, however, larvae are reared with constant and abundant food availability and continuous light, and therefore appetite-controlling mechanisms become crucial to avoid continuous ingestion of prey, short gut transit times of ingested food, reduced time for digestion, low digestive efficiency, and nutrient absorption (359). This is of particularly interest for altricial-gastric species, which lack a fully developed and functional stomach prior to metamorphosis (360–364).

Some studies have started to explore the ontogeny expression of several appetite regulators (71, 240, 365, 366), and their detailed spatial and differential distribution in fish larvae (159). Key factors in appetite control are present very early in fish development, such as *npy* at zygote stage in blunt snout bream (*Megalobrama amblycephala*) (367) and at blastula stages in orange-spotted grouper (170), *ghrl* (240) and *ox* (71) at cleavage stage, and *gastrin* (240) at blastula stage in Atlantic cod. In Atlantic halibut, only *ghrl* and *cart* mRNA expression levels were significantly modified throughout development, while ontogeny did not affect *npy*, *pyy*, and *pomc-c* expressions levels in the brain of the developing larvae (35). Ghrl was widely distributed in the GI-tract and present in the anterior GI-tract before the gastric glands and pepsinogen production appeared in newly Atlantic halibut hatched yolk-sac larvae (368). Notably, increased levels of *ghrl* in the GI-tract during metamorphosis were correlated with stomach development (360, 369). *cart* mRNA expression levels decreased at the initiation of halibut metamorphosis, while *cart* levels in whole larvae of Atlantic cod increased during the corresponding developmental phase (365). In Atlantic cod, *cck*, *npy*, and *ox* show a similar pattern of a moderate but consistent decrease from 3 days post-hatching (dph) until 60 dph (42, 365). The differences in *cart* expression between Atlantic halibut and Atlantic cod larvae are intriguing and may be a result of different factors, including the use of whole cod larvae versus halibut head and differences in developmental rate (370, 371).

Many of the neuropeptides involved in appetite control in higher vertebrates and adult teleost are present in the brain of fish larvae, suggesting a role of these genes in appetite control also in the early stages (35, 159, 168, 372–374). In the recent study of Le et al. (159), the development expression patterns of *npy*, *cart*, and *ox* genes were analyzed in brain regions of Atlantic cod, from start of exogenous feeding until juvenile stage. Both spatial and temporal expression patterns of orexigenic and anorexigenic factors during larval ontogeny indicated a progressive development of the brain regulatory networks that control appetite. In addition, the wide distribution and co-expression of *npy*, *cart*, and *ox* in hypothalamus, led the authors to propose that this is the main area for appetite control in fish larvae, comparable to mammals and adult fish (6, 374–376). However, it remains unclear to what extent these appetite-regulating genes are functional at these early developmental stages.

Few have assessed the response of these factors in terms of feed intake (35, 40) or different diets (40, 42, 377). In Atlantic cod larvae, Kortner et al. (42) showed that the expression levels of *cck* and *npy* were diet-specifically modulated and followed the same expression profile as the genes coding for digestive enzymes, suggesting a close connection between appetite control and digestion processes. Recently, two studies in Senegalese sole larvae have analyzed the effect of fatty acids ingestion in the control of food intake (378, 379). The administration of several fatty acids (leate, linoleate, α-linolenate, or eicosapentaenoate) in sole post-larvae enhanced the expression of the anorexigenic neuropeptides *cart4* and *pomcb* and decreased the orexigenic *npy*, with no major discrepancies between the different fatty acids tested (378). However, the transcriptional analysis of several anorexigenic: *pyya*, *pyyb*, *glp1*, *cckl*, *cart1a*, *cart1b*, *cart2a*, *cart4*, *pomc-a*, *pomc-b*, *crf*; and orexigenic: *gal*, *npy*, *agrp2* factors showed a dissimilar response to feeding times and dietary fatty acid composition (cod liver oil, linseed oil, soybean oil, or olive oil) that was generally not in agreement with their putative function (40). For example, the changes observed for sole *npy* in developmental stages 16 and 34 dph were not consistent. At 16 dph *npy* expression levels increased before feeding, as expected, but then continue to increase up to 3 h after feeding (40), which is counterintuitive for an orexigenic factor (1, 12). At 34 dph, *npy* expression was only affected by the dietary fatty acid profile. This was similar to the results obtained by Kortner et al. (42), where cod *npy* was diet-specifically modulated in larvae at 16 dph, but no evident changes were found at 29 dph. Furthermore, in Atlantic halibut larvae, *npy* levels increased 5 h after refeeding (35). The differences observed between species may suggest that the Npy is still not fully functional in appetite regulation in larvae, possibly reflecting a yet underdeveloped appetite-regulating system. Furthermore, the response of *npy*, *pyy*, *pomc-c*, and *cart* to food deprivation and refeeding in Atlantic halibut larvae did not appear to be coordinated (35), lacking a consistent expression pattern to explain their contribution to appetite control in early larvae as it was for Senegalese sole larvae (40). In addition, the differences observed between both studies in Senegalese sole larvae may be explained by the different approaches used: use of complex diets fed through the whole larval and post-larval stage (379) versus a tube-fed single meal of pure fatty acids solution (378).

Altogether, these studies support the hypothesis that a feedback signaling system from the GI-tract to the CNS is still not fully established in the early larval stages. This, however, does not rule out that developing fish larvae may have their own specific system of appetite regulation adapted to their feeding ecology or that larvae possess a rudimentary, still developing, regulatory system. Fish larvae are often considered as "feeding machines" because they can ingest food at rates above their own weight daily (357, 380–382). This suggests that larvae are constantly hungry and motivated to feed, although several studies have shown that some fish larvae exhibit a circadian prandial pattern and do not feed constantly (383–385). Given the complexity of appetite-controlling mechanisms and how difficult

it is to interpret results due to the lack of specific information on the roles played by some of the potential anorexigenic and orexigenic factors in fish, it remains a challenge to elucidate the appetite-control system in fish larvae with different digestive tract morphologies and feeding strategies. A better understanding will greatly increase our basic knowledge on larval physiology and help to improve larval rearing regimes and feeding protocols in hatcheries.

The Voracious Feeders

Several species have an aggressive and voracious feeding behavior, most of them usually being carnivorous top predators. Well-known examples include Perciformes such as bluefish (*Pomatomus saltatrix*), bluegill (*Lepomis macrochirus*), cobia, groupers, tilapia and African cichlids, salmonids (e.g., rainbow trout), pikes (e.g., Northern pike *Esox lucius*), some characids (e.g., dourado and piranhas), as well as elasmobranchs, i.e., sharks and rays (338).

Within the teleosts, several studies have examined the effects of fasting and feeding on the expression of a few appetite regulator genes. However, there are no data on how endocrine mechanisms might regulate the increased feed intake in these voracious fish, and no comparative study has been performed between voracious species and a "gentler" herbivore/omnivore species (e.g., cyprinids, some flatfish species).

In response to fasting, it appears that most voracious fish display a similar trend to what occurs in non-aggressive species [e.g., the omnivorous goldfish and pacu (*Piaractus mesopotamicus*)], i.e., increases in expression of orexigenic factors [e.g., *ox* in dourado (73) and piranha (225), and *ghrl* in piranha (386)] and decreases in expression of anorexigenic factors [e.g., *cart* in piranha (225)]. However, few studies have examined periprandial changes in voracious fish. Taking the example of orexin, its expression appears to increase around feeding time and decrease after feeding, similar to what is seen for other fish species, such as orange grouper (70) and tilapia (387). In dourado, *ox* expression is similar before, during, and after feeding, suggesting a constant state of feeding/searching behavior. In addition, *ox* expression levels in fasted fish increase at mealtime and dramatically at post-feeding time, suggesting that dourado have a high motivation to search for food that persists after meal time (73). In contrast, pacu, a fish from the same order (Characiformes) as dourado, shows high *ox* levels at pre-feeding, and these tend to decrease at mealtime and post-feeding. Moreover, if pacu is not fed at the scheduled mealtime, *ox* levels increase at mealtime but return to basal levels within 1 h, suggesting that the fish have "given up" on searching food (388), which is reflected by their calm behavior (Volkoff, personal observation).

Voracious fish are often aggressive during feeding. Although aggression is often related to reproduction, in these species it also occurs outside the reproductive context (389). Interestingly, early studies in cichlid fish (*Tilapia heudelotii macrocephala*) and in bluegill have shown that electrical stimulation of the hypothalamic region elicited both feeding and aggressive responses (390, 391). The brain monoaminergic system, especially serotonin [5-hydroxytryptamine (5-HT)], plays a key role in controlling aggressive behavior (392). 5-HT has been reported to inhibit aggressive behavior in several voracious species, e.g., trout (393) and pikeperch (*Sander lucioperca*) (394). Interestingly, surface Mexican tetra (*Astyanax mexicanus*) species are aggressive predators, in particular during feeding episodes, whereas blind cave forms of this species exhibit reduced aggressiveness and have a tendency to continuously search for food. These differences in foraging and aggressive behaviors are related to 5-HT network modifications within hypothalamic neurons (395, 396). 5-HT also has anorexigenic actions in rainbow trout (397) and in mammals (387) and has been shown to interact with appetite regulators. For example, the behavioral effects produced by orexin administration, i.e., increased locomotion and feeding, are blocked by 5-HT antagonists (398). It would therefore be valuable to compare 5-HT levels between voracious and non-voracious fish.

Intra-species differences (sometimes referred to as personality/motivation) in basal locomotor and feeding activities are often observed between individuals. These differences might be due to different expression levels of appetite regulators or monoamines. For example, in tilapia, low serotonergic activity in the hypothalamus is correlated with a personality characterized by high feeding motivation (399). Similarly, in salmonid fish, subordinate individuals characteristically exhibit higher plasma cortisol levels than dominant ones (400). There are most likely different causes for voraciousness in fish, and more direct studies are needed to explain the underlying mechanisms of the appetite-controlling networks that result in these large differences in feeding behaviors.

How Important Is Vision? (The Blind Mexican Cavefish)

Although most fish rely in part on vision to feed (401), this sense is not essential for some species. The best example is that of fish living in cave environments, which are characterized by constant darkness and food scarcity (338, 402). Cavefish such as the Mexican tetra are often blind and have specialized anatomical features to better locate food and maximize food intake (396, 403, 404). Such adaptations include well-developed olfactory bulbs (405), taste buds (406), and lateral line neuromasts (407–409). In addition, these fish display behavioral adaptations for detecting prey and increasing feeding efficiency: they are opportunistic feeders, show increased swimming/exploratory and feeding behaviors (410), do not sleep (411), and do not exhibit schooling behavior (403, 412, 413). This enhanced food-finding efficiency is present not only in adults but also in young larvae when the yolk has been depleted (414). Overall, surface fish placed in the dark are less efficient at finding food than cavefish (415–417).

To cope with a particularly food-limited habitat compared to most surface fish, cavefish have developed behavioral (increased appetite, with ingestion of large amounts of food during feeding events) and metabolic adaptations. The latter include reduced basal metabolic rate, increased metabolic efficiency, starvation resistance (reduced weight loss during fasting), and increased body fat composition (403, 413, 418).

Peripheral injections of known orexigenic factors in cavefish, such as OX, GHRL, and apelin, increase not only food

consumption but also the whole brain mRNA expressions of orexigenic factors (e.g., GHRL injections induce an increase in *ox* brain expression), whereas injections of CCK reduce food intake and induce a decrease in the whole brain expression orexigenic factors (e.g., *apelin*) (67, 79). Peripheral injections of OX greatly increase locomotor activity and *ox* brain mRNA levels in cavefish. Basal *ox* mRNA levels in whole brain are higher in cave fish than in surface fish (Buenos Aires tetra, *Hyphessobrycon anisitsi*, a characid surface species closely related to *Astyanax*) (405), suggesting that the higher overall locomotor/feeding activity in cavefish compared to the surface forms might be mediated by an increase in *ox* levels (67, 79). Coding mutations in *mc4r* also contribute to the increased appetite and starvation resistance of cavefish compared with surface fish (419).

Cavefish are avid feeders and become very active around feeding time when appetite increases (420). Brain *ox* mRNA expression levels increase before and decrease after a scheduled mealtime (67), suggesting that orexin acts as a short-term hunger signal and is linked to food anticipatory activity. Conversely, the brain expression of the anorexigenic *pyy* increases after feeding (67), suggesting a role for Pyy as a short-term satiety factor. However, *cck* brain expression does not display periprandial variations in cavefish (67), which might contribute to a less rapid satiety and longer bouts of feeding.

Short-term food restriction increases *ox* brain mRNA transcription levels in cavefish (67), indicating a role in the long-term regulation of feeding in cavefish and perhaps triggering an increased motivation to seek food. However, as opposed to most surface fish examined to date, short-term fasting does not increase brain mRNA levels of *pyy* and *cck*, suggesting that the anorexigenic systems are inhibited during fasting, perhaps to slow down digestion/gastric emptying of food in the gut or to maintain a hunger state that would favor food-seeking behavior.

FUTURE

Many of the studies on appetite-controlling systems in teleosts are based on domesticated fish that have been bred in captivity for generations (e.g., salmon, carp, and cod). These fish, which are submitted to optimal habitat (e.g., no predators, constant optimal photoperiods and temperatures) and feeding (e.g., satiation, minimal food-seeking behavior) conditions might have present modifications in their feeding behavior and systems controlling appetite, as compared to wild fish exposed to suboptimal conditions. This phenomenon has been shown in domesticated rats that eat more than wild individuals (421). Comparisons between wild and captive populations might reveal important information on the effects of domestication on feeding behavior. Therefore, observations of feeding behavior and sampling of fish in their natural environment would be valuable.

Overall, within a few model species, only a few appetite-regulating hormones (e.g., leptin, Npy, and Cck) have been studied more in detail. In addition, there are very few studies on the mechanisms of action of these hormones, including at the level of their target cells and their receptors. Many questions related to the concepts "set-point" in energy homeostasis and stimulus for synthesis/secretion of these hormones, i.e., whether it is direct nutrient sensing by the hormone-producing cells or stimulation of these cells by another hormone/neurotransmitter or both, also remain to be answered. Also, many of these hormones are expressed both in the CNS and in peripheral tissues and the relative importance of each, as well as their interactions in controlling the appetite, are poorly understood.

One of the major limitations in the field of appetite endocrinology in fish is that the vast majority of studies have been constrained to the analyses of transcript levels. Although the existence of a proportional relationship between mRNA and protein expressions measured from a tissue have long been assumed, recent data show that this is not always the case (422). The development of fish-specific hormone assays and protein expression techniques is crucial for a better understanding of appetite-regulating mechanisms in fish. In addition, most studies analyze large portions of specific tissues (e.g., whole brain, whole hypothalamus, or whole intestine), which might also bias results, as, for example, specific regions (e.g., proximal versus distal intestine, or specific hypothalamic nuclei) might have different functions and respond differently to feeding conditions.

Although it is often observed that growth is directly related to food intake, many gaps exist on our understanding of how these two functions are connected in fish. The recent development of GH-transgenic fish is promising for the exploration of this field. Thus, the development of emerging techniques such as gene editing (CRISPR/Cas9 system) will be a great tool to study the role of appetite regulators in fish. Targeted mutagenesis using CRISPR/Cas9 system has been successfully used in several species, including zebrafish (423), salmon (424), and African cichlids (425), but so far only a few studies have used this technique to examine the role of appetite regulators on fish models, e.g., leptin receptor mutations in zebrafish (302).

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Non-Mammalian Vertebrates: Distinct Models to Assess the Role of Ion Gradients in Energy Expenditure

*Caroline E. Geisler, Kyle P. Kentch and Benjamin J. Renquist**

School of Animal and Comparative Biomedical Science, University of Arizona, Tucson, AZ, United States

Animals store metabolic energy as electrochemical gradients. At least 50% of mammalian energy is expended to maintain electrochemical gradients across the inner mitochondrial membrane (H^+), the sarcoplasmic reticulum (Ca^{++}), and the plasma membrane (Na^+/K^+). The potential energy of these gradients can be used to perform work (e.g., transport molecules, stimulate contraction, and release hormones) or can be released as heat. Because ectothermic species adapt their body temperature to the environment, they are not constrained by energetic demands that are required to maintain a constant body temperature. In fact, ectothermic species expend seven to eight times less energy than similarly sized homeotherms. Accordingly, ectotherms adopt low metabolic rates to survive cold, hypoxia, and extreme bouts of fasting that would result in energy wasting, lactic acidosis and apoptosis, or starvation in homeotherms, respectively. Ectotherms have also evolved unique applications of ion gradients to allow for localized endothermy. Endothermic avian species, which lack brown adipose tissue, have been integral in assessing the role of H^+ and Ca^{++} cycling in skeletal muscle thermogenesis. Accordingly, the diversity of non-mammalian vertebrate species allows them to serve as unique models to better understand the role of ion gradients in heat production, metabolic flux, and adaptation to stressors, including obesity, starvation, cold, and hypoxia.

Keywords: ectotherm, endotherm, energy expenditure, membrane potential, mitochondrial membrane potential, H^+ gradient, Ca^{++} gradient, Na^+/K^+ gradient

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***Correspondence:**
Benjamin J. Renquist
bjrenquist@email.arizona.edu

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INTRODUCTION

The ease of genetic manipulation, the standard husbandry demands, and the well-established experimental methods have resulted in rodent model predominating the obesity field. Yet, the diversity of non-mammalian vertebrates, including both endothermic (birds and some fish) and ectothermic (amphibians, reptiles, and fish) species that have adapted to a variety of environments, provides unique opportunities to better understand the regulation of energy expenditure. The heterogeneity of species and environments to which they have adapted yield an abundance of unique attributes that can better help us understand energy expenditure. Herein, we review research in non-mammalian vertebrate control of electrochemical gradients and application of this work to mammalian species.

Basal metabolic rate is seven- to eightfold higher in mammals than in similarly sized ectotherms maintained at 37°C (1, 2). Because ectothermic species have low levels of basal heat production, they

Abbreviations: UCP, uncoupling protein; ANT, adenine nucleotide translocase; NADH, nicotinamine adenine dinucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

are sensitive models to identify the role of ion flux in altering heat production and basal metabolic rate. For example, egg brooding female pythons use whole body skeletal muscle contractions to facilitate endothermy and maintain a body temperature 9–13°C above ambient temperature (3, 4). This contraction-induced thermogenesis increases the oxygen consumption 22 times above baseline (5). In rat pups (22 days), maintenance of body temperature in an environment 9–13°C, colder than thermoneutral, increases oxygen consumption just two times above baseline (6). While in penguin chicks, a temperature drop of 10°C below the shivering threshold only increases oxygen consumption by 27%, while a temperature 25°C below the shivering threshold doubles oxygen consumption (7). Thus, the low basal metabolic rate of ectothermic vertebrate species allow for a uniquely sensitive model to perturbations in metabolic rate, while the endothermic bird provides a comparative model to better understand perturbations and adaptations that affect whole body and skeletal muscle energy expenditure in the absence of endothermic brown adipose tissue.

Herein, we review the lower vertebrate literature on energy expenditure with a focus on three ion gradients: the H^+ gradient at the inner mitochondrial membrane, the Ca^{++} gradient of the sarcoplasmic reticulum, and the Na^+/K^+ gradient across the plasma membrane. We further apply findings from the lower vertebrate literature to our current understanding of mammalian energy expenditure and its potential application to human health.

H^+ GRADIENT

The H^+ gradient is maintained in the inter-mitochondrial membrane space, created by electron transport chain activity (**Figure 1**). This gradient is dissipated by H^+ ion leak across the inner mitochondrial membrane, which can be exacerbated by uncoupling proteins (UCP) and adenine nucleotide translocases (ANT), or production of adenosine triphosphate (ATP) through ATP synthase. Accordingly, the mitochondrial density, inner-mitochondrial membrane surface area, expression and activity of electron transport chain proteins, and the expression of UCPs and ANTs that allow for H^+ leak across the inner mitochondrial membrane are integral to heat production and energy expenditure.

Compared to endotherms, reptilian tissues have fewer mitochondria with less inner mitochondrial membrane surface area per mitochondria, resulting in 50% less inner mitochondrial membrane across which H^+ can leak (1). Because the organs that are rich in mitochondria are smaller in ectotherms, whole body mitochondrial membrane surface area is four times greater in mammals than in reptiles of the same body size (8). The H^+ gradient is established by electron transport chain activity and depressed by the release of H^+ out of the inter-mitochondrial membrane space. With four times less membrane surface area, ectotherms have lower electron transport chain activity and H^+ ion leak.

Electron Transport Chain

The decreased electron transport chain activity in ectotherms is not a result of decreased activity of proteins integral to oxidative phosphorylation (2, 9). ATP synthesis, expressed as a percentage of mitochondrial respiration, is similar in reptiles and mammals at similar body temperature (2). Inner mitochondrial membrane cytochrome *C* oxidase (complex IV) content and activity is also similar in fish and cattle (9). Moreover, when corrected for tissue protein content, cytochrome *C* oxidase activity is similar in

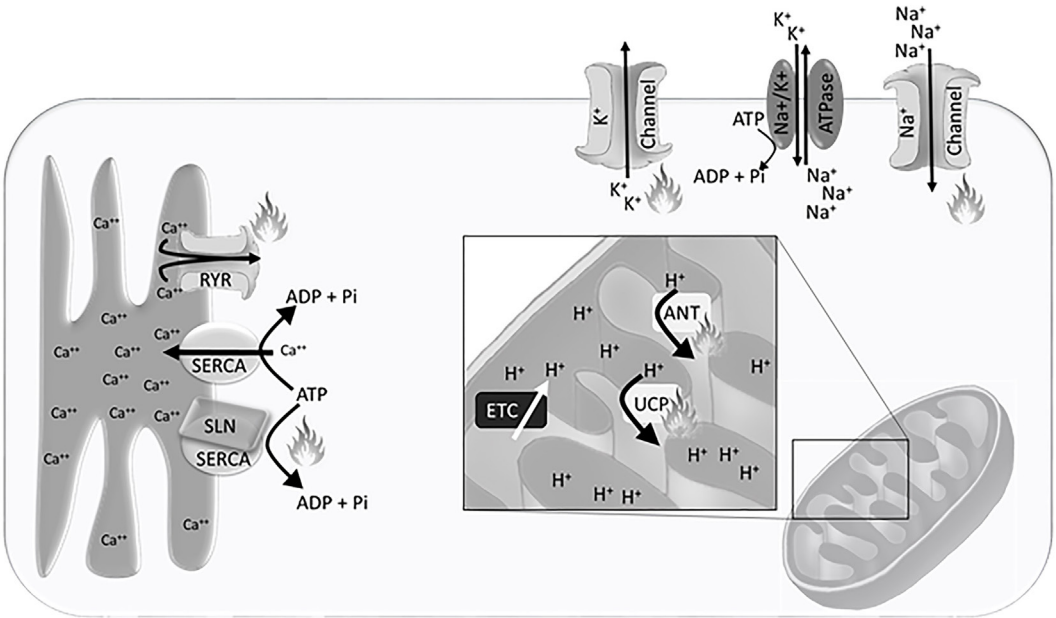

FIGURE 1 | Schematic representation of the Ca^{++} gradient at the sarcoplasmic reticulum, H^+ gradient at the inner mitochondrial membrane, and Na^+/K^+ gradient at the plasma membrane. Flames indicate sites of heat generation from ion leak and non-functional adenosine triphosphate (ATP) hydrolysis. Abbreviations: SERCA, sarco/endoplasmic reticulum Ca^{++} ATPase; RYR, ryanodine receptor; SLN, sarcolipin; ETC, electron transport chain; UCP, uncoupling protein; ANT, adenine nucleotide translocase.

reptiles and mammals (10). Thus, the decreased electron transport chain activity in ectotherms is a result of decreased mitochondria numbers, mitochondrial size, mitochondrial membrane area, and a resulting decrease in total electron transport proteins rather than altered activity of the mitochondrial machinery.

The electron transport chain represents an important point of manipulation for energy expenditure in ectotherms. During hypoxia and cold exposure, electron transport chain activity can be depressed to accommodate the decreased cellular energy demand imposed by these conditions. Thus, in species not dependent on internal heat production, limiting cellular respiration is an adaptive response to environmental stress. In fact, ectothermy allows for adaptive whole body cooling and metabolic depression to prevent metabolic acidosis in response to hypoxia (11). Four months of *in vivo* hypoxia results in a severe decrease in adenosine diphosphate (ADP) stimulated respiration (State 3) and a decrease in ADP-independent respiration (State 4; H^+ leak) from isolated skeletal muscle mitochondria (12). Hypoxia induces a characteristic rise in plasma lactate in frogs maintained at 7°C, while there is no change in plasma lactate in frogs maintained at 1.5°C (13). The rise in lactate at 7°C is a result of glycolytic flux exceeding the mitochondrial capacity for oxidation under hypoxic conditions. In turn, frogs or toads challenged with hypoxia chose to reside at lower temperatures, decrease their metabolic rate, and limit the metabolic disturbances associated with a lack of oxygen (13–15). In alligators, hypoxia leads to hypothermia in an adaptive effort to depress metabolic demands (16). Long-term exposure to hypoxia and 3°C water results in an adaptive decrease in skeletal muscle mitochondrial oxygen consumption by inhibiting flux through the electron transport chain and limiting H^+ ion leak across the inner mitochondrial membrane (12, 17–19). Indeed, ectothermic species exploit the decrease in metabolic demand that accompanies low body temperatures to adapt to acute or chronic hypoxic environments (14).

Chronic hypoxia, due to a high altitude habitat, also regulates mitochondrial respiration. In a comparison of two closely related lizard species that live at different elevations, species adapted to high elevations had lower oxidative capacity and less ADP-independent respiration than species that inhabit lower elevations (20). Thus, adaptation to chronic hypoxia increased the efficiency of H^+ ion gradient energy capture as ATP. Of note, mitochondria from lizards adapted to high elevations are less able to increase respiration in response to an increase in incubation temperature (20). Suggesting that there is an adaptive cost to the tight coupling between the H^+ ion gradient and ATP synthesis.

In endotherms, cold exposure increases electron transport chain activity to promote endogenous heat production. Acute, 24 h, cold exposure in the chick increases expression of cytochrome *C* oxidase and NADH ubiquinone oxidoreductase (complex I) (21). Chronic cold exposure similarly increases cytochrome *C* oxidase activity as a result of increased mitochondria number and increased inner mitochondrial membrane area/mitochondrial volume (22). In small mammals, the primary thermogenic organ is brown adipose tissue. Chronic, 4 week, cold exposure in the tree shrew increases the cytochrome oxidase activity in brown adipose tissue to six times that observed in thermoneutral conditions (23). The same 4-week cold exposure increases liver cytochrome oxidase activity 2.7 times (23). In mice, skeletal muscle electron transport activity is increased by cold acclimation (24). Mice that lack UCP1 and the thermogenic potential of brown adipose tissue, display an increase in skeletal muscle mitochondria to accommodate an increased thermogenic role for skeletal muscle (24). Together these studies suggest that cold exposure increases electron transport chain activity and mitochondrial density similarly in endothermic avian and mammalian systems. The necessity to use a UCP1 knockout mouse to better assess the role of skeletal muscle in thermogenesis recommends that birds, which lack brown adipose tissue, may be better suited models to understand the role of skeletal muscle thermogenesis in response to environmental or dietary stimuli.

H^+ Leak

H^+ leak across the inner mitochondrial membrane uncouples the electron transport chain from oxidative phosphorylation, preventing the capture of energy as ATP and enhancing its release as heat. In rodent-based studies, heat production through H^+ leak is primarily associated with the inducible thermogenesis observed in brown adipose tissue. However, the leak of H^+ in liver and skeletal muscle mitochondria has been estimated to be responsible for 20% of basal energy expenditure in the rat (25). In fact, it accounts for 50% of respiration in perfused rat skeletal muscle (25). By comparing the respiration rate of mitochondria collected from rat and frog heart in the absence of ADP, Akhmerov showed that H^+ ion leak was six- to sevenfold greater in isolated rat mitochondria than in isolated frog mitochondria (26). This may be in part explained by the inner mitochondrial membrane surface area per mitochondria being larger in mammals than in reptiles of a similar size (1). However, it may also be due to differences in the H^+ gradient and membrane lipid composition.

Proton leak increases with mitochondrial membrane potential across species. Still, at the same mitochondrial membrane potential, H^+ ion leak is approximately fivefold greater in the rat than in the bearded dragon (2). Other ectothermic species mute H^+ leak by maintaining a lower mitochondrial membrane potential. In the rainbow trout, mitochondrial proton leak at a given membrane potential is higher than that observed in mitochondria from rat or pigeon (27). This is because membranes in cold-water fish species have a high degree of polyunsaturated fatty acyl groups to maintain membrane fluidity at low temperatures. The increase in polyunsaturation also increases ion permeability (28). To limit proton leak, the rainbow trout has low electron transport chain activity eliciting a low mitochondrial membrane potential. Accordingly, total proton leak is lower in the rainbow trout than in the endothermic rat and pigeon. Thus, proton leak can be manipulated by either altering electron transport chain activity and mitochondrial membrane potential or by changing H^+ ion permeability.

Although proton leak is typically associated with heat production, increasing proton leak can also allow for metabolic flexibility. Accordingly, fish express UCP1, 2, and 3 (29, 30). In the carp, UCP1 is primarily expressed in the liver and expression is downregulated by a decrease in water temperature (30). The liver plays a central role in synthesizing and distributing glucose, ketones, and lipids to the rest of the body. Therefore,

an increase in water and body temperature would increase the metabolic demand of non-hepatic tissues and necessitate expression of UCP1 to regenerate FAD^+ and NAD^+. In line with increased gluconeogenic demand, hepatic phosphoenolpyruvate carboxykinase (PEPCK) enzyme activity, essential for greater gluconeogenic flux, is increased in the Antarctic eelpout in response to a 5°C increase in water temperature (31). Thus, the direct relationship between temperature and UCP1 expression may encourage the regeneration of hepatic FAD^+ and NAD^+, essential for flux through β-oxidation to produce the acetyl-CoA necessary for ketogenesis while sparing amino acid oxidation. Accordingly, in the fish upregulation of UCP1 is not an adaptation to increase hepatic heat production, but instead to promote metabolic flexibility. Skeletal muscle primarily expresses UCP3 in both fish and rodents (30, 32). Interestingly, UCP3 is also similarly upregulated by fasting in both rodents and fish, while fasting increases proton leak from skeletal muscle mitochondria in the cane toad (15). As we proposed for UCP1 in liver, it has been proposed that this upregulation of UCP3-mediated H^+ leak is essential for the increased reliance on fatty acid oxidation during a fast (32). Thus, studies of UCP regulation in fish can improve our understanding of UCP-mediated changes in metabolic flux without the demand for heat generation.

Conversely, some ectothermic fish species appear to manipulate H^+ leak to warm-specific tissues above environmental temperature. Indeed, cold acclimation increases UCP1 expression in the optic tectum, a brain region important for sight, in the carp (33). This warms the brain's optic center to maintain eyesight in cold environments, through a mechanism similar to the Ca^{++} cycling-based heater organ in deep diving marine species (34, 35). In sharks, skeletal muscle endothermy is thought to increase swimming rate, allow for thermal niche expansion, and increase digestive enzyme activity and digestive system passage (36). The mako shark uses red muscle fibers for endothermia. Mitochondria from mako shark red muscle fibers and liver display increased succinate stimulated respiration and maintain membrane potentials that exceed that of mitochondria from two ectothermic sharks. Although mitochondria from all of these shark species have similar levels of proton leak at a given membrane potential, the increased proton gradient in mako sharks drives H^+ leak and heat production (37). Together these studies suggest that heat production through H^+ leak may be used in some ectothermic species to warm specific tissues or maintain a set minimal body temperature required for survival.

In endotherms (birds and mammals), proton leak decreases with increasing body mass and explains 67% of the variability in standard metabolic rate (27, 38, 39). Proton leak is robust in birds. In fact, liver mitochondrial proton leak in all birds studied, including the much larger emu and goose, exceeded that of the rat (39). Across bird species, the ratio of monounsaturated fatty acids in phospholipids increases with body mass (39). The increase in monounsaturated fatty acyl groups may play a role in the decreased proton leak associated with increased body mass. In fact, membranes rich in monounsaturated fatty acyl groups are typical in ectothermic species with low levels of membrane ion permeability (40).

In rodents, studies aimed at understanding the endothermic response to cold have focused on the role of UCP1-mediated H^+ ion leak in brown adipose tissue (41). Brown adipose tissue is integral to the maintenance of homeothermy in newborn infants (42–44). However, in humans, the passage from newborn through childhood and into adulthood results in a decrease in the amount of UCP in adipose tissue, suggestive of a reduction in metabolically active brown adipose tissue (45). The recent identification of brown adipose tissue depots near the clavicle in the adult human has spurred a resurgence in research aimed at better understanding the role of brown adipose tissue in energy expenditure of adult humans (46, 47). Yet the relative mass of brown adipose tissue in the adult human is minimal ($168 \pm 56\ cm^3$). Accordingly, animal models that lack brown adipose tissue may provide a better understanding of the role of H^+ leak in the thermogenesis of other tissues (48).

Birds, which lack brown adipose tissue, are uniquely suited to address the role of H^+ leak in the endothermic response to environmental stimuli (cold or fasting). Avian UCP is expressed in skeletal muscle and increases in cold acclimated ducklings and penguins (49, 50). Proton leak across the inner mitochondrial membrane is higher in skeletal muscle from penguins that have been exposed repeatedly to cold water over 20 days. Teulier et al. observed that cold exposure of ducklings increased whole animal metabolic rate and ADP-stimulated oxidative phosphorylation. Surprisingly, despite the upregulation of avian UCP expression, there was no effect of cold acclimation on the efficiency of ATP synthesis or non-phosphorylating respiration. Accordingly, Teulier et al. propose that avian UCP primarily increases heat production by increasing aerobic skeletal muscle metabolic flux, rather than enhancing H^+ leak (51).

In addition to the UCPs, the ANT may encourage proton leak through the inner mitochondrial membrane (52). Avian ANT protein expression increases in response to repeated cold water immersion (50). Therefore, both ANT and UCP are upregulated in cold acclimated birds and may be integral to the maintenance of body temperature through upregulated skeletal muscle thermogenesis. During a fast, the maintenance of body temperature may partially depend on mitochondrial H^+ leak. Skeletal muscle avian UCP expression increases with glucagon treatment in ducklings (49). In the hummingbird, which undergoes torpor every night to endure an extended fast, skeletal muscle UCP expression increases during torpor and likely serves to help the bird re-warm from the torpid state (53). Thus, skeletal muscle is an integral site of mitochondrial proton leak, a key source of heat production in endotherms.

Ca^{++} CYCLING

Non-mammalian vertebrates have been integral to understanding the thermogenic potential of Ca^{++} cycling. Both fish and birds use Ca^{++} cycling to generate body heat independent of locomotion. To understand the unique physiological adaptations that each have evolved, we must first understand the basis of the Ca^{++} cycle. Within skeletal muscle the sarco/endoplasmic reticulum Ca^{++} ATPase (SERCA) pumps Ca^{++} from the cytosol into the sarcoplasmic reticulum against the concentration gradient, while hydrolyzing an ATP to ADP and inorganic phosphate. The hydrolysis of ATP releases some energy as heat. The remainder

of the energy is released as heat when Ca^{++} leaks from the sarcoplasmic reticulum into the cytosol down the electrochemical gradient (**Figure 1**). This cycle is essential for calcium oscillations in muscle that allow for contraction.

Marlin, swordfish, and tuna all have an endothermic "heater" organ that raises the temperature of the brain and eye (35, 54–56). This heater organ is a highly vascularized, modified eye muscle rich in mitochondria and cytochrome oxidase activity (35, 54–56). By maintaining the central nervous system and eyes at temperatures above ambient temperature, the heater organ improves eye sight and central nervous system function in cold ambient temperatures (35, 56–58). Warming the retina improves temporal resolution up to 10-fold, giving these deep diving, visual predators a crucial advantage over prey species (56). In addition to being mitochondria rich (68% of total cell volume), the heater organ contains a great deal of sarcoplasmic reticulum rich in SERCA1b and the ryanodine receptor 1 (34, 55). This encourages heat production from ATP hydrolysis as the SERCA pumps Ca^{++} into the sarcoplasmic reticulum and additional heat production as that Ca^{++} flows through the ryanodine receptor 1 down the concentration gradient back into the cytosol (34). The lack of contractile proteins uniquely positions the heater organ to take full advantage of heat generation by calcium cycling without motor consequence (58).

Cold exposure increases basal metabolic rate in birds, rodents, and humans (59–62). However, unlike rodents, which rely heavily on brown adipose tissue for thermogenesis, birds lack brown adipose tissue and rely more heavily on skeletal muscle thermogenesis. In the Muscovy duck, *in vivo* cold exposure increases *ex vivo* skeletal muscle oxygen consumption by 25% (63). Acute cold exposure of ducklings increases cardiac output and directs blood flow toward thermogenic sites, resulting in a 130% increase in skeletal muscle blood flow (64), indicating that skeletal muscle is the primary thermogenic organ in the duckling. In fact, birds rely on skeletal muscle calcium cycling to maintain body temperature at low environmental temperatures. Accordingly, acute 24-h cold-exposure increases skeletal muscle Ca^{++} ATPase activity (21). Cold acclimation over 5 weeks in ducklings increases sarcoplasmic reticulum SERCA1, SERCA2, and ryanodine receptor expression and activity as evidenced by increased Ca^{++} uptake and ryanodine binding (65, 66). The increase in Ca^{++} cycling leads to an increased resting metabolic rate, ATP demand, and oxygen consumption in cold acclimated birds (63, 67, 68). This is accommodated by increased expression of components of the electron transport chain, including cytochrome *C* and NADH ubiquinone oxidoreductase (21).

Interestingly, cold exposure stimulates lipolysis in birds, rodents, and humans (21, 62, 69). Mobilized lipids provide a source of carbons to meet this increased metabolic demand associated with maintaining body temperature. Accordingly, in the mouse, fatty acid translocase (cd36) knockout prevents the maintenance of body temperature in response to cold exposure (70). Moreover, cold acclimation in the sparrow increases pectoralis cd36 protein expression by 46% (71). In addition to acting as a carbon source, these lipids may modulate the degree of Ca^{++} cycling. Long-chain acyl carnitines accumulate in the skeletal muscle of cold acclimated ducklings. Palmitoyl (16 C) carnitine activates Ca^{++} release from duckling skeletal muscle sarcoplasmic reticulum (72). Thus, this skeletal muscle non-esterified fatty acid accumulation and the potential downstream consequences are conserved from birds to rodents and humans (62).

Data from the partially endothermic fish species and homeothermic avian species has established the thermogenic potential of skeletal muscle and the role of skeletal muscle calcium cycling in heat generation and body temperature maintenance. More recently, studies focused on sarcolipin (SLN), a protein that uncouples ATP hydrolysis from sarcoplasmic reticulum Ca^{++} sequestration, have elucidated a mechanism by which mammals use this calcium sequestration machinery to generate heat (73). Normally, SERCA hydrolyzes ATP and uses the majority of that energy to pump Ca^{++} from the cytoplasm against a concentration gradient into the sarcoplasmic reticulum, while releasing the remaining energy as heat. By decreasing Ca^{++} sequestration, SLN increases the heat released per mol of ATP hydrolyzed by more than 50% (74–76). The Periasamy laboratory has conducted a set of elegant studies to investigate the role of SLN in the response to thermogenic and dietary challenges (77, 78). They first showed that UCP1 knockout mice, which lack the thermogenic potential of brown adipose tissue, express more SLN in skeletal muscle (78). SLN overexpression in skeletal muscle increases oxygen consumption (77). This increase in skeletal muscle oxygen consumption translates to whole body energy metabolism, as SLN overexpressing mice lose weight when pair fed with wild-type mice. Furthermore, when challenged with a high fat diet, these SLN overexpressing mice eat more yet gain less body weight than wild-type mice (77). These findings in the mouse may translate to humans, as obesity alters methylation in the promoter of the ryanodine receptor 1 gene (79). In fact, this effect on the RYR1 promoter is the most prominent methylation response to obesity, most heavily affected. Moreover, the normal pattern of methylation can be restored with weight loss (79).

Fish and birds have been essential in establishing the role of calcium cycling and the SERCAs in energy expenditure and heat production. Deep water fish have developed a unique tissue with specified function to maintain brain and eye temperatures during exposure to deep cold ocean waters, while endothermic birds establish the potential for skeletal muscle to act as a thermogenic organ. New research reporting that calcium cycling is important for body weight regulation in rodents provides an exciting new avenue for drug development to combat obesity. Humans and birds both express higher levels of SLN than mice, recommending that birds may be a valuable model organism for assessing the role of SLN in body weight regulation.

PLASMA MEMBRANE POTENTIAL

The maintenance of the plasma membrane potential is a critical component of cellular homeostasis and requires the tight regulation of intracellular ion concentrations. Passive ion channels and active transport pumps establish Na^+, K^+, and Cl^- electrochemical gradients across the plasma membrane that are essential for fundamental cellular processes. Transport of molecules both into and out of the cell, hormone secretion, muscle contraction, and neuronal communication are all dependent on the maintenance

of resting membrane potential and utilization of these energetically favorable ion gradients. The Na^+/K^+ ATPase is the primary active pump driving cellular membrane potential (**Figure 1**).

Na^+/K^+ ATPase activity represents a major cellular energy demand and significant portion of resting metabolic activity. At the whole animal level, the Na^+/K^+ ATPase accounts for ~25% of ATP consumption in mammals (80). However, this can vary widely by tissue. In the liver, Na^+/K^+ ATPase activity constitutes ~10% of cellular energy use, while this rises to 60% in brain and kidney (81). Comparing the relative contribution of energy consuming processes between endotherms and ectotherms, 60 and 54% of cellular respiration is directed toward ATP production, of which ~13.3 and ~18.5% is consumed by the Na^+/K^+ ATPase in rat and lizard hepatocytes, respectively (2, 82). Since the respiration rate of rat hepatocytes is about four times that of lizard hepatocytes, 5.6 times more ATP is allocated toward Na^+/K^+ ATPase activity.

While endotherms and ectotherms maintain similar Na^+/K^+ ATPase densities across tissue type, the molecular activity of the pump is four to five times higher in endotherms (83). Accordingly, the plasma membrane passive permeability to both Na^+ and K^+ is four- to ninefold greater in endotherms than in ectotherms at the same temperature (84–86). Thus, to maintain established ion gradients, the leakier cell membranes of endotherms require more active Na^+/K^+ ATPase. Increased total ion flux in endotherms may be an evolutionary adaptation to increase heat production. The resulting higher activity of the Na^+/K^+ ATPase, in part, accounts for the greater level of energy expenditure in endotherms.

The lipid composition of the plasma membrane has a primary role in regulating Na^+/K^+ ATPase activity. Among ectotherms, the degree of membrane polyunsaturation is significantly correlated with Na^+/K^+ ATPase activity (87). In membrane crossover experiments, reconstitution of the Na^+/K^+ ATPase of an ectotherm (cane toad or crocodile) in the more polyunsaturated membrane of an endotherm (rat or cattle) increases pump activity by 40–180% while reconstitution in the reverse direction decreases Na^+/K^+ ATPase activity by 40–250% (88, 89). Thus, the inherent properties of the plasma membrane strongly regulate Na^+/K^+ ATPase activity. Interestingly, membranes of cold water fish have higher levels of unsaturated phospholipids than their warm water counterparts (90). In carp liver slices, exposure to decreasing temperatures immediately inhibits the synthesis of saturated fatty acids and stimulates desaturase activity (91). Across fish species, acclimation to low temperatures increases the degree of membrane fatty acyl unsaturation, stimulating Na^+/K^+ ATPase activity to compensate for the cold-induced decline in enzyme activity (92). Ectotherms adapt to temperature by changing membrane composition, representing a key regulatory mechanism by which activity of membrane-integrated enzymes, including the Na^+/K^+ ATPase, are altered to maintain membrane potential in the face of variable environmental conditions. Still, temperature changes have a more robust effect on active pump processes than passive ion leak, giving rise to the possibility of temperature disrupted ion gradients. In fact, in skeletal muscle of cane toads, bullfrogs, and black racer snakes, intracellular Na^+ concentrations are higher at 20°C compared to 30°C, suggestive of decreased Na^+/K^+ ATPase activity with increasing environmental temperature (93). In cold-adapted species, activity of the Na^+/K^+ ATPase is less temperature sensitive, limiting temperature-dependent changes in membrane potential. In fact, the molecular activity of the Na^+/K^+ ATPase from an Antarctic octopus is 400% greater than that of the temperate octopus at 10°C (94).

Many ectotherms that overwinter underground or are exposed to prolonged water submersion have developed strategies to tolerate hypoxia. Turtles can spend over half of their life in an overwintering state and survive sustained periods of anoxia (95). In the western painted turtle hepatocyte, 28% of total cellular ATP is utilized by the Na^+/K^+ ATPase in normoxia (96). In response to anoxia, the Na^+/K^+ ATPase activity decreases by 75%, but accounts for nearly three-fourth of total cellular ATP turnover. Suppressed Na^+/K^+ ATPase activity is partially mediated by adenosine signaling which is robustly stimulated during anoxia (97). Ion gradients and plasma membrane potential are maintained during anoxia. Thus, passive ion flux must be downregulated in anoxia to match the decrease in active ion transport. In the turtle, neurons adapt to low oxygen by limiting K^+ leak through a fivefold reduction in the open probability of Ca^{++} gated K^+ channels (98). However, not all ectotherms can maintain ion gradients when challenged with low oxygen. In rainbow trout hepatocytes, an anoxia-intolerant species, cellular ATP content, and Na^+/K^+ ATPase activity are rapidly reduced by anoxia while K^+ efflux rates exceed K^+ influx up to eightfold (99). In the anoxia-tolerant goldfish, anoxia diminishes K^+ efflux to match the decline in Na^+/K^+ ATPase activity, resulting in a net flux of K^+ close to 0. Therefore, downregulating ion leak and allocating a greater percentage of total cellular ATP to the Na^+/K^+ ATPase appear to be principal strategies in maintaining membrane potential during metabolic arrest in anoxia-tolerant species.

This comparison between anoxia tolerant and intolerant fish species recommends that hypoxia-induced cellular damage may be limited by inhibiting K^+ efflux channels within cells. Indeed, hypoxia induces K^+ efflux in mammalian tissues (100–102). In fact, inhibition of K^+ efflux across the plasma membrane prevents neuronal apoptosis in rats subjected to transient middle cerebral artery occlusion to induce hypoxia and ischemia or in serum-starved apoptosis-induced mouse neocortical neurons (103). In addition, the role of K^+ efflux in hypoxia-induced apoptosis may be used to better understand the resistance of cancer to hypoxia-induced apoptosis. Several human cancers express low levels of K^+ channels, preventing K^+ efflux, and resulting in resistance to apoptosis (104). As in the anoxia tolerant goldfish, this diminished K^+ efflux, allows cancer cells to maintain a nearly normal membrane potential in the face of depressed Na^+/K^+ ATPase activity. Thus, the strategies developed by anoxia tolerant species may be used to limit hypoxia induced tissue damage caused by cardiac arrest or arterial occlusion, while the sensitivity of anoxia intolerant species may provide insight into treatments that will diminish the hypoxia resistance observed in cancer.

CONCLUSION

Maintaining ion gradients demands at least 50% of mammalian resting metabolic energy expenditures. By adapting body

temperature to the environment, ectotherms are not reliant on ion flux down the mitochondrial membrane H^+, sarcoplasmic reticulum Ca^{++}, or plasma membrane Na^+/K^+ gradients for internal heat generation. Because they are not as stringently dependent on the energetic costs of endogenous thermoregulation, ectotherms can manipulate these ion gradients to adapt to a wide range of environments and stressors. Like mammals, avian species are constrained by the demands of homeothermy. Yet, they lack the endothermic brown adipose tissue depots that constrain interpretation of rodent-based findings. In turn, having adapted to a range of environments, avian species provide an ideal comparative model to understand the regulation of ion flux to manipulate heat generation. The diversity of non-mammalian vertebrate species and environmental niches to which they have adapted provide unique insight into ion gradients that are lacking in studies of mammalian species. Ectothermic fish manipulate H^+ and Ca^{++} leak to warm-specific tissues, providing ideal models to further investigate the regulation of ion leak. Avian species have been essential in establishing the importance of skeletal muscle mitochondrial density and Ca^{++} ATPase activity in thermogenesis. Recent work highlighting the role of SLN in mammalian body weight homeostasis validates the application of these avian studies to understand mammalian physiology. Finally, studies that compare the ability of anoxia tolerant and intolerant fish species to maintain plasma membrane potential may be applied to prevent the damage associated with stroke- or cardiac arrest-induced hypoxia and ischemia. In addition, these studies can be applied to exacerbate sensitivity to hypoxia in cancer. The vast number of lower vertebrate species and the varied environments to which they have adapted allow for unique research opportunities that can be exploited to expand our knowledge of mechanisms underlying metabolic flux, energy expenditure, and body weight regulation.

AUTHOR CONTRIBUTIONS

CG, KK, and BR all made substantial contributions to the conceptual framework of this review, participated in the drafting and revising of the manuscript, approved this final version for submission, and agreed to be accountable for all aspects of the work.

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REVIEW
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Comparative Physiology of Energy Metabolism: Fishing for Endocrine Signals in the Early Vertebrate Pool

Iris van de Pol, Gert Flik and Marnix Gorissen*

Department of Animal Ecology and Physiology, Institute for Water and Wetland Research, Radboud University, Nijmegen, Netherlands

Energy is the common currency of life. To guarantee a homeostatic supply of energy, multiple neuro-endocrine systems have evolved in vertebrates; systems that regulate food intake, metabolism, and distribution of energy. Even subtle (lasting) dysregulation of the delicate balance of energy intake and expenditure may result in severe pathologies. Feeding-related pathologies have fueled research on mammals, including of course the human species. The mechanisms regulating food intake and body mass are well-characterized in these vertebrates. The majority of animal life is ectothermic, only birds and mammals are endotherms. What can we learn from a (comparative) study on energy homeostasis in teleostean fishes, ectotherms, with a very different energy budget and expenditure? We present several adaptation strategies in fish. In recent years, the components that regulate food intake in fishes have been identified. Although there is homology of the major genetic machinery with mammals (i.e., there is a vertebrate blueprint), in many cases this does not imply analogy. Although both mammals and fish must gain their energy from food, the expenditure of the energy obtained is different. Mammals need to spend vast amounts of energy to maintain body temperature; fishes seem to utilize a broader metabolic range to their advantage. In this review, we briefly discuss ecto- and endothermy and their consequences for energy balance. Next, we argue that the evolution of endothermy and its (dis-)advantages may explain very different strategies in endocrine regulation of energy homeostasis among vertebrates. We follow a comparative and evolutionary line of thought: we discuss similarities and differences between fish and mammals. Moreover, given the extraordinary radiation of teleostean fishes (with an estimated number of 33,400 contemporary species, or over 50% of vertebrate life forms), we also compare strategies in energy homeostasis between teleostean species. We present recent developments in the field of (neuro)endocrine regulation of energy balance in teleosts, with a focus on leptin.

Keywords: leptin, insulin, gills, metabolism, aerobic scope, oxygen, teleost, fish

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****Correspondence:***
Gert Flik
g.flik@science.ru.nl

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INTRODUCTION

Among contemporary vertebrate species, none are as abundant as the teleostean fish. With an estimated number of 33,400 species, fish comprise roughly half of all vertebrates (1). The earliest vertebrates originated approximately 530 million years ago (Mya) in the Panthalassan, Paleo-Thetys, and Iapetus oceans (2, 3). The rise of primitive fishes in the Ordovician was followed by an unparalleled

radiation of the aquatic vertebrates in the Devonian period (the "Age of fish"), and next, ~380 Mya, after the water–land transition, terrestrial tetrapods radiated (4, 5).

Before the teleost–tetrapod split, at least two whole (or large-scale) genome duplication (WGD) events occurred (6). These duplication events have been paramount in expanding the functional gene repertoire of all vertebrates and facilitated functional divergence of genes. The "Ohno-mechanism" (2) states that of a pair of duplicated genes, one can retain its original function while the other one is silenced to become a pseudogene (is no longer expressed) or acquire a new function, and this is called neo- or sub-functionalization (7). These phenomena may also explain why fish lineages escaped from five major mass extinctions (and many smaller ones) that raged on earth and challenged all life forms (8).

Within the class of the Actinopterygii (ray-finned fishes), a third genome duplication occurred around 350 Mya (9); in cyprinid and salmonid lineages, even a fourth major duplication occurred (10, 11). An attractive hypothesis is that the phylogenetic timing of these events suggests that fish-specific genome duplications accommodated the extent of radiation and phenotypic diversification seen in the teleostean lineage (12). However, ecophysiological factors appear to be primary drivers of the rate of salmonid diversification, not the salmonid WGD *per se* (13). Indeed, a more extensive study shows that there is no direct association between the teleostean WGD and the *rate* of diversification in lineages (14). Whatever mechanism prevails, of all extant actinopterygian fishes (except for ~44 basal non-teleostean species), the teleosts (the largest group of bony fishes) comprise, with about 96%, the majority of all fishes (9, 12, 15, 16).

Modern bony fish are found in virtually all aquatic niches imaginable (17) and even in terrestrial environments (lungfishes, mudskippers) and display amazing adaptations in energy homeostasis. Fish have adapted to extremely challenging environments offering them either high tolerance or strong acclimation capacity to unfavorable conditions (18). Indeed, as energy homeostasis requires regulation of the balance between anabolism and catabolism, between energy intake and expenditure, we find adaptations in both. Energy intake essentially equals food intake, expenditure concerns adenosine trisphosphate (ATP)-consuming processes and thermogenesis. Breakdown of energy carriers (sugars, fatty acids, and protein) will free energy that is then temporarily stored in ATP. This molecule is, therefore, called the "currency of energy" (19). Under anaerobic conditions, in a fermentation pathway, glucose breakdown to pyruvate yields only two ATP's, while under aerobic conditions and operationalizing the citric acid cycle a total of up to 38 ATP's are formed per glucose molecule.

In the first part of this review, we discuss key physiological aspects of energy metabolism that determine how energy is budgeted: metabolic strategies, thermal physiology, and aerobic scope are addressed with a special attention to the earliest vertebrates, fish. We (non-exhaustively) look at changes in the regulation of the metabolic demand depending on an organism's thermal physiology. In the second part, we discuss the consequences of these different physiologies for the endocrinology of energy metabolism, with a focus on insulin and, mainly, leptin. These hormones are key in manipulating energy stores, i.e., the regulation of energy intake and expenditure, on the long term.

LIVING IN AN AQUATIC NICHE

The inefficiency of metabolism under hypoxia (a regular phenomenon in water bodies) or anoxia must have favored adaptations toward optimization of branchial oxygen uptake mechanisms, oxygen carrying capacity by adjustment of hematocrit, hemoglobin content or oxygen affinity, or facilitating anaerobic metabolism. Fish, gill-bearing vertebrates, threatened by hypoxia invest in adaptations that boil down to adjustments in oxygen provision and rely secondarily on modifications of metabolic pathways.

When oxygen levels become limiting or 0, fish living in such niches exhibit strong metabolic suppression and use fermentation pathways for ATP production as escape; goldfish (*Carassius auratus*), bitterling (*Rhodeus amarus*), and crucian carp (*Carassius carassius*) avoid lactate accumulation by pyruvate dehydrogenase-mediated production (in their muscle compartment) of acetaldehyde and ethanol, which is excreted *via* the gills. By doing so, potentially lethal lactic acidosis is avoided (20, 21).

Antarctic icefishes (with many representatives in the families of *Nototheniidae* and *Channichthyidae*) live at ambient seawater (SW) temperatures of −1.9°C and below [having evolved antifreeze proteins that prevent ice crystals in their bodily fluids from growing and by doing so, prevent cryodamage (22)] in normoxic seawaters. Although more oxygen dissolves in colder water, the bioavailability of oxygen is much lower (23). This is due to lower O_2-diffusion rates in the cold, reducing an organism's capacity to take up oxygen and ensure an adequate oxygen supply. Icefishes have lost hemoglobin (Hb) (and lack red blood cells) and often also myoglobin (Mb). This remarkable feature is not, as was widely assumed, an adaptation to prevent their blood from becoming too viscous. In fact, in terms of energetic cost, it would be a disadvantageous adaptation, as icefishes pump a far greater blood volume per time unit than "red-blooded" teleosts of equal body mass (24). Instead, the loss of Hb and Mb includes the loss of a primitive function of these proteins: the oxygenation of NO to NO_3^- (25). Indeed, icefishes have high concentrations of circulating NO, which stimulates vasodilation, angiogenesis, and mitochondrial biogenesis. Hence, elevated NO levels might have been the evolutionary driver of unique adaptations in the oxygen delivery system of icefishes [reviewed in Ref. (24)]. The very-low aerobic metabolism of these carnivores depends merely on oxygen diffusion over an enlarged gill surface from water to blood and from blood to tissues from a large vessel bed; a large heart, with cardiocytes and with an extreme mitochondrial density, pumps the blood through the vessel bed that is expanded to compensate for the limited oxygen carrying capacity of the plasma (26).

The scaleless carp (*Gymnocypris przewalskii*) meets extreme conditions, including chronic mild hypoxia, in Lake Qinghai [6 mg O_2 L^{-1}; 9–13 ppt salinity; pH ~9.3 (27)] on the Tibetan Plateau at 3,200 m. When challenged with extreme hypoxia (0.3 mg O_2 L^{-1}), its gills are remodeled by lamellar expansion in a matter of hours to increase (diffusional) oxygen uptake (27).

The inherent consequences for hydromineral disturbances are counteracted by changes in ion channel and aquaporin expression in osmoregulatory organs (28).

The crucian carp survives over six dark winter months of anoxia by strong metabolic suppression. The mechanisms of hypoxia/anoxia-tolerance of this fish are only partly understood; preferentially, the fish stores energy in glycogen, and the glycogen volume determines the duration of hypoxia it can survive. Preceding the switch to anaerobic metabolism (see above), the crucian carp remodels its gills in response to the imminent anoxic conditions. An extensive gill remodeling takes place under control of hypoxia-inducible factor 1α (HIF-1α) (29): the gas exchange surface increases due to a reduction of filamental epithelial thickness resulting in a further protrusion of lamellar epithelium. This comes with a cost as energy consuming osmoregulatory adjustments are required to compensate diffusional flows of Na^+ and Cl^- (reminiscent of the situation in the scaleless carp) over the expanded gill surface. The investment in an expansion of the machinery to obtain oxygen has high priority to keep on fueling aerobic metabolism. Next, anaerobic metabolism offers an escape when oxygen diffusion becomes limiting to fuel the citric acid cycle.

The Magadi tilapia (*Alcolapia grahami*) from Lake Magadi in Kenya is described as the "hottest fish on earth": it thrives in highly alkaline (pH 9.8), hypersaline (880 mOsmol kg^{-1}) waters with temperatures over 40°C (critical temperature: 45.6°C, i.e., above this temperature loss of equilibrium and often death occurs). The concentration of oxygen in SW (at 35‰ salinity, comparable to the water in which this fish lives and 101.1 kPa pressure[1]) will drop from 7.2 mg L^{-1} at 20°C to 5.3 mg L^{-1} at 40°C, and, thus, although a large water body in principle provides an infinite oxygen source for the fish, its uptake mechanisms need adjustment to compensate for this roughly 30% drop in water oxygen content. Indeed, upon comparison with the same species kept at lower temperatures, compensation was seen in a very high mass-specific gill area to facilitate oxygen uptake, a high metabolic rate (of which an estimated 50% seems needed for acid–base regulation), and a high mitochondrial respiration rate. Moreover, because of the basic environment and its consequences for ammonium excretion, this fish is 100% ureotelic, i.e., it does not produce ammonium but the energetically costlier urea as waste (30). The rates of O_2 consumption and swimming performance at 39°C in laboratory setting indicate that this tilapia exhibits the greatest metabolic performance recorded in any fish, in the basal metabolic rate (BMR) range of a similar sized shrew (31).

PHYSIOLOGY

Metabolic Rates

Metabolism (*sensu lato*) can be defined as "the complex of physical and chemical processes involved in the maintenance of life" (32). Metabolic rate is a measure for the amount of energy used per unit of time by an organism, generally assessed as rate of oxygen consumed per hour [e.g., O_2 consumption in millimoles per hour (33, 34)]. In fish and other bradymetabolic animals (see Data Sheet S1 in Supplementary Material), and these are mostly ectotherms, the standard metabolic rate (SMR) and maximum metabolic rate (MMR) are generally used as ratios to define the "scope for activity" (35). SMR refers to the minimal rate of energy expenditure in an organism at rest. Basically, this equals the BMR in the tachymetabolic birds and mammals. However, SMR can be influenced by ambient temperature (36). MMR indicates the highest possible rate of energy expenditure, e.g., during sustained and aerobic maximal activity. In between these states, the routine metabolism is defined as energy expenditure during spontaneous activity in common life processes (35). Note that only the aerobic metabolism is taken into account when metabolic rate is measured *via* oxygen consumption.

Ectothermy Meets Endothermy

The majority of animal life forms are ectothermic (see Data Sheet S1 in Supplementary Material for discussion on terminology). The environment determines the body temperature of ectotherms for the major part and, with that, the pace of biochemical reactions and rates of physiological processes. If we go back to the Magadi tilapia, we have seen, to the best of our knowledge, the upper limit of metabolic performance of an aquatic ectotherm. Note that the maximum performance of this fish comes to 35% of that of a similar sized mammal (31). After the water–land transition of vertebrates (~390–360 Mya) and the evolution of endothermy, new niches of the terrestrial environment became available. True endothermy (by this we do not mean regional endothermy, as we will discuss below) evolved independently in two different clades: (i) the diapsid clade that gave rise to extant birds and (ii) the synapsid clade that gave rise to the contemporary mammals (37). Animals that invest in endothermy (birds, mammals) can stay active independent of meteorological conditions, but do so at phenomenal cost [i.e., it requires lots of fuel (38)].

One could argue that ectotherms function at the mercy of ambient temperature; however, they are not passive and have the potential to regulate their body temperature. By shifting thermal preference and actively migrating toward warmer or colder environments, ectotherms are capable of sophisticated and adaptive behavioral thermoregulation (39, 40). To cope with challenges like infections or stressors (41–43), some fish migrate to warmer water, i.e., behavioral fever, a process that should be regarded as an intrinsic feature of ectothermic physiology.

Indeed, ectothermy comes with many benefits: it is energetically more economical, since the energy demand per unit mass, compared to an endothermic animal of the same size, is four to five times lower (44). Therefore, ectotherms require less time for foraging, and by doing so reduce their energy demand even more as a result of less locomotor activity. Moreover, as ectotherms need not invest in maintenance of a relatively high and constant body temperature, they can allocate more energy to growth and reproduction (44).

Here, we seem to reach a paradox, as endothermy also has advantages over ectothermy. Besides relative independence from ambient temperatures, energy metabolism is significantly enhanced in a warm body, which allows for high and fast

[1] http://www.engineeringtoolbox.com/oxygen-solubility-water-d_841.html.

muscular activity, fast metabolism, and growth during circadian and seasonal fluctuations. Notably, locomotion requires much more energy in terrestrial than in aqueous environments [the energetic cost for transport is about 10 times higher in terrestrial, running or walking, vertebrates than in equal-sized swimmers (45)], which might have been an important determinant in the evolution of endothermy. In addition, endothermy provides a stable thermal environment, securing optimal enzyme activities (46) and facilitating the ultimate parental care seen in mammals (47, 48).

What drove the evolution of endothermy? Three major hypotheses addressed this question. Crompton et al. (49) proposed that endothermy evolved in a two-step process: first, a more or less constant body temperature was acquired; next, the body temperature and metabolic rate increased. McNab (50) argued that early reptiles with a large body mass were *de facto* inertial homeotherms. Following an evolution of fur and decreasing body size, with a modest increase in mass-specific metabolic rate, inertial homeothermy became true endothermy. The most widely accepted theory, however, was put forward by Bennett and Ruben (38), who argued that higher body temperatures and endothermy have evolved secondarily to the selection on enhanced maximal aerobic capacity. This is known as the aerobic scope hypothesis. The evolution of endothermy is maybe the single most debated topic in comparative biology. The proposed hypotheses are not mutually exclusive. For this review, we focus on the aerobic scope hypothesis, as it conveniently joins aspects of metabolic demand and energy balance.

Bennett and Ruben (38) showed that the ratio between resting and maximal metabolic rates in vertebrate ectotherms and endotherms is roughly the same [on average 10 (51)], although the resting metabolic rate in endotherms is around 10 times higher than in ectotherms of similar body mass, i.e., the factorial aerobic scope is comparable for ectotherms and endotherms, but the absolute aerobic scope (the difference between resting and maximal metabolic rates) is much greater in endotherms. As a result, endotherms have, in general, more energy available for processes other than resting metabolism. The aerobic scope hypothesis holds aerobic metabolism at its center, which is important in sustained muscular activity. Still, a reptile can outrun a mammal, achieving great muscular power through anaerobic metabolism (52). This burst activity is time limited, as lactic acid builds up; some lizards and amphibians are highly tolerant to these metabolic waste products (53, 54).

Clarke and Pörtner (37) modified the original aerobic scope hypothesis: they regard a higher body temperature as the mechanism by which a greater aerobic scope was achieved, rather than as a consequence. They point out that an endotherm is not merely a warm ectotherm, since several processes are linked to the evolution of endothermy, such as the modification of mitochondrial membranes and heat retention (insulation by feathers, hair, and fat) mechanisms. In their view, the evolution of endothermy is considered a gradual process in which the driving force was selection for increased aerobic scope (37).

The magnitude of aerobic scope is greatly influenced by temperature. An optimal aerobic scope, associated with a species' thermal niche specialization, is of vital importance for their performance and fitness [reviewed by Pörtner et al. (55)]. The first indication for thermal intolerance in ectotherms is a decreased aerobic scope due to a mismatch in oxygen supply and demand in tissues (56). Even before critical temperatures (here defined by the switch from aerobic to anaerobic metabolism) are reached, failure of circulatory and ventilatory systems occurs, which affects all higher functions (e.g., locomotor activity, behavior, growth, and reproduction) (57). Hence, even slight decreases in aerobic scope can result in lowered performance and enhanced mortality. The mechanisms that are crucial for aerobic scope and related to thermal intolerance are best studied in cold-adapted eurytherm and stenotherm fishes. These special adaptations allow them to withstand seasonal and permanent cold, respectively (58).

Eurythermal fishes are those that tolerate a wider thermal range than stenothermal fishes, and this relates to their ability to increase mitochondrial density or capacity in the cold to prevent hypoxia in tissues (58, 59). This is energetically costly, since they need to upregulate their SMR for mitochondrial maintenance. Stenotherm fishes have a permanently low SMR, associated with specialized cold-adapted mitochondria. These are found in extremely high densities in aerobic tissues, but do not increase overall aerobic capacity (60). Sidell (61) explained that the high density of enlarged mitochondria, seen in the icefishes lacking Hb and Mb, forms an interwoven membrane network, acting as a "lipid highway" for oxygen delivery. Selection for energy savings may have narrowed the thermal window for stenotherm fishes to survive with minimal aerobic capacity and energy expenditure, but allows them to preserve metabolic energy for processes like growth and reproduction, which are temporarily suspended in cold-acclimatized eurytherm fishes (55, 58).

Where is the heat coming from in an endotherm? During generation of ATP in the mitochondria, highly energetic electrons pass through the electron transfer chain and protons are pumped from the mitochondrial matrix into the intramembranous space. Following build-up of that gradient, protons flow back over the mitochondrial inner membrane and drive an H^+-ATP synthase. As membranes are somewhat leaky for protons, some protons passively diffuse over the membrane and, without being used for ATP synthesis, emit energy as heat. Controlled uncoupling *via* the mitochondrial uncoupling proteins (UCPs), of the proton flux from ATP generation was thought to be the main source of heat in endotherms (62). However, more recent research shows that UCPs are not the exclusive actors in proton conductance, as the observed proton leak contributes proportionally and equally to the SMR in ectotherms and endotherms (63).

In mammals, five UCPs are present. In mammals UCP1 is responsible for non-shivering thermogenesis, whereas UCP2 and UCP3 are thought to be involved in maintaining the resting metabolism. In fish, we find orthologs of UCP1, UCP2, and UCP3, illustrating that the presence of UCPs is not restricted to endothermic animals (64, 65). In common carp (*Cyprinus carpio*), hepatic *ucp1* transcript abundance was significantly downregulated in response to cold (opposing the function of UCP1 in mammals). Note that the fish liver is a primary storage site of energy. In red muscles, *ucp3* is predominantly expressed and increases up to fivefold in response to fasting (65). As fasting leads to high lipid oxidation and reactive oxygen species, UCP3

is suggested to protect mitochondria and cells from damage *via* mild uncoupling activity or fatty acid anion export (66–68). The omnipresent UCP2 may play a similar scavenging role. Certainly, UCPs are evolutionary much older than originally thought; they were already present in fish ~420 Mya (65).

Although most fishes are strictly ectothermic, there are (of course) also fish that attain a regional endothermy in important organs and tissues, such as muscles, eyes, visceral organs, and the brain (69). We follow the nomenclature proposed by Clarke and Pörtner (37) (see Data Sheet S1 in Supplementary Material), and name this process heterothermy. An exceptional group of teleosts capable of heterothermy are the *Thunnini* (tuna).

Tuna are obligate ram ventilators and they are not sufficiently buoyant, which means they have to swim continuously to maintain a constant water flow over their gills (to fuel their relatively high metabolic activity) and to prevent sinking (70). Tuna have a specialized red muscle (RM) that is constantly metabolically active to power this so-called cruise swimming. Since the byproduct of all metabolic processes is heat, tunas have developed a way to retain the heat that is generated by RM and use it to elevate their body temperature regionally (69).

In tuna, the circulation to and from RM is structurally arranged in so-called *retia mirabilia* (literally: wonderful nets) (71). In these parallel to one another organized vascular bundles with very thin vessel walls, arterial and venous vessels lay side by side with the blood flowing in opposite directions, so that they function as countercurrent heat exchangers. Hence, a stable thermal gradient is established, which warms the cool arterial blood coming from the gills. The heat conserved is used to warm other body parts (72). When tuna dive during predation from pelagic zones with high temperature (e.g., 20°C) to more benthic zones where temperatures may drop to around 4°C, the biochemistry of brains, eyes, and swimming muscles is guaranteed of a rather constant temperature.

Considering that the SMR of tuna is 2–10 times higher than in most other fish species of comparable size and activity (69), this demonstrates that metabolism, and more specifically metabolic rate, is at the basis of thermal regulation in both ecto- and endotherms. The production of heat and having heat-retaining mechanisms are equally important. In general, maintaining the temperature of (part of) the body above the ambient temperature, is energetically costlier than keeping the body at lower temperatures, due to higher standard or basal metabolic rates. However, it also results in enhanced ATP production, which in turn allows for higher locomotor activity, growth rate and, completing the circle, metabolic rate. To understand causality, one needs to take a closer look at the regulation of energy intake and expenditure, and a comparative endocrine view may facilitate this, as we will do in the next section. Yet, it is clear why temperature is regarded as the abiotic master factor (73).

ENDOCRINOLOGY

Regulation of Energy Balance

Metabolism, thermoregulation, and aerobic performance are intertwined and have one nexus in common: they are all dependent on food intake, food being the source of chemical energy for these related processes. In general, and despite mismatches in energy intake and expenditure on the short term, on the long term, they are carefully balanced and regulated by several endocrine systems, which together guarantee energy homeostasis (74).

A plethora of research on mammals is available in this field, not in the least to gain a better understanding of disturbances in energy homeostasis, which are pivotal for the current obesity pandemic (75, 76). In a healthy animal, the control system that matches energy intake to energy expenditure is remarkably accurate. A striking example is put forward by Seeley and Woods (75), who calculated that it takes only a mismatch of 46 kJ per day (0.55% of the daily energy intake!) for an adult human male to gain a pound in a year. Such tight regulation of energy balance can only be achieved by an accurate integration of signal molecules from stored and currently available fuel, in accordance with internal set points reflecting energy availability.

Where these signals come from and how they contribute to the regulation of food intake is of ongoing research interest. It has long been recognized that specific hypothalamic nuclei are crucial in monitoring energy balance (77), and these seem to be mainly tuned by signals regarding the storage of fat (i.e., lipostatic regulation). Another theory about hypothalamic tuning of energy balance through signaling of carbohydrate storage [i.e., glucostatic regulation (78)] has largely been abandoned in the past decades, as it has become clear that neurons are well protected from fluctuating glucose levels and usually feeding takes place when plasma glucose levels are within normal physiological values (75).

The link between body-fat stores (adiposity) and food intake was formalized by Kennedy (79), when he postulated that signals distributed in proportion to the total amount of body fat influence the control of food intake by the brain. This lipostatic regulation is based on a negative feedback system involving communication on the total amount of fat by adiposity signals and the central nervous system (CNS) (80).

Since adipose tissue is poorly innervated by the peripheral nervous system, research has been directed mainly to humoral signals. Adiposity signals (see below) should fulfill three criteria, *viz*.,: they should circulate in proportion to the total body fat, they should reach specific nuclei within the CNS [i.e., cross the blood–brain barrier (BBB)], and they should produce predictable changes in energy balance by altering food intake and energy expenditure dose-dependently (74).

Insulin and Leptin: Adiposity Signals in Mammals

In the 1980s, ample evidence identified insulin as a major adiposity signal (81, 82). Porte and Woods (81) showed that intracerebroventricular (icv) infusion of insulin in baboons induced drastic weight loss and decreased food intake dose-dependently. In additional studies, insulin receptors were found in key brain areas involved in control of food intake, including the hypothalamic arcuate nucleus (ARC, see below) (83, 84). Insulin is shuttled over the BBB *via* receptor-mediated transport (85). Insulin, produced by pancreatic β-cells, is best known for its involvement

in carbohydrate metabolism; in response to high plasma glucose levels, insulin enhances the uptake of glucose by adipocytes, liver, and skeletal muscle cells, storing energy as glycogen or triglycerides (86). Insulin circulates in proportion to the amount of adipose tissue, although its levels fluctuate greatly when food is ingested and absorbed, in accordance with its hypoglycemic actions. However, baseline insulin levels and the magnitude of the fluctuations following food intake are directly proportional to body adiposity (87).

A second adiposity signal, leptin, a type-I α-helical cytokine, is encoded by the *obese* (*ob*) gene and was discovered in mice in 1994 (88) and has gained unparalleled momentum in research on energy homeostasis. It is a potent anorexigenic hormone produced in peripheral white fat and meets the aforementioned criteria for an adiposity signal. In addition, receptors for leptin are present in brain areas involved in the regulation of food intake, especially in the ARC (75).

Two subpopulations of neurons in the ARC integrate peripheral signals regarding energy homeostasis: the "anabolic" population expresses neuropeptide Y and agouti-related peptide, is orexigenic, and inhibited by leptin (89), whereas the "catabolic" population expresses pro-opiomelanocortin and cocaine and amphetamine regulated transcript, is anorexigenic, and stimulated by leptin (90). In line with this model, leptin administration in rodents was shown to decrease body mass dose dependently (91, 92). During fasting, plasma leptin levels fall dramatically and over prolonged periods of food deprivation, changes in the activities of the gonadal, adrenal, and thyroid axes are observed (93). Postprandially, leptin does not increase significantly, nor does it lead to termination of a meal by itself, indicating that leptin is largely involved in long-term regulation of feeding behavior and energy balance (94).

Leptin administration is an effective treatment for obesity in mice and humans with genetic leptin deficiency (95–97). However, in most cases of obesity, circulating leptin levels are already high and leptin therapy is not effective. Indeed, leptin resistance and the consequent lack of anorexic signaling in the ARC is commonly associated with obesity (98). Interestingly, leptin resistance in the ARC does not cause obesity, but it contributes to its persistence, as it develops secondarily after adiposity and body mass increase (99). More recent research revealed that vagal afferent neurons (VAN), apart from their role in meal termination *via* short-term gut–brain signaling, are involved in the long-term regulation of food intake (100–102). The authors showed that leptin resistance developed in VAN, before hypothalamic leptin signaling became disturbed (100). Moreover, when the leptin receptor is knocked out in VAN specifically, mice displayed increased food intake, body mass, and adiposity, indicating that the absence of leptin signaling in VAN is an important factor in the onset of hyperphagia and obesity (101).

Both adiposity signals (insulin and leptin) exert their function centrally through signaling *via* the ARC neurons, which results in reduced food intake and increased energy expenditure (103, 104). Arguably, leptin, for a while thought to be the panacea to beat obesity (105), received major attention in obesity research, whereas the role of insulin was less appreciated. This might be due to the opposing actions of insulin in peripheral tissues and the CNS; its hypoglycemic and anabolic function (energy storage) peripherally; and its catabolic effect (steering energy expenditure) in the hypothalamus (106). In the periphery, however, obesity eventually leads to inflammation of adipose tissue, and the following interplay between leptin signaling and the inflammatory cytokines secreted by macrophages seems to contribute to insulin resistance (107).

The complementary, and sometimes redundant, roles of leptin and insulin in the central regulation of energy homeostasis are best illustrated in a condition known as diabetic hyperphagia. When pancreatic β-cells become non-functional, complete depletion of insulin leads to strong hyperglycemia and an inability to store energy peripherally, with considerable weight loss as a result. However, without the catabolic insulin action in the hypothalamus and the co-occurring decrease in plasma leptin levels, increased compensatory food intake is initiated, counteracting energy wasting and preventing more rapid weight loss (106). Administration of either leptin or insulin to the CNS in rats with streptozotocin-induced diabetes blocks this compensatory hyperphagia almost completely (108, 109).

In fact, one can state that leptin and insulin are equally important adiposity signals involved in a negative feedback loop, as has been shown in many studies before (**Figure 1**) (75, 103, 110, 111). We focus on leptin mainly in this review, because leptin received the lion's share of attention in recent research; however, we recognize the significance of the undervalued role of insulin. As Seeley and Woods (75) point out, insulin provides the brain with information not only about fat storage (long-term energy) but also about glucose availability (short-term energy). From this perspective, although indirectly, glucostatic regulation of food intake is indeed taking place. Thus, insulin signaling to the brain could provide the link that integrates glucostatic and lipostatic peripheral signals, allowing for a precise monitoring and accurate regulation of energy balance.

Insulin and Leptin: Adiposity Signals in Fish?

The insulin signaling pathway and its role in energy metabolism is evolutionary conserved and serves fundamentally the same physiological functions from invertebrates to mammals; it is found in phylogenetically distant invertebrate species, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, where it is involved in energy storage (106, 112–114). Interestingly, these animals express homologs of key players in the insulin signal transduction pathway, but do not seem to actively regulate carbohydrate fluxes. They do regulate fat stores: insulin appears to limit energy storage in *C. elegans* (mutants for insulin signal transduction had increased fat deposits), which indicates a catabolic function of this protein in early evolution (106).

In elasmobranchs (mostly carnivores), protein and lipid metabolism are the primary energy sources (115), and although infusion of mammalian insulin resulted in severe prolonged hypoglycemia in spiny dogfish (*Squalus acanthias*) (over 15 times lower than time-zero levels), these fish did not exhibit any symptoms of illness (116). Apparently, strict glucose regulation is not as vital in these animals as it is in later vertebrates. The structure

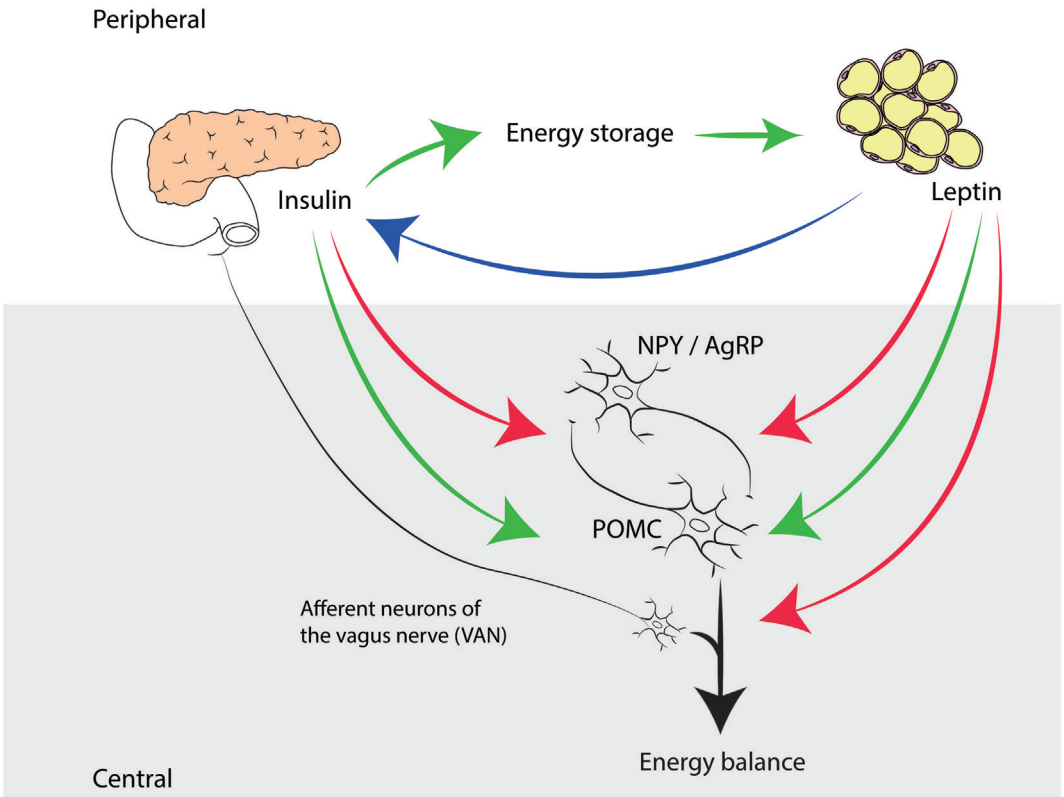

FIGURE 1 | **Complementary roles of insulin and leptin in energy homeostasis in mammals**. Insulin, produced by pancreatic β-cells, has an anabolic effect in peripheral tissues by promoting glucose storage, which has a positive effect on body weight and adipose tissue. Leptin, produced by adipocytes, signals to the hypothalamic arcuate nucleus, stimulating POMC expressing neurons (enhancing energy expenditure) and inhibiting NPY/AgRP expressing neurons (promoting food intake). In addition, leptin signals to VAN, which, according to recent insights, directly influences energy balance. Insulin also has a catabolic effect on the CNS, by exerting the same functions as leptin in the ARC. Note that insulin levels are proportional to adiposity (indicated by a blue arrow).

of insulin and its receptor is also highly conserved among vertebrates (117). Fish insulins, as their mammalian orthologs, consist of an α- and β-chain, linked by disulfide bridges (118); when we align the zebrafish an human insulin genes, we find a sequence of 60%.

As in mammals, it is important to distinguish between peripheral and central functions of insulin. The role of insulin in the periphery as an anabolic hormone has gained more attention, as it was long, but erroneously thought that fish were glucose intolerant [reviewed in Ref. (117)]. He argues that metabolic rate is a factor commonly overlooked in the studies performed on glucose clearance in fish. Acknowledging the fact that the metabolic rate of fish is about 10 times lower than in mammals, it may not come as a surprise that glucose turnover is rather slow (119). For several fish, it was shown that amino acids are a more potent stimulator of insulin secretion than glucose (120, 121), and fish are in general much slower in clearing a glucose load, compared to mammals (117). Notwithstanding, hyperglycemia does induce hyperinsulinemia *in vivo* (122–124). Significant differences between species exist: in the herbivorous carp glucose is more potent in stimulating insulin secretion than it is in carnivorous species, such as trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) (125).

Contrasting results have been found in studies that address the central role of insulin. Fish brains are likely insulin-sensitive organs, as they contain insulin receptors (126–128). In channel catfish (*Ictalurus punctatus*), 24 h of icv injection of insulin appeared to have no effect on food intake (128). In contrast, icv administration of insulin in rainbow trout elicited a reduction of food intake after 26 h (129). Moreover, it has been shown in rainbow trout that insulin levels drop dramatically after 6 weeks of fasting, a response also seen in mammals (130). These conflicting outcomes could be the result of the longer administration time in the trout study or, more likely, central insulin effects could be species dependent. Progressive insight suggests that fish, like mammals, have specialized neurons involved in glucose sensing, which modulate the hypothalamic neurocircuitry controlling food intake (131).

Whether insulin has similar functions in fish as in mammals in communicating adiposity status to the CNS is not evident. In studies on rainbow trout, insulin stimulates lipogenesis in the liver and, more recently, in white mesenteric adipose tissue (132, 133). Traditionally, researchers considered the fish liver to be the main lipogenic tissue (134). However, Polakof et al. (133) showed that in rainbow trout fed a carbohydrate-rich diet and provided with

insulin, lipogenesis in adipose tissue was significantly enhanced. The researchers hypothesized that the increased lipogenic capacity helps to regulate glycemia when excess carbohydrates need to be processed. In addition, these results are indicative for the utilization of lipids, stored in white adipose tissue, as an energy source. In juvenile chinook salmon, circulating insulin levels increased with a high-fat diet, and body adiposity clearly reduced food intake compared to fish that were fed a low-fat diet (135). However, no direct association between insulin levels and adiposity was found, but insulin levels correlated with dietary fat content.

More than a decade after the breakthrough identification of the mammalian *ob* gene and its protein product leptin (1994), the first non-mammalian ortholog was characterized in a teleostean fish [common carp (136, 137)] and many other fish species followed, including zebrafish (*Danio rerio*) (138), Atlantic salmon (*Salmo salar*) (139), and tiger pufferfish (*Takifugu rubripes*) (140). More recently, the first non-vertebrate functional leptin homolog was identified in *Drosophila* (141). The *Drosophila* cytokine Unpaired 2 has structural and functional similarities with mammalian leptin, is secreted by the fat body and signals *via* a similar intracellular messenger signaling pathway to reduce growth and alter energy metabolism. The canonical paper by Zhang et al. (88) addressed the *ob* gene as "evolutionary conserved" after demonstrating its presence in evolutionary distant species like *Drosophila* and eel (*Anguilla rostrata* LeSueur). However, once fish leptin was cloned and sequenced in 2004 (Huising et al.; accession numbers AJ868357 and AJ868356), the primary sequence identity between fish leptins and mammalian leptins turned out to be less than 25% (136), at variance with the earlier proposed "evolutionary conservation." However, conserved gene structure, phylogenetic analyses, the conservation of essential cysteine residues, and tertiary structure, as well as synteny, firmly demonstrated true orthology of teleostean leptins (136, 138, 140). This indicates that if one wants to study the original function(s) of leptin(s), fish should be addressed.

The organization of hypothalamic nuclei involved in energy metabolism and food intake is conserved throughout the vertebrate lineage (142, 143), yet reports on leptin's physiological role(s) have not been consistent for different fish species, which may not be surprising considering the vast number of species and niche adaptations. Most studies, however, recognize the organs and tissues with a high-fat content (liver or muscle) as the main expression sites for leptin in fish, not adipose tissue like in mammals (136, 138–140, 144, 145). In contrast with mammals, the liver is one of the major energy depots (glycogen and fat) in fish, together with muscle tissue and mesenteric fat (146). Moreover, lipid catabolism is the main source of energy in many fish species (147). Next to being the primary energy storage site, hypodermic fat in endotherms serves a second function, *viz.* insulation (148). No such insulation is required in an ectotherm, which then may explain differential leptin functioning (see below).

In some fish, leptin inhibits food intake through an interaction with hypothalamic orexigenic and anorexigenic genes, as it does in mammals. For example, in goldfish, administration of heterologous (murine) leptin caused a decrease in food intake, with lower doses needed when applied centrally than when applied peripherally to exert the same effect (149). In rainbow trout, recombinant homologous leptin injected intraperitoneally (144) and recombinant human leptin, administered icv, reduces food intake (150).

In common carp, two *leptin* paralogs were characterized initially, designated *leptin-I* and *leptin-II*, which, given their similarity, are probably the result of the tetraploidization following the "recent" cyprinid WGD ~16 Mya (11, 136, 138). These were later renamed *leptin-a* paralogs. The common carp *leptin-a* paralog did not respond to 6 weeks of fasting, nor to feeding to satiation, which feeding regime caused the experimental fish to grow twice as fast as controls. Only transient postprandial increase and decrease were observed in hepatic *leptin-a-II* and *leptin-a-I* mRNA, respectively (136).

Reduction of food intake is an early response of fish when exposed to stress in general (18) and hypoxia in particular (151, 152); in mammals, leptin levels are known to increase in hypoxic conditions (153). Indeed, the mammalian *ob* gene contains hypoxia response element (HRE) sites which can be bound by HIF-1α, a transcription factor that regulates the expression of hypoxia-sensitive genes (153–155). Analyses of the promotor region of zebrafish *leptin* and the *leptin receptor* (*lepr*) revealed putative HRE in both genes (156). Therefore, a follow-up study with common carp was done to elucidate the relation between leptin, hypoxia, and the hypothalamic regulation of food intake. It was shown that hepatic *leptin-a-I*, *leptin-a-II*, and *lepr* expression in common carp is indeed stimulated in hypoxic conditions, which is congruent with a reduction in food intake (156). This fits with a well-known strategy among fishes to deal with hypoxia (152, 157, 158), since appetite suppression leads to precious energy and oxygen savings by reducing the cost of specific dynamic action (i.e., the metabolic energy cost of digestion) (159). These results were subsequently confirmed by a study in zebrafish, in which chronic hypoxia and HIF-1α induced a rise in hepatic *leptin* mRNA levels (160).

The Bernier laboratory (2012) compared hypothalamic gene expression between hypoxic and fasted (pair-fed to the hypoxic groups) carp, which led to the suggestion that during hypoxia, leptin counteracts the suppression of *pomc* and upregulation of *agrp*, characteristic for fasted carp (136), since *agrp* levels were not affected in hypoxia and the suppression of *pomc* was attenuated (156). These data provide grounds for the involvement of leptin in re-establishing energy balance during chronic hypoxia and indicate a broader physiological role for leptin beyond the signaling of nutrient status.

LEPTIN: STATE OF THE ART

More than 20 years after the discovery of leptin, a picture emerges of a pleiotropic cytokine, apart from its well-known roles in regulation of appetite and energy balance in mammals, which relates energy status to adaptive responses of multiple physiological systems within vertebrates. In a comprehensive review on mammalian leptin physiology, Friedman (161) elaborates on leptin involvement in the entire neuro-endocrine axis: he points out that already in 1991, Bray noted that *ob/ob* mice are infertile, euthyroid sick, hypothermic, and diabetic (162). In addition, *ob/*

ob mice have increased corticosterone levels and immunological and hematological abnormalities. Most of these abnormalities are linked with starvation, not with obesity (163); the lack of leptin signaling to inform the brain that adequate fat stores are present, elicits physiological responses that reduce energy expenditure and stimulate appetite, similar to when the organism is starving (161).

In fish, leptin functions appear to share similarities, but also differences with their mammalian orthologs. Leptin and its receptor have now been cloned for all major vertebrate classes, including the notoriously elusive leptin in birds (164–166). The aim of this section is to provide a concise overview of the functional divergence and evolution of the leptin system, and to gain insights in recently discovered leptin functions in fish.

Evolution of Leptin and Leptin Receptor Genes

After the initial cloning of leptin in carp, multiple *leptin* paralogs were found in zebrafish (138) and in the Japanese ricefish [*Oryzias latipes*, aka medaka (145)] and named *leptin-a* and *leptin-b*. Indeed, also in common carp a *leptin-b* paralog was found (167) and the first characterized carp leptins were renamed *leptin-a-I* and *leptin-a-II*. The *leptin-a* and *leptin-b* paralogs most likely find their origin in the third, fish-specific, genome duplication, as they are found in evolutionary distant species, such as zebrafish (*Cypriniformes*) and medaka (*Beloniformes*), that shared their last common ancestor ~296 Mya (168).

However, reflecting the diversity among teleostean fishes, leptin phylogeny appears to be even more complex (**Figure 2**). Also in the genome of salmonids, four leptin paralogs (*leptin-aI/II* and *leptin-bI/II*) are present. These paralogs originated as a result of the salmonid WGD 88–103 Mya (13, 139, 169). In more modern fish, e.g., the Tiger puffer (Tetraodon family), only a single *leptin* gene is found, which suggests that this lineage experienced genome reduction, after the split from the *Beloniformes* ~186 Mya (170).

As some of the leptin paralogs share very low primary amino acid sequence identity even within a species [e.g., zebrafish leptin-a and leptin-b amino acid identity is only 24% (138)], it is likely that these leptin genes acquired different functionality (neofunctionalization or sub-functionalization) (165). Testimony to this reasoning is that *leptin-a* and *leptin-b* are expressed differentially (at least spatially); *leptin-a* is mainly expressed in the liver (138, 139), while the ovary is the main expression site for *leptin-b* (138). In addition, calculations on binding energy suggest that in both medaka and zebrafish, leptin-a has a higher binding energy for the leptin receptor than leptin-b (172).

Surprisingly, in all currently available teleostean genomes only one *lepr* gene is found (165). In salmonids [Atlantic salmon; Rønnestad et al. (139), and rainbow trout; Gong et al. (173)], several splice variants have been identified, but, as in the mammalian situation, only one of these variants contains the full length sequence required for intracellular signal transduction. In Atlantic salmon, four truncated and one full length leptin receptor variants were identified, the latter of which was ubiquitously expressed, including in gills, gonads, and brain (139), whereas in rainbow trout at least three splice variants gave rise to functional circulating leptin binding proteins (LepBP1, LepBP2, and LepBP3) (173). Together, these findings suggest a complex mode of leptin functioning in teleostean fish, with both inter- and intraspecies differences in the interaction of leptin (paralogs) with the leptin receptor, and in the modulation of endocrine and/or paracrine signaling pathways.

Recent Discoveries on Leptin Physiology

In the past 5 years, research on leptin functioning in teleostean fish has been carried out not only in the field of feeding and appetite regulation (156, 174–177) but has extended to other endocrine regulatory systems, including sexual maturation (169, 178), energy expenditure and metabolic rate (179, 180), osmotic adaptation (181, 182), and glucose homeostasis (183), all of which we will discuss below. In addition, the involvement of leptin in reproduction (184), the growth hormone (GH)–insulin-like growth factor axis [GH–IGF axis (185)], hypothalamic neurotensin networks (186), and the immune system (187) has been studied.

Feeding and Appetite Regulation

The early observations by Huising et al. (136, 137) that *leptin-a* in common carp is regulated by feed intake postprandially are strengthened by a study by Klaren et al. (174). Juvenile common carp were either fed once daily, or demand fed (*via* operating a pendulum connected to an automatic feed dispenser). An interaction effect between time of day and feeding regime was observed for hepatic *leptin-a* transcript abundance (174), indicating an effect of short-term feeding status on *leptin* expression.

Hypoxia-induced elevation of *leptin* mRNA levels, congruent with a reduction in food intake, has been demonstrated in carp (156). MacDonald et al. (176) investigated the effect of hypoxemia on hepatic leptin expression, plasma leptin levels, and food intake in rainbow trout. To establish the hypoxic state, fish were infected with a pathogenic hemoflagellate, *Cryptobia salmositica*, which was followed by an immediate drop in hematocrit. After 14 weeks, infected fish had consumed 75% less food compared to controls, an observation accompanied by an initial, transient, 17-fold increase in hepatic *leptin-a1* expression, and a sustained increase in plasma *leptin-a1* levels. Non-infected fish, pair-fed to the infected group, did not exhibit differences in liver *leptin-a1* expression and plasma leptin compared to non-infected fish fed to satiety. Thus, *leptin-a1* expression appears to be stimulated by hypoxemia, not feed restriction (176), consistent with the results obtained in carp.

Accordingly, no role for leptin in the regulation of food intake in rainbow trout was observed by Jørgensen et al. (177). Plasma leptin levels did not change upon fasting for 4 months, compared to controls, nor after consumption of a large meal. Levels of *leptin-a1* mRNA increased in the belly flap of the fasted fish and stayed high, even after re-feeding, which suggests a tissue-specific role of leptin in long-term fasting. Transcript levels of *pomc* were elevated in fasted fish, possibly to serve as a satiety signal to reduce energy expenditure when food is scarce. However, no causative role in appetite regulation was found for leptin and hypothalamic neuropeptides (177).

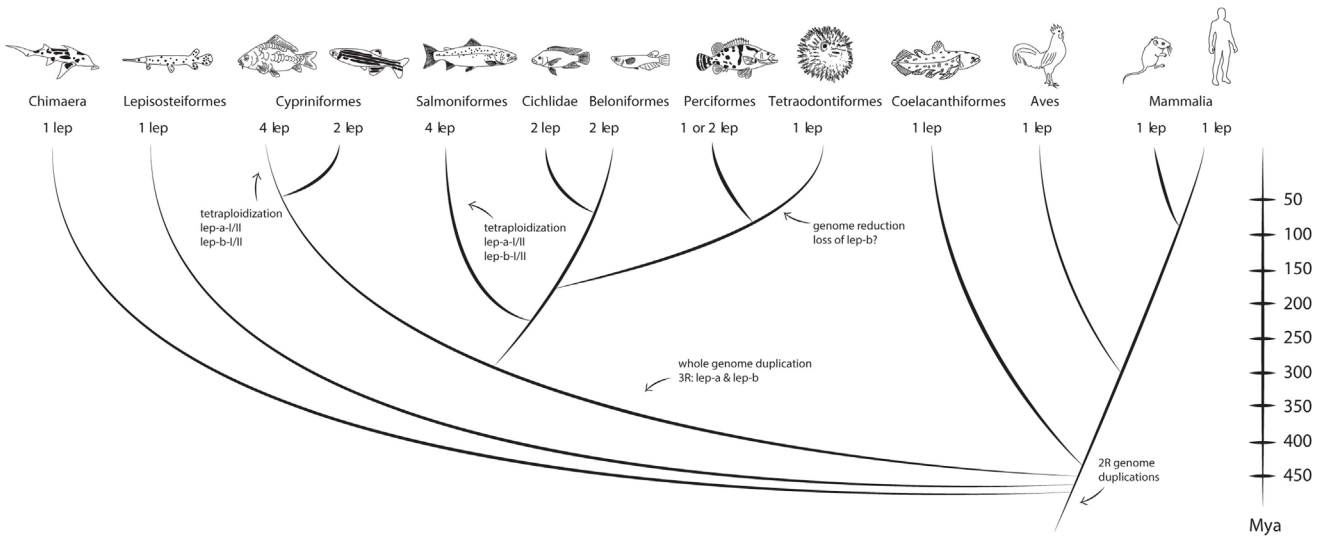

FIGURE 2 | **Vertebrate leptin phylogeny**. Fish and mammals share a common ancestor that lived ~450 Mya. Depending on whole genome duplications one, two, or even four leptin paralogs are found in a species. Recently, the chicken leptin ortholog was found (171). Divergence times come from Near et al. (170). Figure adapted from Gorissen and Flik (165).

To study leptin functioning in medaka, Chisada et al. (175) generated the first fish model deficient of a functional leptin receptor, by inducing a homozygous mutation in the *lepr* gene. Loss of function of the leptin receptor was suggested by disrupted leptin signaling: the expression of appetite-regulating genes differed between mutant and wild-type fish. Independently of feeding, the mutants exhibited constant and upregulated mRNA levels of orexigenic *npya* and *agrp*, but diencephalic *pomc1* expression was downregulated. The post-juvenile and adult mutants displayed hyperphagia, resulting in a high growth rate in the post-juvenile stage, but not in an altered final body size compared to wild-types. In addition, mutants had large deposits of visceral fat, whereas wild-type fish had none (175). These results suggest a stage-specific influence for leptin in food intake, growth, and fat allocation in medaka.

We see that leptin serves different functions with respect to feeding and metabolism among teleosts. Possibly, this is due to differences between species in lipid metabolism and energy storage sites (188).

Sexual Maturation

In fish, reproduction is dependent on a healthy energy status. Recently, the focus has been on the role for leptin in sexual maturation in Atlantic salmon. A possible role for leptin in the sexual maturation of male parr (freshwater, FW, stage) has been proposed by Angotzi et al. (169), since they observed higher hepatic *leptin-a1* mRNA levels during mid-spermatogenesis compared to immature fish. However, plasma leptin levels did not differ, so the physiological relevance of these findings is not clear (169).

A seasonal study on the link between leptin and energy balance during sexual maturation of the same species revealed that hepatic *leptin* expression was upregulated during mid-spermatogenesis with a 7.7-fold increase of *leptin-a1* and a 49-fold increase of *leptin-a2* during final maturation (178). For the first time in fish, an upregulation of *lepr* mRNA was observed in the testis from mid- to late spermatogenesis. In non-maturing control fish, the hepatic expression of *leptin-a1* and *leptin-a2* and brain *lepr* was downregulated in early spring, coinciding with the start of growth and fat accumulation. The incidence of sexual maturation was also assessed in a feed-restricted group (fed a low-fat diet at a 50% ration of the control group). This resulted in a 53% decrease in sexual maturation incidence and a major upregulation of both liver *leptins* and pituitary *lepr*. It appears that hepatic *leptin* expression and *lepr* expression in the testis are affected by early sexual maturation in male Atlantic salmon. In addition, the results suggest that leptin does not signal as an adiposity signal in Atlantic salmon, as there is an inverse relationship between fat stores and leptin expression (178). Interestingly, zebrafish lacking either leptin paralog or the leptin receptor show no difference in fecundity compared to wild-type fish (183). Apparently, leptin is involved in sexual maturation, but is not critical for reproductive capability.

Energy Expenditure and Metabolic Rate

Leptin affects metabolic rate in zebrafish embryos (179). Translation of *leptin-a* was inhibited by injection of an antisense morpholino oligonucleotide (MO) in the 1–8 cell stage, and oxygen consumption and total acid production were used as indicators of metabolic rate. Morphants consumed significantly less oxygen until 48 h post-fertilization (hpf) followed by a lower acid production, compared to wild-type controls. Co-injection of recombinant zebrafish leptin-a and antisense MO rescued these effects. In addition, a significant decrease in heart rate was seen in morphants, and developmental abnormalities in, *inter alia*, the

eyes and the inner ear (189). Taken together, these results suggest that, as in mammals, leptin influences metabolic rate in fishes.

Another study in zebrafish embryos showed that leptin, insulin, and α-MSH, increase energy expenditure dose-dependently (180). An assay to measure metabolic rate was developed based on the reduction of non-fluorescent resazurin by NADH2 to fluorescent resorufin (alamarBlue®). To validate the results, a compound known to inhibit hypermetabolic effects of leptin in mice [etomoxir, a carnitine palmitoyl transferase I inhibitor (190)] was tested and shown to block the leptin-induced increase in energy expenditure. These results indicate that leptin's involvement in the endocrine regulation of energy expenditure is conserved in a teleost (180).

SW Adaptation

When fish move between FW and SW, they are challenged by opposite osmotic gradients. Proceeding from a plasma osmolality of 300 mOsmol kg^{-1} this gradient in FW (10 mOsmol kg^{-1}; 20°C) results in an osmotic pressure of 706.8 kPa, while in SW (1,000 mOsmol kg^{-1}; 20°C) this gradient results in an osmotic pressure of −1,706.1 kPa. Two type-I α-helical cytokines, *viz.* prolactin and GH, play a key role in dealing with these gradients, in FW and SW, respectively. Interestingly, in both cases, cortisol contributes in a synergistic way to hyper- and hypo-osmoregulation (191). Thus, in response to SW exposure, a fish needs to adjust its hydromineral balance, an adaptive and energy demanding process that is associated with enhanced glucose (192) and fatty acid utilization (193). Protein utilization, on the other hand, decreases, as amino acids are retained and function as osmolytes, to maintain cell volume (191, 192). Maybe not surprisingly, considering the energetic cost of osmoregulation, also leptin is involved.

Indeed, in the euryhaline Mozambique tilapia (*Oreochromis mossambicus*), during a 72-h SW challenge, plasma glucose levels were significantly elevated (with a maximum at 12 h after transfer), accompanied by a 25-fold increase in hepatic *leptin-a* expression at 4 h, and elevated *lepr* mRNA levels at 12 h, compared to FW controls (181). To test whether leptin stimulates hepatic glycogenolysis, FW tilapia were injected with recombinant, homologous leptin-a, which resulted in a similar increase in plasma glucose levels as observed during the salinity challenge. Liver glycogen levels were significantly depleted, indicating that leptin-a induced hepatic glycogenolysis necessary for glucose mobilization, to meet increased energy demands during hyperosmotic adaptation (181).

Due to the absence of species-specific antisera, no plasma leptin levels were analyzed in the study described above. In their next study, however, the authors developed and validated an assay to measure plasma leptin-a levels in the Mozambique tilapia (182). This study further identified interactions between prolactin, the pituitary hormone key for adaptation to FW (194, 195), and leptin-a in the euryhaline tilapia. Leptin-a appeared to be the dominant paralog in this species (determined by qPCR analysis of tissues) and is primarily produced by the liver. Hypophysectomized tilapia had higher plasma leptin-a, and hepatic *leptin-a* mRNA levels. These effects could be restored to control values by administration of ovine prolactin. As leptin was found to stimulate *prolactin* expression in the pituitary *in vitro* (196), a negative feedback regulatory model for leptin-a and prolactin seems likely: leptin-a stimulates the expression and secretion of pituitary prolactin (both *prl1* and *prl2*), the prolactins in turn inhibit hepatic *leptin-a* expression, which then translates into a decrease in circulating leptin-a levels (182).

Plasma prolactin and pituitary mRNA levels decrease rapidly upon SW exposure (195). Douros et al. (182) presented tilapia with a 24-h SW challenge, which inactivates the pituitary prolactin cells. During SW acclimation, again a major increase in liver *leptin-a* transcript abundance was observed. Therefore, the authors proposed a mechanism in which the sudden decline in prolactin levels alleviates the continuous inhibition of leptin-a. Prolactin may therefore, *via* leptin-a, be a key glucose regulator in the adaptation to SW (182).

Growth hormone serves to control somatic growth both in FW and SW, and prepares the fish for SW entry by increasing ionoregulatory capacity (197); moreover, GH is particularly well known as permissive for SW adaptation (191). Tilapia leptin-a decreases pituitary *gh* mRNA and hypophysectomy increases *leptin-a* expression, which is rescued by GH replacement. Additionally, during fasting leptin-a enhances hepatic *gh receptor 1* & *-2* and *igf1* & *-2*, to prepare the hepatosomatic growth axis in case feeding resumes (198). We now better understand the GH-IGF axis and its control by leptin-a in the euryhaline tilapia, as leptin, the energy signal, directly steers the endocrine growth axis and, together with cortisol, controls the expensive energy expenditure related to SW adaptation.

Both studies by Baltzegar et al. (181) and Douros et al. (198) on hyperosmotic adaptation in tilapia provide original evidence that leptin-a acts as a potent hyperglycemic factor in tilapia, which is functionally distinct from leptin's actions in mammals. It is, therefore, an attractive hypothesis that the functional divergence of the leptin protein among vertebrates reflects fundamental differences in metabolic regulation between ectotherms and endotherms (181). The interplay between these three type-I helical cytokines, *viz.* prolactin, GH, and leptin, once again strengthens the epithet *pleiotropic* of this group of hormones [reviewed in Ref. (137)].

Glucose Homeostasis

In an elegant series of experiments, Michel et al. (183) demonstrated a role for leptin in glucose homeostasis and disproving a role for leptin as an adipostat in zebrafish. To do so, they created a zebrafish with a dysfunctional leptin receptor. These mutant zebrafish did not exhibit increased adiposity or hyperphagia compared to wild-type controls. In addition, no effect of genotype on length or body mass was found in different life stages and fertility appeared to be normal in these mutants (183).

Given the profound diabetes observed in leptin receptor-deficient mice (*db/db*) (199), and leptin being an important hyperglycemic factor in tilapia (181), it is very tempting to speculate that leptin receptor deficiency in zebrafish would have an effect on glucose homeostasis. Indeed, it seems that, at least in zebrafish, a role for leptin in glucose homeostasis is more pronounced than a role as an adipostat. In the zebrafish leptin receptor mutant, leptin is not required for adipostasis, reproductive functions, or appetite regulation. However, several aspects of glucose homeostasis were

altered in mutant fry compared to controls: a small increase in whole body glucose content was found, the expression of the preproinsulin gene *insulin-a* (*insa*), not *insb*, was enhanced in endocrine pancreas tissue, the number of β-cells was 25% higher than in controls, and the expression of key enzymes involved in hepatic glucose metabolism was altered (183). A 3-day exposure of mutant larvae to metformin [a drug known for its beneficial effects on hepatic glucose homeostasis and insulin sensitivity in diabetes patients (200)] normalized the number of β-cells to wild-type levels at 5 days post fertilization. Mutant *leptin-a* and *leptin-b* zebrafish (generated using CRISPR technology) confirmed that lack of leptin-a signaling *via* the leptin receptor is responsible for the increased number of β-cells (183).

With respect to the involvement of leptin in the regulation of food intake and adipostasis, the zebrafish and medaka studies [Michel et al. (183) and Chisada et al. (175), respectively] present opposing results. Whereas Michel et al. (183) concluded that leptin plays a role in glucose homeostasis in zebrafish, but not in adipostasis, Chisada et al. (175) concluded that leptin exerts a powerful influence on food intake regulation and fat allocation in medaka. Although medaka and zebrafish are evolutionary distant species [~296 Mya apart (168)], which could explain differential functions of leptin, the effect of differences in genetic background and raising density in the medaka study cannot be ruled out. A study on knock-out medaka with proper genetic background controls should resolve this issue.

LEPTIN AND STRESS

Re-establishing energy balance is pivotal for vertebrates to realize general homeostasis and cope with environmental or physical disturbances (18, 201, 202). To cope with a (potential) stressful event, vertebrates have to adjust neural, endocrine, and immune mechanisms (203), that, together, modulate energy metabolism. This is an allostatic response; i.e., the ability of an animal to acquire "stability through change" (204, 205). Allostasis is essential in attaining homeostasis (203), and leptin has been annotated as an allostatic hormone (206). This description embraces leptin functioning as a pleiotropic hormone, involved in redistribution of energy, independent of context.

The stress response is largely conserved from fish to terrestrial vertebrates (18, 201), and recently reviewed with respect to fish (202). As both leptin and corticotropin-releasing factor (CRF) are important modulators of energy balance, a link between these hormone systems was predicted soon after the discovery of leptin. Indeed, icv injections of recombinant leptin in fasted rats resulted in increased *Crf* mRNA levels in the hypothalamus, decreased *Npy* expression, and a reduction in food intake (207, 208). Leptin receptors appeared to be concentrated in the ARC (207), thus leptin exerts its anorexic effect, at least in part, mediated by indirect stimulation of CRF *via* the ARC and paraventricular nucleus in mammals (208).

Nutritional state is a crucial component in stress axis activity (209). The contribution of leptin and CRF to the regulation of the stress axis and energy homeostasis is dependent on shared signaling pathways with complementary effects centrally and peripherally. The ultimate result of the stress response is the production and release of glucocorticoids that stimulate the induction of gluconeogenic enzymes in the liver and lipogenesis (210). In mammals, peripheral leptin functions directly at the level of the adrenal gland, where it reduces cortisol release and blunts the adrenal corticotropic hormone (ACTH)-induced rise in cortisol levels (211). Thus, leptin stimulates CRF release from the hypothalamus, but counteracts the peripheral effects of glucocorticoids by inhibiting cortisol release from the adrenal gland.

Also in fish, leptin has been shown to modulate the stress response at multiple levels (156, 212). We have already considered the upregulation of hepatic *leptin* expression during chronic hypoxia in common carp (156), and another study with this species demonstrated that recombinant (human) leptin decreased regulated, CRF-mediated, as well as constitutive ACTH release, and lowered basal cortisol secretion from the head kidney (212). Leptin may then serve as a master signal to downplay the stress response and decrease energy expenditure, as these two processes are intimately linked. This situation is strongly reminiscent of the role of leptin in the GH-IGF axis, as well as the interaction with prolactin.

SYNTHESIS AND PERSPECTIVES

Vertebrates have adapted to essentially all niches found on earth, aquatic, terrestrial, and aerial. The conquest of and adaptations to these niches come with niche-specific energetic consequences. The earliest vertebrates evolved in aquatic niches, and their well-lubricated integument is an adaptation to save energy spent on transportation. Swimming is energetically cheaper than flying and running (the most expensive mode of transport). Efficient (aerobic) production of ATP requires a guaranteed oxygen uptake machinery, which is found in high sophistication in the gills of extant fish. The delicate barrier of the gills that facilitates oxygen diffusion comes with a cost: the large branchial surface holds the danger of unwanted water and ion flows, in hypo- or hypersaline waters. Accurate hydromineral balance is secured by the energetically expensive Na^+/K^+-ATPase. Fish play with the surface area required for oxygen uptake, but also show metabolic suppression when hypoxic or anoxic conditions arise.

Although several trials with regional endothermy are found in fish, with the transition to land the evolution of true endothermy is seen. The terrestrial environment required more expensive modes of transportation, facilitated by the large metabolic scope inherent to endothermy. Heat loss through air is considerably less than through water. The keratinized skin was equipped externally with feathers or hair and internally with a hypodermic insulating fat layer to retain heat. At the same time, the fat tissue is the major energy depot, which secures the energy requirements of endothermy. In many terrestrial animals, the fat is the largest endocrine tissue, production site, and target of humoral factors key in energy metabolism. Of note, in some icefishes fat can make up 50% of total body mass, fat that serves a role in buoyancy and vertical migration (213).

In the first part of this review, we have non-exhaustively discussed different metabolic strategies. Metabolism, specifically

metabolic rate, is at the basis of thermal regulation in both ecto- and endotherms. Metabolism, thermoregulation, and aerobic performance affect each other and all depend on food intake, food being the source of chemical energy. Diametric differences in energy metabolism resulted in different endocrine mechanisms, regulating energy balance and food intake. In the second part of this review, we discussed these endocrine mechanisms in a comparative way, with a focus on insulin and (mainly) leptin.

Up till now, major differences in leptin function between fish species were reported. There is urgent need to find a "common denominator" in teleostean leptin physiology. Most of our current knowledge arises from studies on cyprinids and salmonids, which reflect only a small share of the teleostean diversity. Therefore, one should study leptin in a truly comparative way, including a broader range of fish species. Furthermore, studies on the endocrinology of energy balance should also include insulin. An interesting new avenue of research is the contribution of VAN to energy balance in early vertebrates, and the effects of insulin and leptin thereon.

Insulin and leptin are evolutionary old and the pinnacle regulators of energy intake, storage, and expenditure. Our recent knowledge, in particular on the involvement of leptin in the entire neuro-endocrine axis, is greatly enhanced by comparative studies between early vertebrates and mammals. The evolution of and the interactions within the type-I helical cytokine family (including leptin, prolactin, and GH) elaborated sophisticated control of the energy balance in challenging niches. In addition, comparative studies keep promise to solve the paradoxical (?) evolution of endothermy.

AUTHOR CONTRIBUTIONS

IP drafted the manuscript. IP, GF, and MG, all edited and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

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Assessing the Functional Role of Leptin in Energy Homeostasis and the Stress Response in Vertebrates

*Courtney A. Deck, Jamie L. Honeycutt, Eugene Cheung, Hannah M. Reynolds and Russell J. Borski**

Department of Biological Sciences, North Carolina State University, Raleigh, NC, USA

Leptin is a pleiotropic hormone that plays a critical role in regulating appetite, energy metabolism, growth, stress, and immune function across vertebrate groups. In mammals, it has been classically described as an adipostat, relaying information regarding energy status to the brain. While retaining poor sequence conservation with mammalian leptins, teleostean leptins elicit a number of similar regulatory properties, although current evidence suggests that it does not function as an adipostat in this group of vertebrates. Teleostean leptin also exhibits functionally divergent properties, however, possibly playing a role in glucoregulation similar to what is observed in lizards. Further, leptin has been recently implicated as a mediator of immune function and the endocrine stress response in teleosts. Here, we provide a review of leptin physiology in vertebrates, with a particular focus on its actions and regulatory properties in the context of stress and the regulation of energy homeostasis.

Keywords: leptin, energy homeostasis, stress, teleosts, metabolism, cortisol, appetite

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***Correspondence:**
Russell J. Borski
russell_borski@ncsu.edu

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INTRODUCTION

Leptin is a class I helical cytokine encoded by the *obese* gene (*ob*) that has typically been characterized as an adipostat, circulating in proportion to the quantity of white adipose tissue and relaying information regarding the energy status of the animal to the central nervous system (1, 2). In mammals, leptin is pleiotropic, regulating a multitude of physiological processes including appetite, lipid metabolism, growth, reproduction, stress, and immune function [reviewed in Ref. (3)]. The function of leptin has been less extensively studied in non-mammalian vertebrates; however, there is growing evidence in teleosts that leptin may play a greater role as a glucoregulatory hormone than an adipostat in this group of vertebrates. Studies on the interactions between leptin and the stress axis as well as the immune system, however, suggest that some of the actions of leptin may be conserved between fish and mammals despite the low sequence conservation between these two groups. Here, we provide an overview of what is known about the role of leptin in regulating energy homeostasis and the stress response in teleost fishes and compare this to the known effects of leptin in mammals and other vertebrate groups.

LEPTIN CHARACTERIZATION, DISTRIBUTION, AND SIGNALING

Orthology in Vertebrates

Leptin was first cloned in the mouse by Zhang et al. (1) and has since been identified in all extant vertebrate groups examined to date. Following the discovery of leptin in the mouse, orthologs were

identified in several other mammalian species (4); however, attempts to isolate a putative leptin sequence in non-mammalian vertebrates were largely unsuccessful. It was not until 2005, over a decade after its discovery in mammals, that a leptin homolog was cloned in a non-mammalian species, the Japanese pufferfish [*Takifugu rubripes* (5)]. This delay was due to the low amino acid identity (often less than 30%) between vertebrate leptin sequences (6) (**Figure 1**). The deduced primary structure of the pufferfish leptin (pLep) shared only 13.2% identity with human leptin; however, three-dimensional modeling suggested a strong conservation of tertiary structure with mammalian leptins, as pLep also possesses four α-helices (5). Further, the amino acid sequence of pLep contained two cysteine residues to form the disulfide bridge between α-helices C and D, a highly conserved element of vertebrate leptins (5).

Shortly after the identification of pLep, a leptin homolog was cloned in an amphibian, *Xenopus laevis*, that shared 35 and 13% amino acid identity with human and pLeps, respectively (7) (**Figure 1**). Putative leptin sequences have also been identified in the tiger salamander [*Ambystoma tigrinum* (8)] and in the green Anole lizard [*Anolis carolinensis* (9)], both of which show low amino acid identity to human leptin. In teleosts, leptin orthologs have now been characterized in striped bass [*Morone saxatilis* (10)], common carp [*Cyprinus carpio* (11)], rainbow trout [*Oncorhynchus mykiss* (12)], zebrafish [*Danio rerio* (13)], Atlantic salmon [*Salmo salar* (14)], orange-spotted grouper [*Epinephelus coioides* (15)], Japanese medaka [*Oryzias latipes* (13, 16)], yellow catfish [*Pelteobagrus fulvidraco* (17)], Nile tilapia [*Oreochromis niloticus* (18)], Jian carp [*C. carpio* var. Jian (19)], Arctic charr [*Salvelinus alpinus* (20)], grass carp [*Ctenopharyngodon idella* (21)], silver carp [*Hypophthalmichthys molitrix* (21)], chub mackerel [*Scomber japonicus* (22)], mandarin fish [*Siniperca chuatsi* (23)], and white-clouds mountain minnow [*Tanichthys albonubes* (24)]. These teleost leptins all have low sequence conservation with mammals, varying from 13 to 25% amino acid identity (**Figures 1** and **2**); however, each one is composed of two

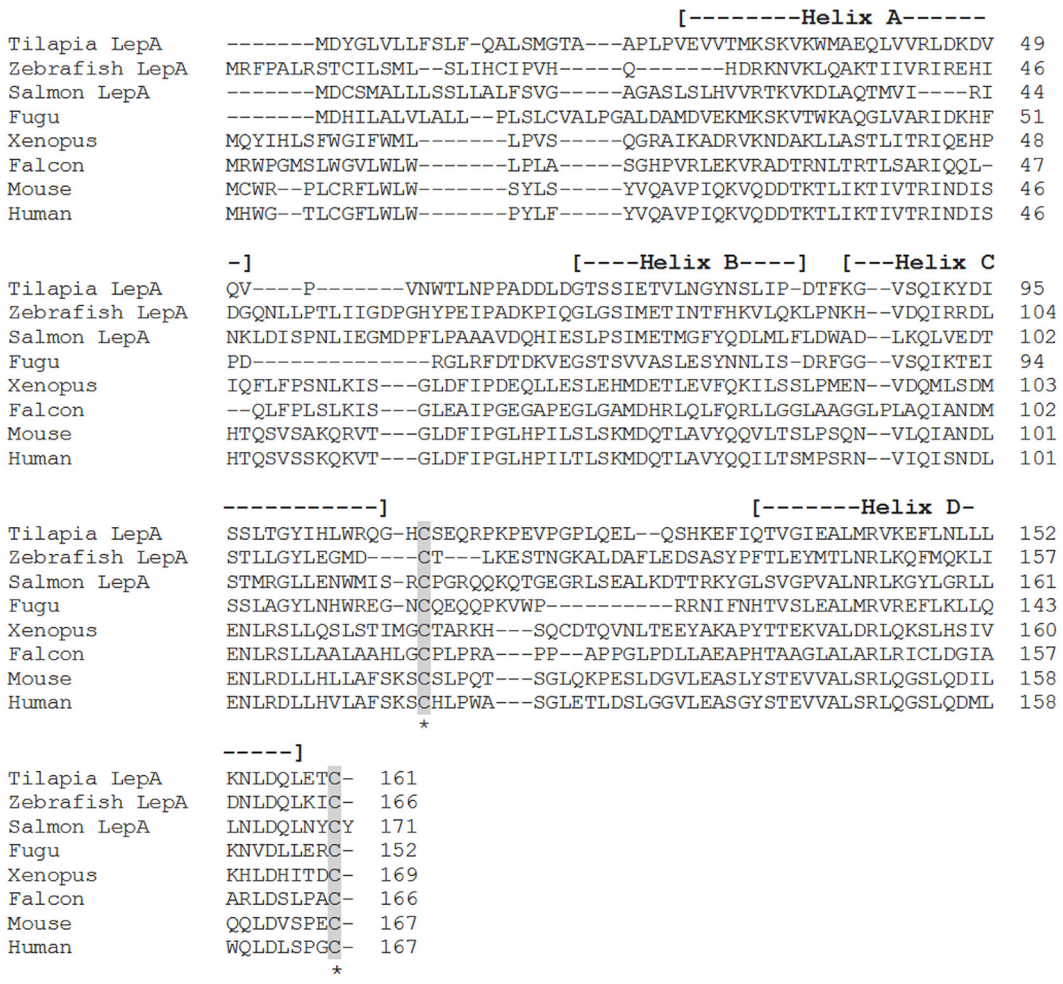


FIGURE 1 | Alignment of teleost leptin A (LepA) with the leptin homologs from other vertebrate classes. Accession numbers: tilapia LepA, AHL37667.1; zebrafish LepA, NP_001025357.2; salmon LepA, ACZ02412.1; fugu, NP_001027897.1; *Xenopus*, NP_001089183.1; falcon, NP_001298279.1; mouse, NP_032519.1; human, NP_000221.1. Shaded areas represent the conserved cysteine residues required for the formation of the disulfide bridge. The four alpha-helices are indicated by dashed lines within the parentheses.

FIGURE 2 | Alignment of teleost leptin B (LepB) with the leptin homologs from other vertebrate classes, tilapia leptin A (LepA), has been included for comparison. Accession numbers: tilapia LepA, AHL37667.1; tilapia LepB, AHL37668.1; salmon LepB, NP_001266063.1; zebrafish LepB, NP_001025357.2; *Xenopus*, NP_001089183.1; falcon, NP_001298279.1; mouse, NP_032519.1; human, NP_000221.1. Shaded areas represent the conserved cysteine residues required for the formation of the disulfide bridge. The four alpha-helices are indicated by dashed lines within the parentheses.

exons separated by a short intron, contains the cysteine residues required for formation of the disulfide bridge, and is predicted to have retained the four-helix tertiary structure characteristic of mammalian leptins. Even within the teleost lineage, there is often low amino acid identity between species (<50%), unless the species are closely related, such as within the salmonids or cyprinids.

Paralogs in Teleosts

In certain teleost species, two leptin paralogs have been identified. This is a common feature of teleostean class I cytokines, resulting from the genome duplication that occurred in the teleost lineage (6, 25). Zebrafish (*D. rerio*), Japanese medaka (*O. latipes*), orange-spotted grouper (*E. coioides*), Nile tilapia (*O. niloticus*), Mozambique tilapia (*Oreochromis mossambicus*), chub mackerel (*S. japonicus*), mandarin fish (*S. chuatsi*), and white-clouds mountain minnow (*T. albonubes*) have all been shown to possess two separate leptin proteins, leptin A (LepA) and leptin B (LepB) (13, 15, 18, 19, 22–24, 26) (**Figures 1** and **2**). The amino acid identity between LepA and LepB within each species is low, ranging from 18 to 30%, and phylogenetic analysis shows that the two genes form separate branches (18, 19, 24, 27). Due to the additional genome duplications that occurred within the salmonid and cyprinid lineages, a number of species including the common carp (*C. carpio*), Atlantic salmon (*S. salar*), goldfish (*Carassius auratus*), rainbow trout (*O. mykiss*), and Jian carp (*C. carpio* var Jian) possess up to four leptin paralogs, two LepA genes, and either one or two LepB genes (11, 14, 19, 28, 29). The two LepA sequences and the two LepB sequences in these species share higher amino acid identities than is seen between the A and B forms (ranging from 71 to 83%); thus, the nomenclature typically used is leptin A1 and A2 and leptin B1 and B2 (13, 14, 28, 29).

Tissue Distribution in Teleosts

Unlike in mammals where leptin is produced predominantly in adipose tissue, teleost leptins often have the highest mRNA expression levels in liver, with most species having low or non-existent leptin expression in adipose tissue. Other sites of

expression in teleosts are the brain, gonads, muscle, and kidney; however, this can vary widely between species (10–24, 26). In some instances, the tissue distribution between paralogs within a single species differs, and it has been suggested that *lepa* is more prominent in the liver, while *lepb* is predominantly expressed in the gonads, thus indicating divergent roles of the two paralogs (13, 29). However, studies on LepB are limited, and this differential expression pattern is not consistent across species, with most showing substantial overlap in the tissue expression patterns for the two forms. Regardless, *lepa* appears to be the predominantly expressed form in most species examined (15, 16, 24) showing 10–100 times greater tissue mRNA copy number than *lepb* and hence likely reflecting the major source of circulating leptin (26).

Receptor and Signaling Pathways

The leptin receptor (LepR) is part of the glycoprotein 130 family of cytokine receptors, which utilize gp130 as a signal transducer to activate signaling pathways within the cell, typically the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (30, 31). Signaling *via* this pathway has been observed in the pituitary of both mammals and frogs, suggesting conservation of this signaling mechanism for leptin across vertebrate groups (30–32). Although sharing low identity with mammalian receptors (<30%), teleost LepRs show genomic synteny with the human receptor and possess the functionally important JAK- and STAT-binding domains that are largely conserved within vertebrates (14–18, 33–35). In teleosts, *lepr* mRNA is ubiquitously expressed, with higher levels typically being observed in the pituitary, hypothalamus, and gonads, suggesting that these are prominent sites of leptin action (14–18, 28, 33–35). Indeed, leptin regulates glucose sensing in the hypothalamus and hindbrain of rainbow trout (*O. mykiss*) both *in vitro* and *in vivo* (36, 37). These actions were attenuated when leptin was administered in combination with either a phosphoinositide-3-kinase or JAK2 inhibitor, indicating involvement of these pathways in leptin signaling (36, 37). Further evidence for leptin signaling *via* the JAK/STAT pathway comes from the increase in Akt and STAT3 phosphorylation observed in trout hypothalamic cells following incubation with leptin (38). The lipid regulatory activity of heterologous leptin on hepatocytes and ovarian follicular cells of yellow catfish (*P. fulvidraco*) is attenuated by JAK/STAT inhibitors, reiterating a role for this pathway in leptin signaling (39). Leptin has also been shown to act on the pituitary of tilapia (*O. mossambicus*) to stimulate prolactin (PRL) release through activation of the extracellular signal-related kinase (ERK) pathway (40) and on the liver of the hybrid striped bass [*Morone chrysops x Morone saxatilis* (41)] and Mozambique tilapia [*O. mossambicus* (42)] to regulate growth hormone (GH) receptors and insulin-like growth factors (IGFs), although the signaling pathways have yet to be determined. Albeit studies assessing the function of leptin in teleosts are limited, existing data suggest that the sites of leptin action and the signaling pathways responsible for eliciting its effects may be conserved with that of other vertebrate systems. Further investigations are required to elucidate the full complement of intracellular pathways mediating leptin action(s).

LEPTIN ENERGY HOMEOSTATIC ACTIONS

Feeding

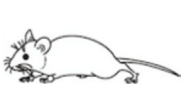
Leptin is renowned for its role in regulating food intake and body mass (43). Secreted primarily from adipose tissue in mammals, leptin serves as a lipostatic signal and conveys critical information regarding metabolic state to the brain (44, 45). As lipid stores accumulate and circulating leptin rises, the hormone enhances energy expenditure and reduces food intake by stimulating anorexic proopiomelanocortin/cocaine and amphetamine-related transcript neurons and inhibiting orexigenic neuropeptide Y/agouti-related protein neurons (46–50). Leptin-deficient pathologies are typically accompanied by hyperphagia and obesity [reviewed in Ref. (45, 49, 51)]. The anorexigenic properties of leptin have been well characterized in the context of leptin deficiency through experimental administration to obese, leptin-deficient *ob*/*ob* mice, as well as leptin-deficient humans, resulting in the reduction of food intake and body mass (52, 53).

In some fishes, leptin demonstrates a marked postprandial elevation [(54, 55); reviewed in Ref. (56, 57)] in accordance with the mammalian paradigm. Further, the administration of leptin *via* injection has been shown to reduce food intake in goldfish [*C. auratus* (58, 59)], rainbow trout [*O. mykiss* (12, 36)], grass carp [*C. Idella* (21)], Atlantic salmon [*S. salar* (60)], and striped bass [*M. saxatilis* (10)]. Properties similar to that of leptin-related pathologies initially observed in the *db*/*db* mouse have also been reported in a LepR-deficient medaka [*O. latipes* (61)]. This mutant line showed consistently elevated hypothalamic activity of orexigenic neuropeptides, suppression of anorexigenic neuropeptides, and increased food intake, suggesting a similar regulatory role for leptin in appetite suppression in fishes. While the anorexigenic properties of leptin would also suggest potentially concurrent lipostatic properties as seen in mammals, no changes in adiposity were observed in leptin receptor-deficient strains of zebrafish (62), and other species exhibit inconsistent correlations between fat deposition and leptin expression, e.g., during fasting leptin rises in fish as adiposity declines, while it declines with fasting and lipid stores in mammals (38, 42, 63–65). Nonetheless, the anorexigenic properties of leptin appear well conserved among vertebrates.

Metabolism

Leptin regulates energy availability in mammals by mobilizing lipid stores (66) and stimulating the oxidation of fatty acids (67). It also induces hypoglycemia by enhancing glucose uptake into peripheral tissues (68) and elevates metabolic rate in muscle and liver (69). Studies on the metabolic actions of leptin in other vertebrate classes are limited (**Table 1**) leading to difficulties in elucidating whether leptin evolved primarily as a lipolytic agent or if its basal metabolic functions are more glucoregulatory in nature. Teleosts appeared relatively early in the vertebrate lineage, and thus, understanding the role of leptin in regulating metabolic pathways in these fish could provide valuable insights into the evolution of energy homeostasis in vertebrates. The existing data in teleosts are equivocal, with lipolytic actions being reported in response to leptin treatment in some species, while in others,

TABLE 1 | Comparison of the source of leptin, response to fasting, and effects on appetite, energy metabolism, glycemia, and metabolic rate in the different vertebrate classes based on current knowledge.

Leptin effects					
Source	Adipocytes	Adipocytes and hepatocytes	Hepatocytes	?	Hepatocytes
Appetite	Anorexigenic	Anorexigenic	Anorexigenic	Anorexigenic	Anorexigenic
Fasting	Levels decline	?	?	?	Levels elevate[a]
Metabolic rate	Elevates	?	?	Elevates	?
Energy mobilization	Lipolytic	?	Glycogenolytic	?	Glycogenolytic Lipolytic[b]
Glycemia	Hypoglycemic	?	Hyperglycemic	?	Hyperglycemic Hypoglycemic[b]

?, unknown.

[a]*Predominant response but does not occur in all species.*

[b]*Response varies between species.*

TABLE 2 | Different effects of leptin on appetite, energy metabolism, and glycemia as well as the response to fasting in various teleost species.

Species[a]	Leptin source	Appetite	Energy metabolism	Glycemia	Fasting	Reference
Grass carp (*Ctenopharyngodon idella*)	Carp	Anorexigenic	↑ lipolytic enzyme mRNA	?	?	Li et al. (21)
Catfish (*Pelteobagrus fulvidraco*)	Human	?	↑ lipolytic enzyme mRNA ↑ enzyme activity ↓ triglycerides	?	?	Song et al. (39); Zhang et al. (70)
Striped bass (*Morone chrysops x Morone saxatilis*)	Human	Anorexigenic	?	?	↓ *lep* mRNA	Won et al. (10)
Tilapia (*Oreochromis mossambicus*)	Tilapia	?	↓ glycogen ↓ lipolytic enzyme mRNA	Hyperglycemic	↑ *lepa* mRNA ↑ plasma leptin A	Baltzegar et al. (71); Douros et al. (42)
Rainbow trout (*Oncorhynchus mykiss*)	Salmonid	Anorexigenic	↓ glycogen	Hyperglycemic	↑ plasma Lep	Murashita et al. (12); Kling et al. (72); Aguilar et al. (36, 37)
Goldfish (*Carassius auratus*)	Human	Anorexigenic	↑ glycogen ↓ lipids	Hypoglycemic	No effect?	de Pedro et al. (58); Vivas et al. (59); Tinoco et al. (28)

The source of leptin indicates whether homologous or heterologous leptin was used in the study.

[a]*Species were chosen to highlight the disparate effects of leptin observed in teleosts.*

leptin instead stimulates glycogen depletion and increases plasma glucose (**Table 2**).

Leptin actions appear to agree with the classic mammalian paradigm in grass carp (*C. Idella*), wherein it induces a decrease in the hepatic stearoyl-coA desaturase-1 mRNA, an enzyme involved in the synthesis of fatty acids, while simultaneously increasing the mRNA level of hormone-sensitive lipase (*hsl)* (21). Fatty acid levels were not measured in these studies; however, an overall effect on lipid regulation cannot be ascertained. Nonetheless, human leptin increases activity and mRNA levels of lipolytic enzymes in catfish (*P. fulvidraco*) hepatocytes and ovarian follicular cells, which paralleled a decrease in overall lipid content, suggesting a lipolytic action of the hormone (39, 70). Further, human leptin increased the mRNA levels of various lipolytic genes, decreased the levels of lipogenic genes, and decreased overall triglyceride content in hepatocytes of the goby [*Synechogobius hasta* (73)]. In contrast, the mRNA levels of hormone-sensitive lipase, as well as lipoprotein lipase (*lpl*), decreased in the liver of Mozambique tilapia (*O. mossambicus*) in response to homologous hormone treatment (71). The latter study also observed a decrease in hepatic glycogen content and corresponding increase in plasma glucose (71), suggesting that leptin has hyperglycemic actions in teleosts and thus may represent a functional divergence from mammalian leptins. This corroborates an earlier study in rainbow trout (*O. mykiss*) in which central administration of leptin also increased plasma glucose while concurrently reducing the glycogen content of the liver (36). Similar effects were observed in lizards, with leptin decreasing hepatic glycogen content and increasing plasma glucose levels (74). Disparate results have been reported in goldfish, however, with human leptin increasing muscle and liver glycogen while depleting liver lipids and lowering plasma glucose, similar to what is observed in mammals (58). The different actions of leptin reported in teleosts could be a function of differences in life history strategies or from using mammalian vs. homologous leptins. Baltzegar et al. (71) reported similar glucoregulatory effects for both recombinant human leptin and tilapia LepA. However, distinct actions on regulation of hepatic *hsl* and *lpl* were observed between the two, with tilapia LepA reducing and human leptin having little effect on the lipases, suggesting that the use of homologous hormone may be essential for determining species-specific effects.

Further glucoregulatory roles for leptin have been demonstrated in the brain of rainbow trout and tilapia. Aguilar et al. (36) demonstrated increases in the glucose and glycogen contents of the trout (*O. mykiss*) hypothalamus and hindbrain in response to an intracerebroventricular injection of human leptin, which were

paralleled by increases in *glut2* mRNA and glycogen synthase activity. Leptin also induced a significant increase in glucokinase activity in the brain (36), suggesting that one of the functions of leptin may be to stimulate glucose uptake and metabolism. In the pituitary rostral *pars distalis* of the tilapia (*O. mossambicus*), homologous leptin induced an increase in the activity of phosphofructokinase, a rate-limiting glycolytic enzyme, and this was correlated with an increase in lactate secretion or overall glycolytic output (75). Although typically believed to be a lipolytic agent, leptin has also been implicated in glucose metabolism in mammals, having been shown to stimulate glycolysis and gluconeogenesis and inhibit glycogenolysis [reviewed in Ref. (76)]. These data suggest that one of the basal functions of leptin may be to regulate glucose uptake and catabolism (e.g., glycolysis) in vertebrates; however, the source of glucose may vary as the hormone can elicit catabolic effects on either lipid or glycogen stores. One explanation for this could be the evolution of endothermy [see Ref. (77) for review of energetics between endothermy and ectothermy]. Mammals exhibit higher metabolic rates that, if fueled by fatty acids and/or glucose that has been synthesized *de novo*, would allow glycogen stores to be conserved in the event the animal is in need of a rapid source of energy. Hence, leptin may promote gluconeogenesis, but not glycogenolysis. Whether leptin alters gluconeogenic pathways in fish remains to be determined.

LEPTIN INTEGRATION WITH THE CLASSICAL ENDOCRINE STRESS AXIS

Endocrine Stress Response

It is apparent that leptin is a catabolic hormone in vertebrates that enhances energy mobilization and suppresses appetite, two processes often linked to stress responses. Hence, the hormone may be integral to the endocrine stress response. Stress impacts virtually all aspects of vertebrate physiology including immunity, reproduction, hydromineral balance, and energy homeostasis (78–80). The adrenergic (humoral and neuronal) and hypothalamic–pituitary–adrenal [interrenal in fish; HPA/hypothalamic-pituitary interrenal (HPI)] axes are central components of the vertebrate stress response and ultimately aid in restoration of homeostasis when disrupted. In all vertebrates, including teleost fishes, acute and chronic stress events are mediated through the sympathetic adrenergic and HPA/HPI axes, two primary components of the endocrine stress response. The two axes release catecholamines (epinephrine/norepinephrine) and glucocorticoids (cortisol/corticosterone), respectively, to allow for the mobilization of energy stores (79, 81, 82).

Upon the perception of a stressor, sympathetic nerve fibers release acetylcholine onto chromaffin cells within the adrenal medulla (mammals) or interrenal tissue (teleosts) to stimulate the secretion of catecholamines and allow for the rapid mobilization of energy stores from peripheral tissues (81, 83–85). Simultaneously, the hypothalamus releases corticotropin-releasing factor (CRF), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then triggers the production and release of glucocorticoids from the adrenal cortex (mammals) or interrenal cells of the head kidney (teleosts) (79, 80, 85). These glucocorticoids then elicit a myriad of metabolic effects such as inducing lipid and protein catabolism and stimulating gluconeogenesis to increase plasma glucose levels (79, 86). In a classic negative feedback pathway, the increase in circulating cortisol then inhibits further release of CRF and ACTH, attenuating the stress response.

Catecholamines and Leptin

Epinephrine is thought to be the primary hormone of the humoral adrenergic system in most fishes (80, 81). As part of the "fight or flight" response, catecholamines exert numerous actions that include rapid mobilization of glucose and free fatty acids through enhanced glycogenolysis and lipolysis, respectively, as well as regulation of respiration and blood flow (79, 81, 87). Leptin is also critical for regulating energy expenditure in vertebrates and responds to various stressors (see Leptin Responses to Stress in Vertebrates), yet little is known about how the hormone interacts with components of the endocrine stress axis, particularly in non-mammalian vertebrates (27). To date, the majority of studies examining the relationship between leptin and the stress axis have been performed in mammals (27, 51). However, studies in lizards [*Podarcis sicula* (74)] and teleosts have indicated that leptin may act as a key metabolic regulator during stress in all vertebrates through mobilization of energy stores (**Figure 3**).

Leptin has been shown to stimulate the release of catecholamines in both porcine (88) and bovine (89) adrenal medullary cells. In addition, leptin increased mRNA levels of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine production (88). This suggests a synergistic relationship between leptin and catecholamines wherein leptin mobilizes energy from lipids while simultaneously stimulating the release of catecholamines to mobilize glucose during periods of stress (90). Interestingly, other studies utilizing human chromaffin cells have shown no significant change in catecholamine release with leptin treatment (91). The contradictory responses observed between human and other mammalian models could possibly be due to differences in methodology (isolated cells vs. whole adrenal tissue), the leptin concentrations used, or simply species differences (88). The regulation of catecholamines by leptin in fishes has not been well characterized. In goldfish (*C. auratus*), chronic leptin treatment resulted in no significant changes in hypothalamic catecholamines (58); however the effects of leptin on circulating catecholamines are yet to be examined.

While leptin exerts a stimulatory effect on catecholamine release in mammals, epinephrine has been shown to directly inhibit leptin secretion (92–95). In addition, increases in intracellular cAMP in medullary cells, one of the second messengers involved in adrenergic signaling, downregulate leptin mRNA (96). Leptin increases intracellular cAMP in addition to stimulating catecholamine release (88), both of which could act in a negative feedback loop to inhibit further leptin release. One theory behind this inhibition is that it is not advantageous for catecholamines to stimulate leptin during acute stress as obtaining energy from lipolysis is too slow for a "fight or flight" response; however, it may play a role in mediating the response to chronic stress (27). The regulation of leptin by catecholamines in fishes and other ectotherms is still unclear. However, both leptin and epinephrine exhibit glycogenolytic and/or lipolytic actions and have been shown to increase during times of stress in fishes (71, 79).

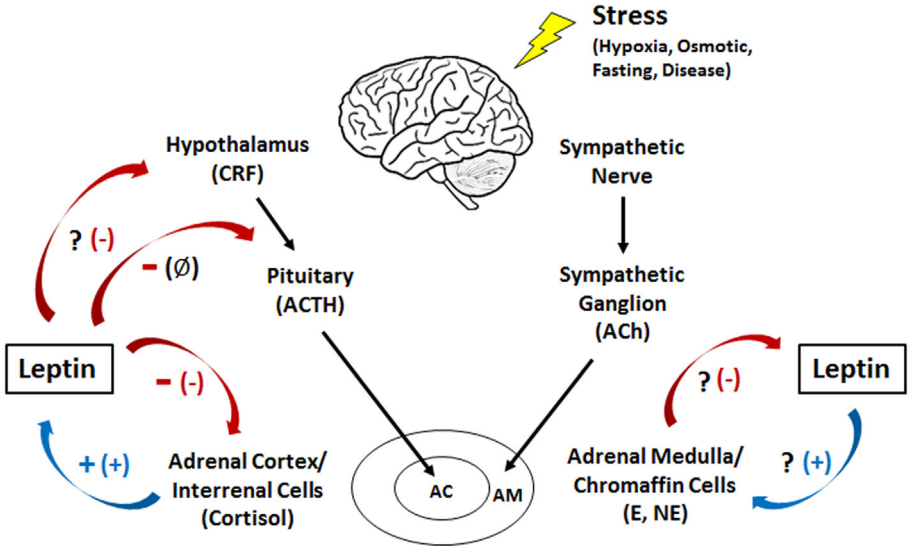


FIGURE 3 | Interactions between leptin and the humoral adrenergic and hypothalamic–pituitary–adrenal/interrenal axes in teleosts and mammals. +, stimulation; -, inhibition; Ø, no effect; ?, an unknown relationship. The mammalian response is represented by the symbol in parentheses. CRF, corticotropin-releasing factor; ACTH, adrenocorticotropic hormone; Ach, acetylcholine; E, epinephrine; NE, norepinephrine; AC, adrenal cortex; AM, adrenal medulla.

Glucocorticoids and Leptin

The human *ob* promoter region possesses glucocorticoid response elements, suggesting that cortisol may elicit some of their actions by inducing leptin transcription (97, 98). Indeed, glucocorticoids elicited a stimulatory effect on leptin synthesis and secretion in rats (99), humans (100), and cultured human adipocytes (101) (**Figure 3**). In addition, the synthetic glucocorticoid dexamethasone increased mRNA levels and stimulated leptin secretion in rat adipocytes (96, 102). Similar results have been observed in teleosts, with cortisol increasing hepatic leptin mRNA levels in rainbow trout (*O. mykiss*) both *in vivo* and *in vitro* (103). In addition, when trout hepatocytes were treated simultaneously with cortisol and RU486, a glucocorticoid receptor antagonist, the increase in leptin mRNA was attenuated (103). Whether a similar response occurs with leptin secretion remains unknown. It has been speculated that since cortisol release is slower than that of catecholamines, the prolonged stressors that elicit cortisol actions would also benefit from the catabolic effects of leptin on lipids and/or carbohydrates reported in fishes, particularly in the liver where leptin is produced and may act locally (26, 27, 39, 71, 73, 104).

Leptin in turn has an overall inhibitory effect on the HPA axis in mammals (98), inhibiting CRF release from the hypothalamus in mice (105) and suppressing cortisol secretion from adrenal cells (106–108) (**Figure 3**). In contrast, leptin has no effect on ACTH secretion from the pituitary, suggesting that it regulates glucocorticoid release indirectly *via* the hypothalamus and directly by acting on the adrenal gland (105). When human adrenocortical cells are incubated with leptin, a dose-dependent decrease in ACTH-stimulated cortisol secretion is observed (91), while in leptin knockout mice (*ob/ob*), circulating levels of glucocorticoids are 85% higher than basal. Injecting these knockouts with leptin, however, reduced the level of glucocorticoids by 40% (109, 110). These data could potentially suggest a synergism between leptin and cortisol wherein cortisol stimulates the secretion of leptin that, in turn, mobilizes energy stores necessary for coping with a stressor. It has also been suggested that the anorexigenic effects of leptin could counteract the weight gain effects of cortisol in mammals (111). Similar results have been observed in teleosts, suggesting that interactions between leptin and glucocorticoids may be conserved in vertebrates. In the common carp (*C. carpio*), leptin inhibited ACTH-stimulated cortisol secretion *in vivo* and caused a dose-dependent decrease in CRF-induced ACTH secretion from the pituitary *in vitro* (6, 112). No changes in circulating cortisol were observed in leptin-injected goldfish [*C. auratus* (59)]; however, it is possible that leptin only inhibits glucocorticoid production when the HPI axis has been activated and circulating cortisol levels are elevated. In general, we do know that teleost pituitary glands are responsive to leptin (6, 26, 40, 42), and as such, it has been postulated that leptin may regulate the stress axis at the level of the pituitary (6, 113).

Currently, there are no other studies in fishes examining the relationship between leptin and the hormones of the stress axis, specifically interactions with catecholamines and glucocorticoids. There is a need to address these gaps as understanding these interactions will help to elucidate leptin's basal function as a putative regulator of the endocrine stress response in these organisms and how these actions may differ from that of the classically described adipostat in mammals.

LEPTIN RESPONSES TO STRESS IN VERTEBRATES

Fasting

Catabolic stress associated with fasting typically leads to down-regulation of leptin expression in mammals (114). The preponderance of evidence in teleosts, however, points to fasting-induced

increases in leptin synthesis and secretion (23, 42, 58, 72, 115, 116); albeit evidence in two species, the hybrid striped bass (*M. chrysops x M. saxatilis*) and red-bellied piranha (*Pygocentrus nattereri*) show that production of the hormone may decline with fasting (10, 117). The general increase in leptin during fasting found in most teleosts presents a functional paradox between the role of leptin as an anorexigenic endocrine signal and the drive to increase food intake during fasting. Leptin could aid to limit feeding to avoid the metabolic costs associated with foraging and digestion (118) during periods of low food availability, or perhaps other orexigenic factors such as ghrelin, whose levels are known to increase dramatically with fasting (119), outweigh the anorexigenic properties of leptin in driving food intake when energy status is low. Regardless, the increase in leptin with fasting is likely critical for promoting the catabolism of energy stores to fuel essential cellular processes. The variability of responses in fishes compared to mammals may be attributed to distinct regulation of energy stores, perhaps suggesting that signaling during altered metabolic states may not be reliant solely on leptin, but an integration of lipostatic, glucostatic, and other metabolic and endocrine signals. Further, as a consequence of genome duplication events in teleosts [reviewed in Ref. (120, 121)], some species possess multiple leptin paralogs that may exhibit different functional properties.

Hyperosmotic Stress

Euryhaline fishes can withstand wide fluctuations in environmental salinity. Through active excretion of ions, they can overcome large increases in plasma osmolality (>150 mOsm) during acute seawater challenge (71). The process of seawater acclimation consumes 20–68% of their total metabolic energy demand (122, 123). Elevated leptin stimulates Na^+ retention and induces hypertension in rats and may be associated with hypertension induced kidney disease in humans (124). Few studies have investigated the role of leptin in osmoregulation in teleost fishes, despite its regulatory interactions with GH, IGFs, and PRL, hormones known to control salt and water balance (26, 40–42). In the Mozambique tilapia (*O. mossambicus*), acute seawater transfer induced significant increases in hepatic *lepa* and *lepr* mRNA levels (71). The authors propose that leptin may work with cortisol to mobilize energy stores by inducing hepatic glycogenolysis and gluconeogenesis, respectively, thereby allowing the organism to fuel the increased energy demands associated with hyperosmotic stress. The hormone had no direct effect on expression of the gill Na^+K^+-ATPase pump, so it remains unclear whether the hormone is ionoregulatory in teleosts. Additional studies suggest that leptin may stimulate the release of PRL, an important freshwater osmoregulatory or Na^+-retaining hormone in teleosts (26, 40). Collectively, the results suggest that leptin may act to mobilize energy for seawater adaptation and promote GH sensitivity and IGF production to enhance seawater acclimation (41, 42, 71). It may also promote synthesis and secretion of PRL for freshwater adaptation (40).

Hypoxia

Oxygen is a necessary component of energy production in all vertebrates, and thus hypoxia represents a severe and potentially lethal stress. As leptin functions at the intersection of the endocrine stress response and metabolism, it is reasonable to postulate that it is involved in the vertebrate response to hypoxia. Indeed, an increase in the transcription of leptin in humans, observed in response to hypoxia and hypoxia-inducible factor 1 (HIF-1), transactivates the human leptin gene promoter (125, 126). In addition, leptin mRNA levels increase in response to hypoxia in a variety of mammalian cell lines (127–129). Interestingly, Meissner et al. (130) reported that short-term hypoxia in rats had no effect on plasma leptin levels or expression in adipose tissue; however, leptin expression was increased in the liver, kidney, and lungs suggesting a unique metabolic role for leptin under hypoxic stress. Leptin has further been shown to attenuate apoptosis under hypoxic conditions and appears to be necessary for behavioral recovery following acute hypoxia (131, 132). Taken together, the data from mammals point to a crucial role for leptin as a multifaceted mediator of energy homeostasis during hypoxia.

The first report of leptin regulation by hypoxia in fishes came from Chu et al. (133). The authors showed that *lepa* expression increased after 4 and 10 days of hypoxic exposure in zebrafish (*D. rerio*) and implicated HIF-1α as a key mediator of this response. In common carp (*C. carpio*), the expression of *lep-a1*, *lep-a2*, and *lepr* in the liver increases in proportion with the length of hypoxic exposure (113). This study also showed that exposure to hypoxia upregulated expression of *lepr* mRNA in the pituitary, suggesting potential integration with the HPI axis (113). In addition, transcriptome data for the tilapia (*O. mossambicus*) shows upregulation of genes responsive to hypoxia in the pituitary following leptin treatment [e.g., chaperone-containing TCP1, chromodomain helicase-binding domain, heat shock protein 90b1, Gene Ontology 0070482/001666 (75)]. Crucian carp (*C. carassius*) expresses multiple isoforms of the LepR in the gill, and the mRNA levels increased in response to hypoxia *in vivo* (134). While there are still significant gaps in knowledge with regards to how leptin is acting to augment organism energetics during hypoxia in fishes, it appears that leptin is indeed regulated by hypoxia in much the same way as mammals, increasing in response to the decreased availability of oxygen for ATP production. The emerging role of leptin in stimulating glycolysis among different vertebrates may fit well with its upregulation during hypoxia or normoxia (Warburg effect).

Immune Function and Disease

Immunity is intimately linked to an organism's metabolism and energy status, and as such, allocating energy to the immune system in states of both health and disease is critical to the overall fitness and survival of an organism (135). Fasting and nutritional deprivation are associated with an increased disease susceptibility, as well as immune system suppression and dysfunction in vertebrates (136–138). Due to its role as a vital neuroendocrine mediator of metabolic state, leptin has been investigated as a regulator of the energetics associated with the innate and adaptive immune responses. In mammals, increases in serum leptin levels occur with inflammation, a response that appears to be modulated by glucocorticoids (139). Further, leptin has been shown to reverse starvation-induced immunosuppression by stimulating

the proliferation of pro-inflammatory cytokine-secreting T cells (140). Despite having been extensively studied in mammals, few studies have explored the interplay of leptin and immunity in teleost fishes or other non-mammalian vertebrates.

The correct allocation of energy to the innate immune system, the first line of organism defense and the most important responder in the acute phase of an infection, is critical to host survival. Leptin signaling has been shown to be necessary for innate immunity in mammals (135, 141), increasing chemotaxis and oxidative function and delaying apoptosis in immune cells (142–146). Leptin increases activation and proliferation and induces production of pro-inflammatory cytokines in phagocytes (147). Similar functions have been observed in the adaptive immune response, wherein leptin acts to stimulate B-lymphocytes by inducing cell cycle entry, preventing apoptosis and causing the secretion of pro-inflammatory cytokines (148, 149). In addition, it has been determined that leptin signaling is necessary for normal rates of glucose uptake and glycolysis in activated T-cells (150). These data suggest that, in mammals, leptin may drive immune activation by increasing the oxidative and overall glycolytic capacities of various immune cells.

Very little work has been done to directly connect leptin to the immune system in teleost fishes. Mariano et al. (151) showed that leptin drove ERK and STAT3 phosphorylation in both adherent and non-adherent trout leukocytes. Additional evidence for a role of leptin in regulating immune function in teleosts comes from MacDonald et al. (152) in which rainbow trout (*O. mykiss*) infected with a pathogenic hemoflagellate exhibited significantly higher mRNA and plasma levels of LepA. The authors determined that leptin was being secreted in response to the hypoxemia associated with the infection to reduce food intake (152). This would serve to prevent the organism from having to allocate energy toward digestive functions while in the diseased state. It is also possible that increases in leptin synthesis and secretion could lead to catabolism of energy stores necessary to meet the energetic demands of fighting the disease. Although limited, the data suggest an integration of leptin with immune function, and future studies should investigate the extent of leptin's involvement in immunometabolic pathways in teleost fishes.

CONCLUSION

In teleost fishes, there is much that remains to be elucidated about the role of leptin in energy homeostasis. Although there is evidence that leptin acts as a glucoregulatory agent in teleosts, there are also reports of leptin having lipolytic actions, particularly in the cyprinid fishes. In mammals, leptin has been implicated in regulating the metabolism of both glucose and lipids, suggesting some conservation of function between the two groups, perhaps sharing roles in promoting glycolysis. However, the increase in leptin levels during fasting presents a functional paradox against its role as an anorexigenic hormone. A further look into the function of leptins in regulating basal metabolism may shed light in this area. As multiple paralogs of leptin have been identified in teleosts, future studies should focus on whether the disparate actions are simply species-specific differences or the result of neofunctionalization between the various leptin paralogs. To date, the studies investigating the involvement of leptin in regulating immunity and the endocrine stress response suggest that such roles may be conserved within vertebrates. However, it is currently unclear by what means metabolic energy stores might be preferentially mobilized by leptin upon exposure to acute and chronic stressors, such as osmotic stress or hypoxia. Further, it remains to be determined how multiple endocrine signals (e.g., catecholamines, glucocorticoids) might integrate with leptin signaling to achieve the appropriate physiological response under such conditions. Studies in teleosts, or other ectotherms, may shed light on potential new functions of leptin that may be well conserved in the vertebrate lineage.

AUTHOR CONTRIBUTIONS

All individuals contributed to writing and reviewing the final version of the article.

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On the Molecular Evolution of Leptin, Leptin Receptor, and Endospanin

Richard Lyle Londraville[1], Jeremy W. Prokop[2], Robert Joel Duff[1], Qin Liu[1] and Matthew Tuttle[1]*

[1] Program in Integrative Bioscience, Department of Biology, University of Akron, Akron, OH, USA, [2] Hudson Alpha Institute for Biotechnology, Huntsville, AL, USA

Over a decade passed between Friedman's discovery of the mammalian leptin gene (1) and its cloning in fish (2) and amphibians (3). Since 2005, the concept of gene synteny conservation (vs. gene sequence homology) was instrumental in identifying leptin genes in dozens of species, and we now have leptin genes from all major classes of vertebrates. This database of *LEP* (leptin), *LEPR* (leptin receptor), and *LEPROT* (endospanin) genes has allowed protein structure modeling, stoichiometry predictions, and even functional predictions of leptin function for most vertebrate classes. Here, we apply functional genomics to model hundreds of LEP, LEPR, and LEPROT proteins from both vertebrates and invertebrates. We identify conserved structural motifs in each of the three leptin signaling proteins and demonstrate *Drosophila* Dome protein's conservation with vertebrate leptin receptors. We model endospanin structure for the first time and identify endospanin paralogs in invertebrate genomes. Finally, we argue that leptin is not an adipostat in fishes and discuss emerging knockout models in fishes.

Keywords: leptin, leptin receptor, endospanin, *in silico* modeling, molecular evolution, obesity, adipostat, fish models

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****Correspondence:***
Richard Lyle Londraville
londraville@uakron.edu

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INTRODUCTION

In 1994, Friedman's laboratory described leptin as a peptide hormone that is synthesized by adipose tissue (1) and soon after it was proposed to regulate appetite and metabolic rate by communicating energy stores to the central nervous system (4–6). In mammals, leptin is synthesized by adipose tissue and released into the blood; there it travels to the hypothalamus and binds to the leptin receptor, which stimulates reduction of appetite and increased mobilization of lipid for metabolism. Through this feedback loop, the brain regulates energy stores to remain relatively constant ["adipostat control" (4–6)]. Control of energy stores is central to an organism's life history, and as such, it is a research focus for comparative biologists. Migratory birds fuel long-distance migration by dramatic changes in lipid stores (7), hibernating mammals accumulate lipid stores to survive long periods of torpor (8), snakes dramatically rework organs to process large and infrequent meals (9), amphibian survival after metamorphosis is tied to adipose stores (10), and fish routinely go months without eating during winter (11). Agricultural scientists also have a great interest in leptin, because manipulating energy acquisition and deposition has potential to influence production of commercially important species (12–14). Therefore, there has been great interest and effort expended toward cloning and characterizing leptin orthologs throughout vertebrates. Recent reviews thoroughly document the progress of the comparative leptin community (15–17). This review will focus on three areas: evolution of genes in the leptin signaling pathway, the status of leptin as an adipostat, and emerging non-mammal

models for studying leptin signaling. These research topics have made significant progress in recent years, and they provide examples of how a comparative approach can inform the study of human leptin (hLEP) endocrinology.

EVOLUTION OF LEPTIN SIGNALING: LEPTIN AND LEPTIN RECEPTOR GENES AMONG VERTEBRATES

Although leptins in domestic mammals were identified soon after leptin in mice (18, 19), the first non-mammal leptin gene took over a decade to discover (2). This was due to the false assumption of sequence conservation among orthologs and was overcome by Kurokawa's insight of gene order conservation or synteny (2). This major advance, along with progress on genome projects, has allowed identification of *LEP*s and *LEPR*s in all classes of vertebrates (**Figures 1** and **2**). It is now clear that the ancestral leptin that gave rise to leptins in tetrapods (birds, reptiles, amphibians, and mammals) is more closely related to coelacanth and shark (*Callorhinchus milii*) leptins vs. leptins from bony fish. In other words, bony fish leptins diverged along their own lineage independent of leptins in higher mammals (**Figures 1** and **2**). After the bony fish and tetrapods diverged, multiple paralogs of fish *lep* evolved. Tetrapods and their closest living relatives for which we have data (gar, coelacanth, Dipnoi not determined) express a single ortholog of leptin (**Figure 1**), with the exception of *Anolis* lizard, which has two *lep*, one of which is not expressed (15).

Bony fish typically expresses two paralogs of leptin, referred to as "A" and "B." These are interpreted as arising during the whole genome duplication event in the Teleost fish lineage; more recent duplications (in salmonids and carps) are subtypes of A and B [see the study by Morini et al. (20) for an insightful discussion of leptin paralog evolution]. Leptin receptors are typically present as single orthologs per species, with the exception of recently identified duplicate *lepr* paralogs in European eel (20) and Asian arowana (*Scleropages formosus*) (acc# XP 018609810 and KPP63040). This duplication event appears to be ancient, but it is unresolved if the duplication of *lepr* was present in the ancestor of teleost fishes and then lost, or if loss of *lepr* predates teleosts (**Figure 2**).

Amphibians express a single paralog of *lep* and *lepr*, with *lep* expressed in multiple tissues, including adipose (3, 21). *Xenopus* responds to homologous recombinant leptin as an anorexigen, but not at all life stages (3). *Xenopus* leptin stimulates hind limb (3) and lung (22) development and may influence life history decisions in spadefoot toad (23). *Xenopus lepr* binds homologous and non-homologous leptins (3) and stimulates phosphorylation of intracellular signal transducer and activator of transcription (STAT) 3 and 5 (24). Less is known about reptile leptins. Several reports indicate that reptiles respond to non-homologous leptin treatment consistent with the mammalian model of leptin function [e.g., reduced appetite (25), reproductive effects (25, 26)]. In addition, studies using non-homologous leptin antibodies have documented leptin-like proteins that respond to seasonal changes in lipid (27–29), which are consistent with mammalian models. Denver et al. reported 2 *lep* (one which may be non-functional) and 1 *lepr* in the genome of the green anole (15). In general, amphibian *lep* and *lepr* expression and *in vitro* and *in vivo* function are more consistent with mammalian models than are similar data for fishes and birds.

What is the significance of multiple leptin paralogs? We assert that leptin-A and -B paralogs have distinct functions in teleosts. The fact that both paralogs (in multiple species of teleosts) are maintained throughout the teleost lineage (**Figure 1**) argues that each paralog has a distinct function. Where expression has been measured, A-type *lep*s are typically expressed at higher message copies and with a more narrow tissue distribution than B-type (16, 30–33), but not in all species (2). If leptin-B paralogs are functional (and not pseudogenes), why is their expression lower and less tissue specific than A? Perhaps leptin-Bs are acting in an autocrine/paracrine manner, similar to that proposed for bird leptin (see below). Supporting this hypothesis is the observation that leptin-B is dramatically upregulated during regeneration of fin and heart in zebrafish (34), and after retinal injury (35), perhaps indicating local vs. circulating action. In addition, leptin-A knockdown in zebrafish embryos (*via* morpholino oligonucleotide) does not elicit a change in expression of leptin-B (36). If the A and B paralogs overlap functionally, one would expect a compensatory increase in B with decreased expression of A. Finally, *in silico* binding simulation of both paralogs predicts significantly lower binding energy of B vs. A to the leptin receptor (37). This indicates that something about the ligand–receptor interaction is different for leptin-B; perhaps it requires a second ligand or a higher local concentration of ligand (as in autocrine/paracrine signaling). To our knowledge, there are no published data on leptin-B protein expression or *in vivo* function other than regeneration (34–35). A leptin receptor reporter assay to assess functional differences between leptin paralogs, similar to that developed for *Xenopus* (24), and specific antibodies to document expression would be useful in addressing these questions.

EVOLUTION OF LEPTIN SIGNALING: IS THERE ANOTHER MAJOR SIGNALING SYSTEM FOR ENERGY STORES IN BIRDS?

Arguably, bird leptin was the most difficult to identify among vertebrates, with over a decade of significant effort from multiple laboratories. A purported chicken leptin gene was reported early on, but independent laboratories were unable to amplify the sequence from chicken tissues, and it was absent in early builds of the chicken genome, despite the presence of a leptin receptor (38–40). The missing bird leptin gene was eventually found within regions of genomes that were refractory to characterization due to their high GC content and repetitive sequence (41–44). The advent of new methods of whole genome sequencing allowed identification of bird leptin in most major lineages of birds. Recently (45), RNAseq experiments in chicken documented highest leptin transcript copy number in brain (hypothalamus and cerebrum) and pituitary, with moderate expression in pancreas and testis, and low expression in liver and adipose [typically high expressing leptin tissues in fish and mammals, respectively (16)]. Further, Friedman-Einat's group speculated that the high

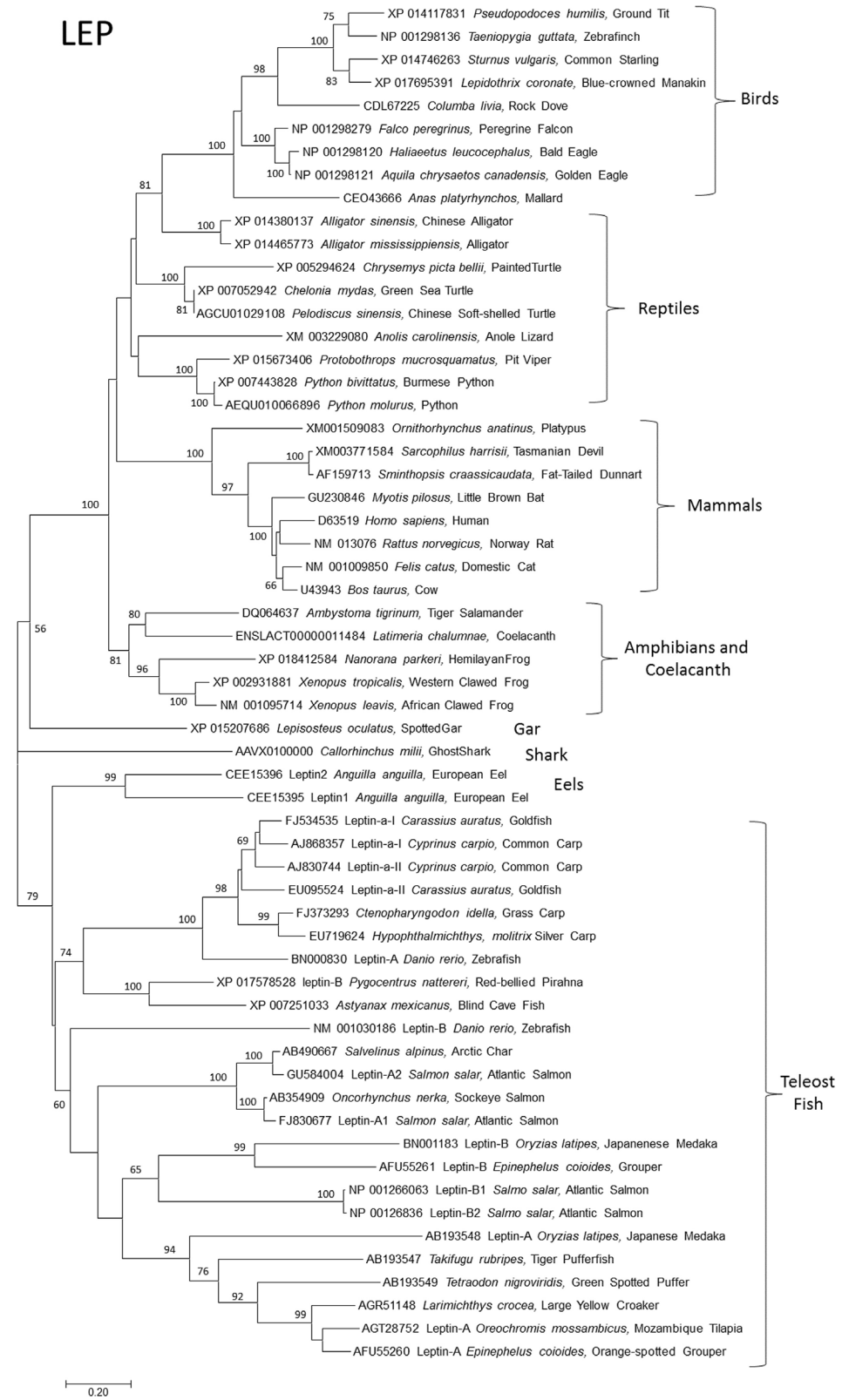

FIGURE 1 | Evolutionary relationships of vertebrate leptins (LEPs). Relationships of 59 amino acid sequences using the neighbor-joining method conducted in MEGA7. Numbers at nodes represent percentage of 500 bootstrap replicates. Nodes with no number indicate bootstrap support of less than 50%. Leptin amino acid sequences were manually aligned in MEGA7 informed by protein structural homologies. GenBank accession numbers represent protein accession.

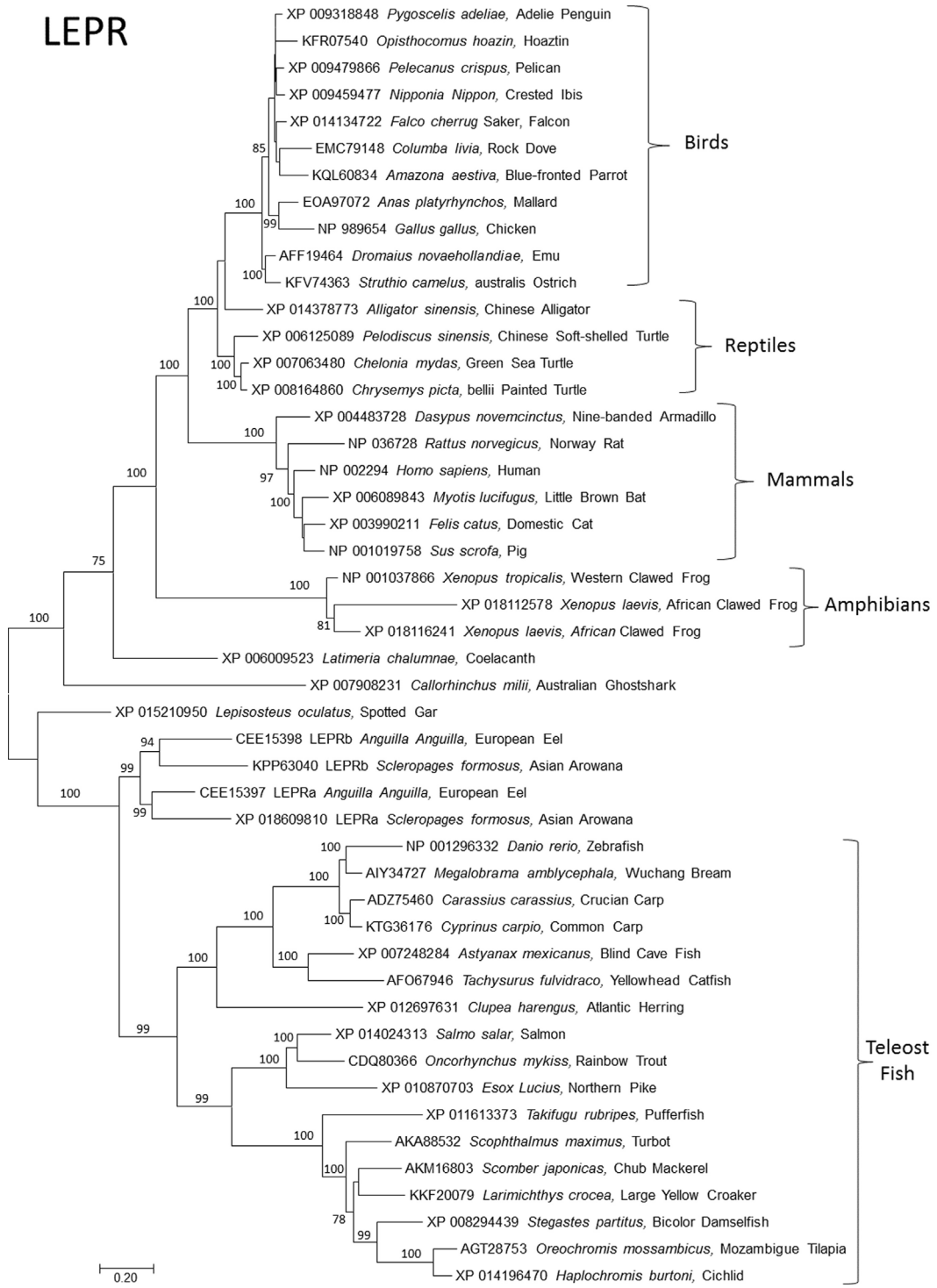

FIGURE 2 | Evolutionary relationships of vertebrate leptin receptor (LEPR). Relationships of 48 amino acid sequences using the neighbor-joining method conducted in MEGA7. Numbers at nodes represent percentage of 500 bootstrap replicates. Nodes with no number indicate bootstrap support of less than 50%. GenBank accession numbers represent protein accessions.

correlation between leptin and leptin receptor transcripts indicated that leptin in birds may not circulate, but instead acts as an autocrine/paracrine factor (45). Several lines of evidence support this hypothesis: bird leptin expression is primarily in CNS (42, 45), bird blood did not activate a sensitive chicken leptin receptor assay, even in birds with extreme adiposity (46), and genes with

high GC content (such as bird leptin genes) are associated with low transcription rates (47). One study that supported a circulating leptin in birds documented that chicken serum and crow blood caused translocation of GFP-labeled STAT 3 to the nucleus in an expressed chicken leptin receptor assay (48). However, potent leptin receptor antagonists tested in chickens effectively block chicken leptin receptor *in vitro* but not *in vivo* (49).

The primary sequences of bird leptins have typically low but recognizable homology with other vertebrate leptins (41, 45), and bird leptin primary structure folds *in silico* into the conserved tertiary structure seen in all leptins (44, 45). Despite this structural conservation (or homology), it otherwise appears that bird leptins do not function similar to leptins in other vertebrates (detailed above). We assert that leptin signaling in birds is fundamentally different than it is in other vertebrates, which suggests that there is a leptin-independent pathway to manipulate energy stores. Birds make large-magnitude changes in adipose stores routinely as a life history strategy. Red knots undergo massive changes in body composition during their 9,000-km migration flights (7), emperor penguin lose ~50% of their body mass during a 4-month fast while incubating eggs on ice (50), and ptarmigan accumulate up to 30% of body mass as lipid in anticipation of winter storms (51). If leptin signaling is reduced/altered in birds, what signals these dramatic changes in lipid mobilization? Other major mammalian adipokine/appetite genes are missing in chickens, including resistin, TNFα, serpine 1, and omentin (52), and ghrelin in falcons (53). Thus, the "usual suspects" for neuroendocrine control of energy stores are either absent or play a fundamentally different role (52).

Concise Methods: Open reading frame (ORF) sequences were obtained for each gene from NCBI gene and aligned to the human ORF using ClustalW (54) in Mega (55). Codon selection was calculated using HyPhy (56) under a Muse-Gaut model (57) and standard Tamura-Nei model (58) for all sites in the *LEP*, *LEPR*, *LEPROT*, and *LEPROTL1*. Conservation scores were calculated using a combination of codon/amino acid fixation rates and dN-dS scores of selective pressure. A score of 2 at any site implies both a greater than 2 SDs above the mean for codon selection and a site that an amino acid is 100% conserved. A score of 0 implies no conservation of the amino acid and below the mean selective pressure (dN-dS). The scores were then put on a sliding window of 21 codons to calculate the top linear motifs within each gene. All numbering throughout the article is based on the full gene sequence of human.

Protein modeling was performed using our previously published LEP–LEPR interaction model (37) combined with I-TASSER- (59) generated extracellular and intracellular domains of LEPR joined by a single-pass transmembrane helix. The endospanin proteins were modeled with I-TASSER (59). Each structure was assessed for structural modeling reliability using a *Z*-score approach of a knowledge-based force field YASARA2 (60) relative to all solved structures of the PDB. Models were generated for both human and mouse and the structures aligned using MUSTANG to calculate sequence and atom alignments [in root mean square deviation (RMSD)]. Each protein was also run for 10 ns of molecular dynamic simulations (MDS) using the AMBER03 force field (61) to assess the average movement in RMSD of the carbon alpha positions throughout the proteins. For all four proteins, evolution was mapped onto protein structures using the sequence alignments above with the ConSurf tools (62). Homology modeling for the *Drosophila* UPD2 and Dome proteins was performed using YASARA (60) and structure scores calculated with the YASARA2 knowledge-based force field. BLAST analysis was performed for invertebrate genomes using all available sequences of ENSEMBL Metazoa BLAST (http://metazoa.ensembl.org/Multi/Tools/Blast?db=core) including Arthopoda, Nematoda, Lophotrochozoa, and Cnidaria. Sequences for metazoa, fungi, and plant endospanin orthologs (*LEPROT* and *LEPROTL1* genes) were also pulled for ENSEMBL annotated orthologs.

EVOLUTION OF LEPTIN SIGNALING: ANALYSIS OF TERTIARY STRUCTURES DETERMINED *IN SILICO*

In the effort to understand the evolution of vertebrate leptin function, often the first data available are sequence data, and we have used these data to model leptins, leptin receptors, and their interaction. Comparing ~100 primary sequences per gene (**Table 1**), we can make some generalizations about structure. Vertebrate leptins demonstrate considerable primary amino acid sequence divergence, but despite this retain high tertiary structure conservation (predicted) when modeled with the hLEP structure (15, 37). We analyzed multiple tertiary structures (generated *via in silico* modeling) and proposed conservation of critical binding sites between leptin and the leptin receptor from fish to human (37). Combining our previous work (37) with our sequence-to-structure-to-function tools (63), we addressed the vertebrate evolution of *LEP*, *LEPR*, and the lesser-studied *LEPROT*. By using a total of 93 vertebrate *LEP* sequences and 89 vertebrate *LEPR* sequences (**Table 1**), we mapped conservation and linear motifs for each gene onto protein structures (**Figure 3**). Leptins contain a conserved disulfide bridge (**Table 1**) and several hydrophobic amino acids that are critical to maintaining the four-helix packing of the protein, even though sequence homology is low (~20%). On the surface of leptins, two linear motifs were identified, one for interaction with the Ig-like domain as suggested by Peelman et al. (64) and the other for the leptin-binding domain of *LEPR*. Utilizing molecular modeling and dynamics, we studied the structural integrity of the leptin protein among many taxa and determined that while sequence is highly divergent, the conservation of several hydrophobic amino acids and the disulfide bridge is sufficient to maintain protein folding in all classes of vertebrates. The leptin receptor conserves protein folding with seven highly conserved and selected linear motifs. There are also 16 conserved sites for posttranslational modification within the receptor (**Table 1**).

We hypothesize that the physiological effects of leptin are induced *via* binding with leptin receptor in a 2–2 molecular interaction, resulting in conformational stability to already dimerized receptors (37, 44, 65–67). There is evidence of higher order oligomerization states such as that of 4:4 stoichiometry (66, 68); however, very little is known about the structural basis for these states. Merging conserved motifs into the model of leptin–leptin receptor interaction, a 2–2 molecular interaction model was created (**Figure 4A**) using previous structures as a guide (66). When viewing the entire leptin receptor protein (**Figure 4A**), docking of leptin to leptin receptor accounted for all motifs. Motif 1 of leptin (red, **Figure 4A**) interacts with motifs 2 and 4 (magenta, **Figure 4A**) of leptin receptor, while motif 2 of leptin (blue, **Figure 4A**) interacts with motif 1 of LEPR (green, **Figure 4A**). Motif 3 of leptin receptor (yellow,

TABLE 1 | Vertebrate *LEP*, *LEPR*, *LEPROT*, and *LEPROTL1* genes analyzed.

Gene	Open reading frame sequences	AA start	AA end	Codons analyzed	Human model *Z*-score	Mouse–human homology (%)	Mouse–human alignment [root mean square deviation (RMSD), Å]	Molecular dynamic simulations carbon alpha (RMSD, Å)	Conserved posttranslational modifications
LEP	93	22	167	13,578	0.28	84.93	0.342	1.33	C117, C167
LEPR	89	29	1,158	100,570	−3	75.35	0.457	2.06	C196, N347, C352, C412, C413, C418, C447, C473, N624, N659, N688, N728, S882, Y986, Y1079, Y1141
LEPROT	150	1	131	19,650	−0.56	94.66	0.346	2.85	–

Z-score is an indicator of how close (number of SDs from the mean) the predicted model fits chemical properties of all previously solved protein structures; RMSD is a measure of average distance between predicted models and native structures.

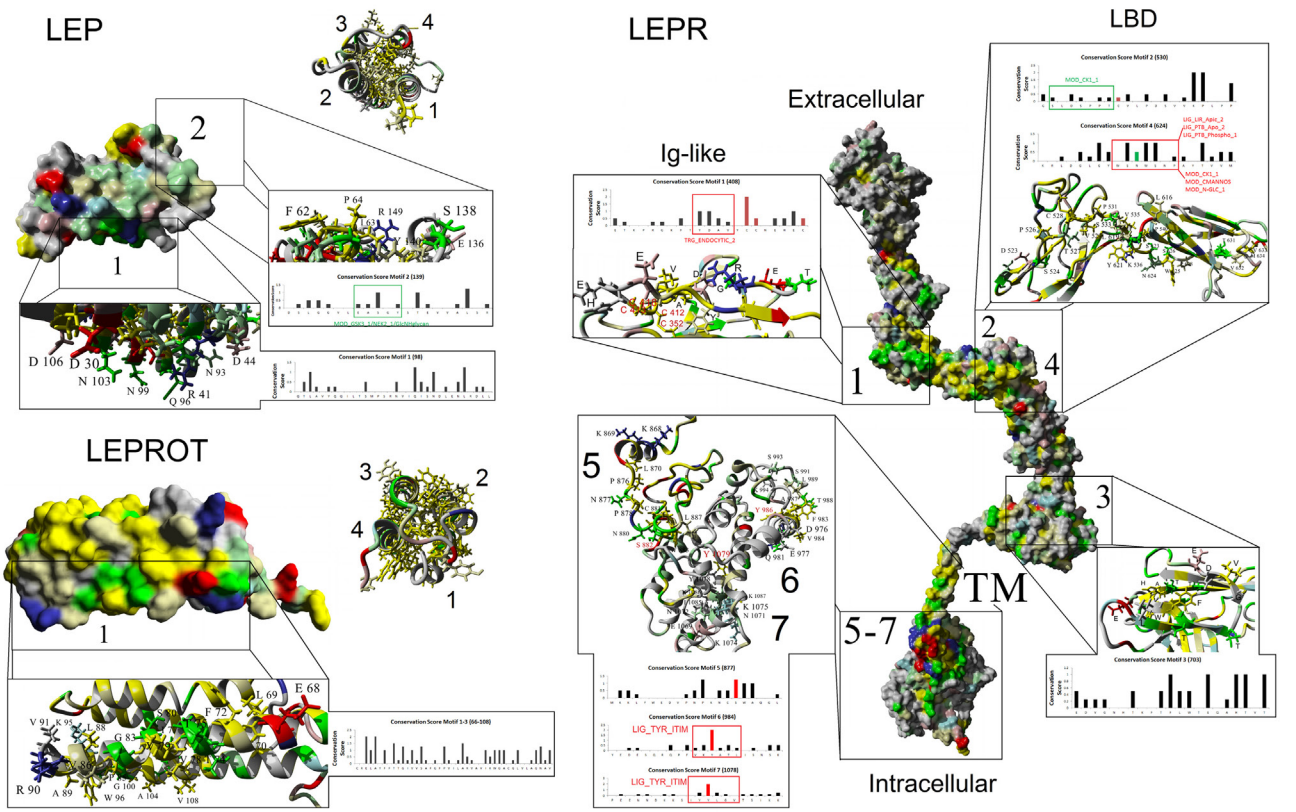

FIGURE 3 | Mapping protein conservation of leptin (LEP), leptin receptor (LEPR), and LEPROT/endospanin. Consurf analysis of LEP (top left), LEPR (right), and LEPROT/endospanin (bottom left) are shown as molecular surface plots of each structure. For LEP and LEPR, a picture of the four-helix bundle with conserved hydrophobic amino acids is shown as a ribbon diagram beside the surface plots of conservation. Top conserved motifs are magnified, identifying conserved amino acids that contribute to each motif. Amino acids are colored as followed: yellow, conserved hydrophobic; red, conserved polar acidic; blue, conserved polar basic; green, conserved hydrophilic; gray, not conserved. Amino acids with known posttranslational modifications are red (disulfide bonds of Cys-C or phosphorylation of Ser-S/Thr-T/Tyr-Y) and green (glycosylation of Asn-N) on the bar graphs of conservation. Predicted eukaryotic linear motifs are boxed and labeled on the bar graphs.

Figure 4A) falls in the fibronectin type III 3 domain, known to control non-LEP-dependent dimerization of LEPR (67, 69, 70). Our models suggest with high probability that this motif contributes to dimerization of the receptor. In this dimer model, LEPR exists on the surface of cells as a dimer controlled by the conserved motif 3, such that the intracellular regions are not in close proximity to each other (**Figure 4B**). On two leptin molecules binding, the receptor is hinged by motif 3 (yellow, **Figure 4C**) to bring together motifs 1, 2, and 4 of LEPR to LEP motifs 1 and 2, resulting in intracellular domains of LEPR brought into close proximity for JAK and STAT activation (**Figure 4C**).

EVOLUTION OF LEPTIN SIGNALING: INVERTEBRATE LEPTIN SIGNALING GENES

Rajan and Perrimon in 2012 described what they thought was a homologous leptin system in *Drosophila melanogaster* (71), through Unpaired 2 (*Upd2*) and Domeless (*Dome*) proteins. Similar to vertebrate *LEPR*s in fish (72), chicken (73), pig (74), cow (75), rat (76), and human (77), the Dome protein of *Drosophila* is critical for germline and follicle cell development through UPD signaling (78). Recent reports of a putative leptin signaling system in *D. melanogaster* through the UPD2 and Dome proteins (71), which are associated with phenotypes from tissue development (79), memory (80), and reproductive systems (78), proposes conserved leptin signaling components in invertebrates. Overlapping functions of vertebrate leptin receptor and Dome proteins suggest possible conserved tertiary structure.

To test homology between vertebrate and invertebrate systems, we modeled UPD2 (**Figure 5A**) and Dome proteins (**Figure 5B**) using our LEP:LEPR models and evaluated conservation of vertebrate motifs in the fly proteins. The UPD2 protein four-helix bundle was homologous to hLEP with some conserved amino acids contributing to packing and others that were surface exposed (**Figure 5A**). Structural alignments of hLEP to UPD2 had

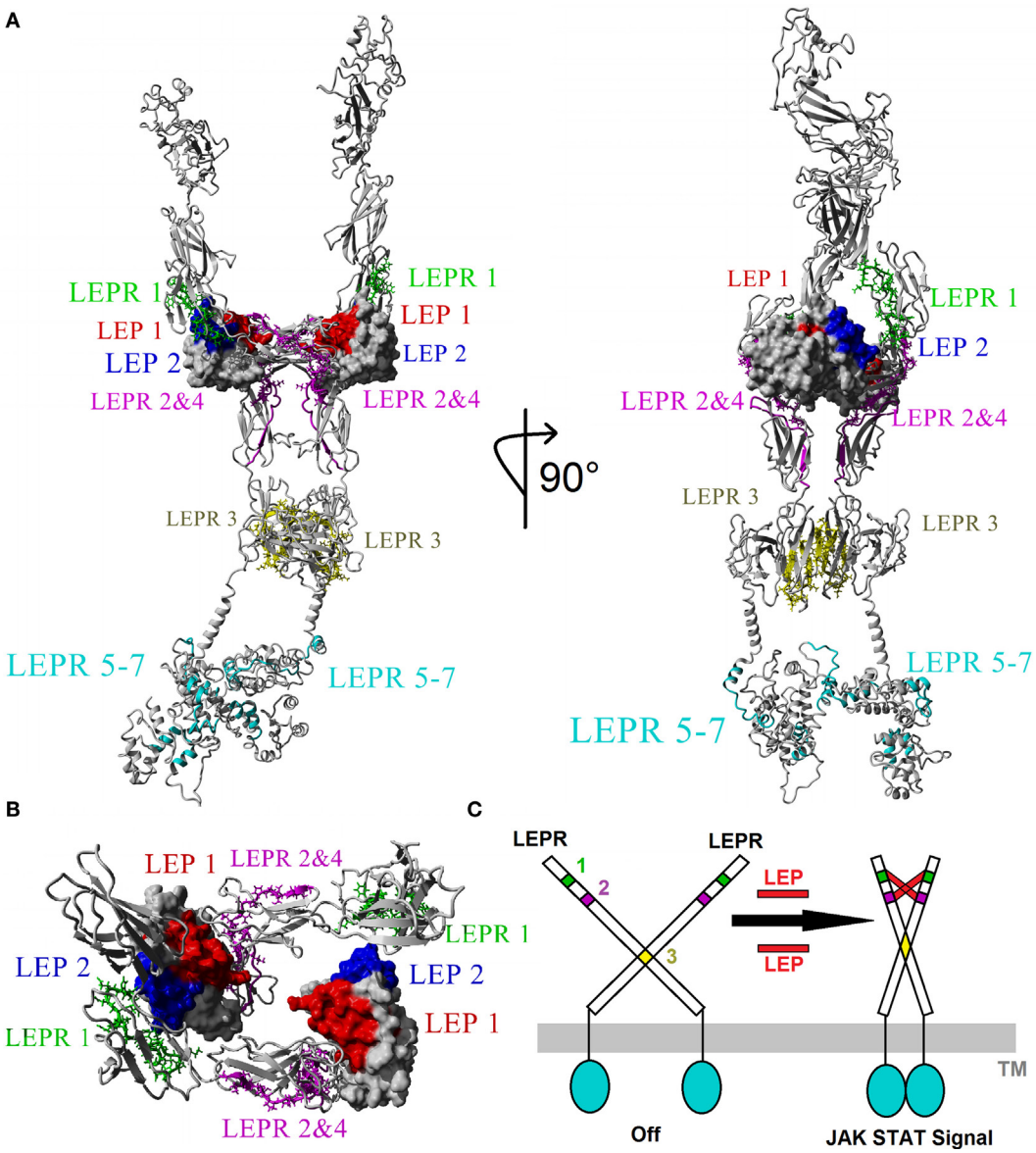

FIGURE 4 | **Modeling the 2xLEP–2xLEPR interaction**. **(A)** Each of the top motifs for leptin (LEP) and leptin receptor (LEPR) are colored in respective color coding. LEP: motif 1, red; motif 2, blue. LEPR: motif 1, green; motif 2 and 4, magenta; motif 3, yellow; motif 5–7, cyan. **(B)** Magnified view of the Ig-like and LBD of LEPR showing the 2–2 interaction model based on vertebrate evolution. **(C)** Model of endogenous dimerized LEPR being activated by LEP binding.

8.24% homology and an alpha carbon average RMSD of 1.627 Å. The UPD2 model had a *z*-score of −1.375, which suggests that the model contains behaviors similar to most known protein structures. Aligning sequence of LEP to UPD2, 9 of 20 amino acids contributing to LEP–LEPR interaction were conserved in UPD2 (cyan, **Figure 5A**), fitting within our expectations based on zebrafish LEP modeling (37). Motif 1 of vertebrate LEP had 8 of 16 conserved amino acids, while motif 2 had 3 of 13 conserved amino acids. These data suggest a high probability of similar fold between leptin and UPD2 with a high number of amino acids conserved that are known to interact with leptin receptor, including motif 1 generated from our vertebrate evolutionary analysis.

The DOME and hLEPR models align with 22.94% homology and an average RMSD of alpha carbons of 0.823 Å (**Figure 5B**). To refine the functional conservation of DOME to hLEPR, we analyzed each of the top seven vertebrate motifs of LEPR. Each of the seven motifs of vertebrate *LEP* were found in the Dome sequence. Motif 1 had 6 of 14 critical amino acids conserved including two cysteine amino acids involved in disulfide bond formation. Motif 2 and 4 involved in the main interaction with LEP had hydrophobic and structural amino acids conserved with the vertebrate sequences. Motif 3 involved in non-LEP-dependent LEPR dimerization had three critical hydrophobic amino acids conserved. Of the intracellular three motifs 5–7, motif 6 was the most highly conserved including the known tyrosine phosphorylation site. To our knowledge, the combination of these seven motifs is not found in any other human protein, thus the high conservation of these motifs in Dome supports the assertion that this is indeed a homolog of vertebrate LEPR.

To probe the existence of the leptin signaling genes in other invertebrate genomes, a BLAST approach for the top motifs was used (**Figure 5C**). BLAST analysis of 54 invertebrate genomes was unable to identify invertebrate homologs. This is likely due to insertions and deletions seen in the motif alignments of Upd2 and Dome (**Figures 5A,B**), decreasing success of BLAST approaches. By using Ensembl Metazoa annotation tools (81), Upd2 homologs were only identified in the 12 sequenced *Drosophila* species, with no other invertebrates having annotated homologs. The Dome protein, however, has homologs found in 48 species of invertebrates according to ENSEMBL (http://metazoa.ensembl.org/Drosophila_melanogaster/Gene/Compara_Ortholog?db=core;g=FBgn0043903;r=X:19676061-19683518;t=FBtr0074756), with 22 being found as similar size of human LEPR and *D. melanogaster* Dome proteins. Outside of invertebrates, no homologs of *Upd2* or *Dome* are yet reported. Contrary to *LEP* and *LEPR*, the *LEPROT* gene is found in many species from invertebrates to plants to fungi (**Figures 5D,E**).

EVOLUTION OF LEPTIN SIGNALING: ENDOSPANIN

Three years after the discovery of the leptin, Bailleul et al. established that the human *LEPR* transcribes a second, non-leptin receptor gene product (82). Initially named leptin receptor gene-related protein (*OB-RGRP*) or *LEPROT* (83), it is transcribed from an alternate AUG within the leptin receptor gene. The alternate start site is out of frame with the leptin receptor transcript, such that it produces a 131 amino acid protein that shares no primary sequence with *LEPR*.

LEPROT [recently renamed endospanin (84)] and its paralog *LEPROTL1* (endospanin 2) are homologous with the yeast vesicle trafficking gene *VPS55* (85). Knockout or disruption of VPS55 in yeast results in generally altered endosomal/vacuole trafficking (85, 86). In vertebrates, endospanin is proposed to specifically regulate endosomal trafficking and surface expression of the leptin receptor. Knockout LEPROT mice express more leptin receptors on the cell surface than wild-type, which makes them hyperresponsive to leptin and resistant to diet-induced obesity (87–89). Further, LEPR protein expression and *LEPROT* genomic copy number are negatively correlated in humans (90), and *LEPROT* may control tissue-specific expression of *LEPR* (91). Both endospanins 1 and 2 are known to interact with Rab13 and Rab8 (92), small G-proteins critical for trafficking between the trans-Golgi network and other cell compartments (93, 94). This suggests that endospanin1/2's role is larger than just regulation of leptin receptor protein.

Is endospanin function conserved among vertebrates? While the BLAST approach did not identify invertebrate *LEP* and *LEPR*, the Ensembl Metazoa annotation (81) identified 48 invertebrate genomes as containing *LEPROT* homologous proteins. Further, 270 sequenced fungi and 44 sequenced plants contain a *LEPROT* homolog. We combined all of these sequences with 150 and 159 vertebrate LEPROT and LEPROTL1 sequences both to build the first tertiary structure prediction for endospanin and to determine critically conserved amino acids throughout eukaryote evolution (**Figures 3** and **5D,E**).

One amino acid is conserved in all 671 sequences studied (red), 13 amino acids are conserved in at least 4 of the 5 taxa (green), 16 in at least 3 of the 5 organism groupings (cyan), and 45 conserved in at least 2 of the 5 organism groupings (gray, **Figures 5D,E**). Using the total of 140 positions in the sequence alignment as shown, red represents 0.7%, green represents 9.3%, cyan represents 11.4%, and gray represents 32.1%, and thus 53.5% of the protein is identified to maintain conservation in at least one of the groupings. This value far exceeds that of LEPR and Dome proteins. Endospanin 1 protein contains a four-helix transmembrane bundle with high conservation of a hydrophobic core of the protein (**Figure 5D**). Noting conserved amino acids on our four-helix model (**Figure 5D**), 12 amino acids were conserved and surface exposed at positions 36, 42, 46, 68, 72, 75, 80, 83, 84, 90, 112, and 120 using the human LEPROT numbering (**Figure 5E**). These residues make up one side of the helix, suggesting possible interaction with another protein at this site.

Another aspect of *LEPROT* genomics likely affects its influence on *LEPR* functional expression (i.e., on the surface of the cell). LEPROT's original designation was as the "leptin receptor overlapping transcript" (82), indicating that *LEPROT* overlapped *LEPR*. Surveying Genbank for *LEPROT* and *LEPR* loci in all vertebrate classes, *LEPROT* overlaps or is adjacent to *LEPR* (within 150,000 bp and no intervening gene) in all cases. The one exception is teleost fishes, where *LEPROT* and *LEPR* are on different chromosomes (**Figure 6**). Gene proximity affects transcription rates (95). Given the high conservation of endospanin sequence,

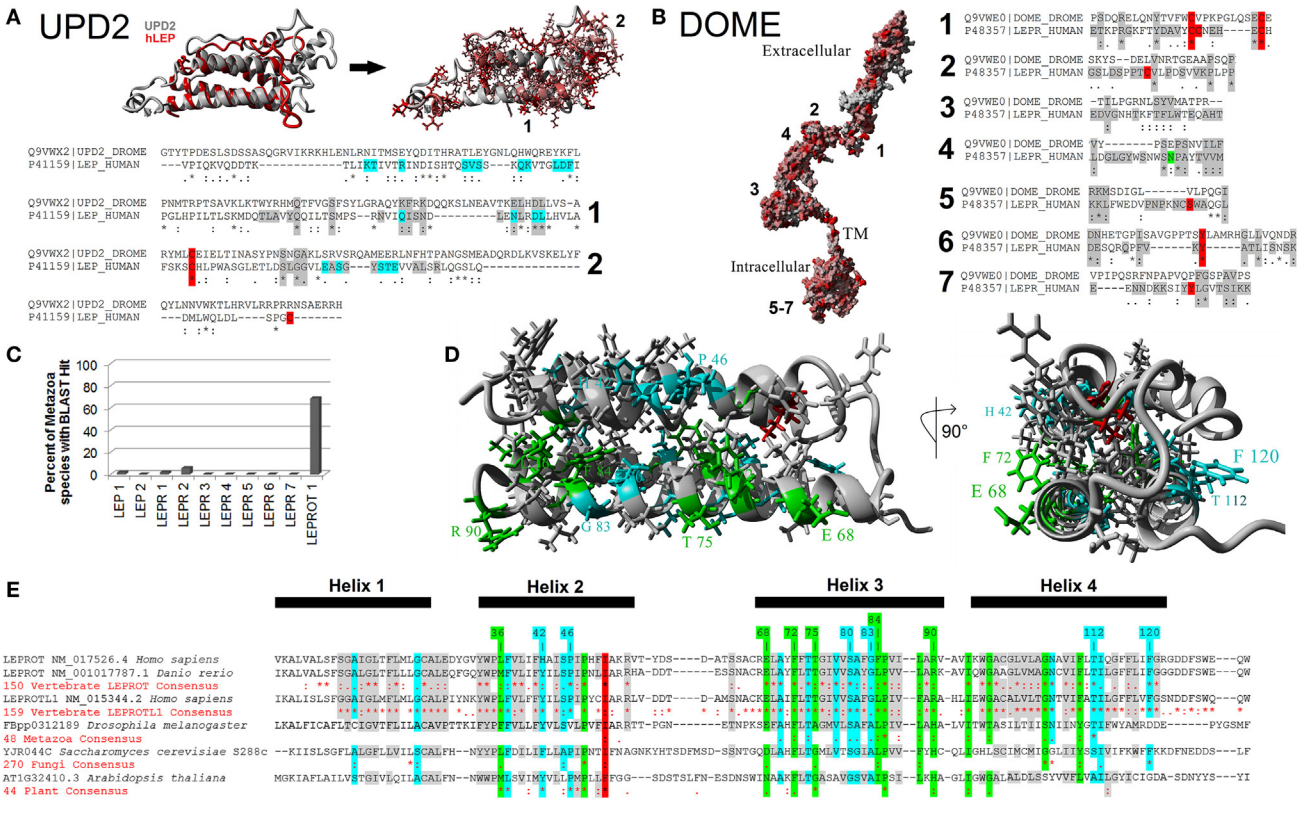

FIGURE 5 | **Defining the metazoan leptin system**. **(A)** UPD2 model (gray) aligned with human LEP (hLEP, red). To the right of the structure overlay are identified amino acids in red that are conserved in the two proteins. Below the models are sequence alignments showing amino acids conserved in the top two motifs (gray), conserved posttranslational modifications (PTMs) (red), and known sites to interact with the receptor (cyan). **(B)** Dome model with amino acids in red conserved with human leptin receptor (LEPR). To the right of the model are sequence alignments showing amino acids conserved in the top seven motifs (gray) and conserved PTMs (red). **(C)** Using the top motifs of LEP, LEPR, and LEPROT, BLAST data for each in invertebrate genomes. **(D,E)** Models of LEPROT, now known as endospanin 1 **(D)**, with conserved amino acids identified **(E)**. Amino acids in red are conserved in all sequences, those in green conserved in at least four of the five groups, those in cyan conserved in at least three groups, and those in gray conserved in at least two groups.

the conservation of its synteny with leptin receptor, and its effect on leptin receptor functional expression (87–89), it is likely that *LEPROT* and *LEPR* coevolved. We assert that because that synteny is broken in teleosts, it may be that control of leptin receptor expression is unique for teleosts among vertebrates.

EVOLUTION OF LEPTIN SIGNALING: WHAT DOES GENE EVOLUTION TELL US ABOUT HUMAN LEPTIN SIGNALING?

Uncovering the evolutionary history of leptin signaling genes and modeling their structure is valuable as a self-contained enterprise, because it sets the stage for understanding functional differences among taxa. However, knowing how these genes are represented among vertebrates also has translational value. Modeling of *Drosophila* Dome as a leptin receptor and finding Dome homologs among other invertebrates provide an avenue for studying leptin signaling in other model systems. How changes in leptin signaling contribute to obesity is certainly complex, with interacting endocrine, neurological, epigenetic, and environmental variables. Added to this complexity is the interaction of multiple leptin receptor isoforms in transporting leptin across the blood–brain barrier. Decreased leptin signaling in the presence of high titers of circulating leptin, or leptin insensitivity/resistance, is often implicated as contributing to human obesity (96, 97). There is growing consensus that reduced blood–brain transport of leptin is a contributing factor to leptin insensitivity in the face of high leptin titers caused by obesity [reviewed in Ref. (98)]. Transport of leptin across the blood–brain barrier is facilitated by leptin receptors with short intracellular domains [commonly referred to as the ObRa paralog, as opposed to the ObRb paralog, which has a complete intracellular domain and is capable of mediating intracellular signaling (99, 100)]. This transport can be inhibited by a soluble form of the leptin receptor (ObRe), capable of binding leptin in serum (101, reviewed in 102). Sequencing cDNAs indicates that these isoforms are the result of alternate splicing of *LEPR* [e.g., Ref. (103)]; however, soluble receptors can also result from cleaving of membrane bound leptin receptors (102). All studies on leptin resistance (to our knowledge) are conducted in mammal models. Comparative study across model systems has the potential to illuminate how receptor paralog diversity contributes to leptin sensitivity. Given that endospanin controls surface expression of long-form LEPR [ObRb (82, 89)], it

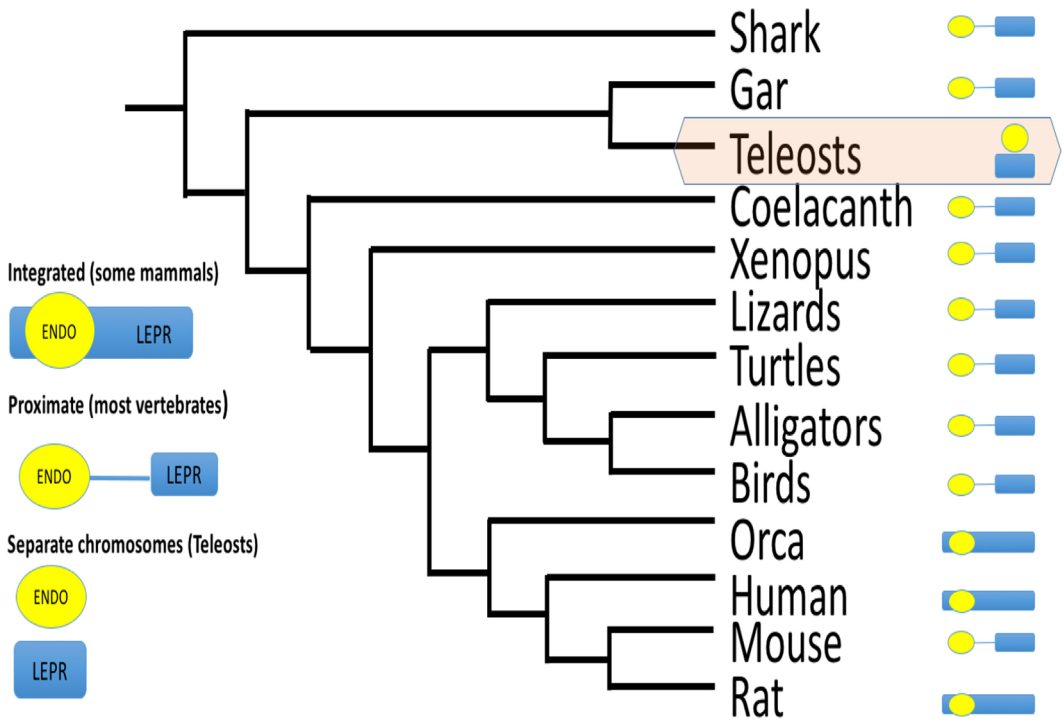

FIGURE 6 | Schematic of gene order for endospanin and leptin receptor (LEPR) among vertebrates. For most vertebrate classes, endospanin (*LEPROT*) is either embedded within the *LEPR* gene, or within 150,000 bp, and without any gene between *LEPROT* and *LEPR*. For teleost fishes only, *LEPROT* and *LEPR* are on separate chromosomes. Data mined from Genbank queries. For example, LEPR search term returns chromosome 1, acc# NC_000001.11 for human *LEPR*, which also maps *LEPROT* within the human *LEPR* sequence, and chromosome 6, acc# NC_07117.6 for zebrafish *LEPR* but chromosome 2, acc# NC_007113.6 for zebrafish *LEPROT*.

may also control expression of the other leptin receptor paralogs. Because endospanin is highly conserved, it represents an opportunity to study leptin sensitivity across models and an avenue to explore for human obesity treatment.

LEPTIN AS AN ADIPOSTAT: LACK OF EVIDENCE IN FISHES

Recently, we made an argument that leptin in fishes does not fit the adipostat model proposed for mammals (16). Importantly, we are distinguishing between leptin's proposed *adipostat* function and its *anorexigen* function. Leptin's anorexigenic function is well documented among fishes (104, 105), amphibians (3), birds (although with non-homologous leptin) (106), lizards (25), and mammals (4). However, central to the adipostat model is the idea that serum leptin is proportional to total adipose stores, because adipose is the major producer of leptin in mammals (4–6). Kurokawa's seminal study first noted that the primary tissue expressing leptin in fish was liver and not adipose (2). This was confirmed in many [e.g., Ref. (31–33)] but not all (107) fish species. Instead of decreasing as fat stores are depleted (as predicted by adipostat), plasma leptin consistently increases with fasting in salmonids (108–110) and flounder (111). Striberny et al. (112) found no evidence that this change in circulating leptin titer was mediated by the CNS. Further, Arctic charr will spontaneously stop feeding in winter even while leptin titers are falling and even if presented with food (11), but will resume eating during the time of year when leptin concentrations are rising (113, 114). The observation that leptin increases at the end of a long fast in fishes runs counter to leptin's documented anorexigenic effects (above). It may be that plasma leptin titers in fasting fish are below the threshold that triggers an anorexigenic response. It is also possible that leptin injections result in supraphysiological concentrations of the hormone in serum, eliciting a response not seen with "normal" leptin signaling (115) and eliciting responses even with artifactual leptins (116). In fishes, increasing serum leptin commonly is interpreted as a signal to mobilize lipid stores in preparation for reproduction, rather than a response to fasting *per se* (113, 114).

Clearly, a decreasing leptin signal during winter and increase prior to reproduction is not consistent with the adipostat model proposed for mammals (2–4). The majority of leptin studies are done on rodents (16), and as such our view of leptin as an adipostat is likely biased by those studies. Rodents have high mass-specific metabolic rates and can only fast for hours, whereas hibernating mammals and ectothermic fishes routinely fast for months. Although leptin is thought to drive the prehibernation anorexia of some, but not all hibernating mammals [reviewed in Ref. (117)], organisms with life histories that are distinctly seasonal (but not necessarily hibernating) may change their set point for leptin sensitivity to accommodate different levels of activity and food availability between seasons (97, 112, 118, 119); thus, an adipostat as described for rodents may not be adaptive for fishes.

Total lipid stores (summing all tissues) clearly are not reflected in serum leptin (as evidenced by fasting fish that increase leptin titers above). However, many researchers (including us) have assumed that liver or gonad is the tissue that contributes to the bulk of serum leptin (because it gives the highest qPCR signal), but that may not be true. Salmonid adipocytes express detectable leptin (110, 120–123), and adipocytes cultured from food-restricted fish secrete significantly more leptin than those from fed fish, reflecting the response of the whole organism (110). Recent knockout models either affect adipose tissue [medaka *lepr* knockout (124)] or do not [zebrafish *lep* knockout (125)]. It may be that the bulk of leptin's serum titer results from expression from liver, but that other tissues express leptin for autocrine/paracrine roles, as proposed for birds (see below). Well-controlled immunological studies, such as those for salmonids and tilapia (110, 126), are needed for a diversity of teleosts, along with how each tissue contributes to the serum/local pool under various physiological conditions.

How does the status of the adipostat model in fishes affect leptin as an adipostat in other vertebrates? We now know that human obesity is influenced by changes in food perception and metabolism after weight loss (127), and therefore a simplistic adipostat feedback loop does not adequately model human phenotypes. Documenting the response of appetite and leptin across vertebrates argues that it is possible to adjust leptin sensitivity, and even presents possible mechanisms for how sensitivity changes (e.g. endospanin).

EMERGING NON-MAMMAL MODELS OF LEPTIN SIGNALING

The obese (*ob/ob)* mouse, a long-standing model of human obesity (128), gained favor for leptin studies after Freidman's laboratory cloned the truncated *LEP* gene (1). Together with the diabetic (*db/db*) mouse, a *LEPR*-deficient model, leptin administration effects have been demonstrated repeatedly. Intraperitoneal leptin injections in *ob/ob* mice causes 30% decrease in body mass, and *db/db* mice are similar to controls (5). Leptin's pleiotropy was detailed using these models, and as a result, we now know that leptin affects reproduction, immune function, bone growth/resorption, and metabolic rate [reviewed in Ref. (129–132)]. The long-term normalizing effects of peripheral leptin injections on hLEP congenital deficiency reflect those in the *ob/ob* mouse [reviewed in Ref. (133)]. There are lines of fish (134) and birds (135) selected for high and low adiposity; however, few *LEP* and *LEPR*-null models are available for comparative (leptin) studies.

Our group used morpholino knockdown to generate zebrafish embryos with reduced leptin signaling (136, 137). We documented severe developmental defects in response to knockdown of *LEPA* or *LEPR*. Morphants were characterized by malformed sensory structures, bent notochord, poor yolk absorption, and low metabolic rate; these effects were rescued by coinjection of recombinant zebrafish leptin (136, 137). Microarray analysis of leptin-A "morphant" and "rescue" expression data identified differentially expressed genes that correspond to leptin signal transduction pathways [GnRH signaling, fatty acid metabolism, glycolysis/gluconeogenesis, MAP kinase, phosphoinositol signaling (138)]. The recent availability of CRISPR technology allowed direct comparison of zebrafish gene knockdown vs. gene knockout. "Morphant" and "mutant" phenotypes generally do not agree when targeting the same gene; typically morphants do not emulate mutant phenotypes (139, 140). Zebrafish morphants targeting (apparently) unrelated genes often share combinations of morphological markers ranging from disrupted eye, ear, and brain development; irregular body/tail curvature; or enlarged yolk (139, 141, 142). Non-specific MO off-target activity upregulates zebrafish *tp53*, which may induce apoptosis and global changes in gene expression (139, 143). For these reasons, we are hesitant to pursue antisense technologies. Similar to recent work by other laboratories, we are opting for knockout technologies as a means to generate comparative null models for many leptin signaling genes. Chisada et al. produced the first *LEPR* mutant fish, using the TILLING approach in medaka (124). Adult medaka *LEPR* mutants are hyperphagic, have elevated NPYa and AGRP, and decreased POMC mRNAs. Liver and muscle lipid does not increase in the *LEPR* mutants, but they accumulate visceral fat as adults (124). The medaka data are consistent with a mammalian adipostat model, but zebrafish are not. Michel et al. recently characterized an established (144) *LEPR* TILLING mutant in zebrafish and also generated CRISPR mutants for *LEPA, LEPB,* and *LEPR* (125). *LEPR*-null adults have no differences from wild-type in adiposity, body size, growth rate, mating success, or feeding behavior. However, *LEPR* mutants have altered glucose metabolism, and both *LEPR* and *LEPA* larvae have increased β-cell number (125).

CONCLUSION AND FUTURE DIRECTIONS

Comparative leptin endocrinology has matured in the 11 years since the first non-mammal leptin was cloned. All major vertebrate classes are now represented in cloned leptins and leptin receptors, and investigation of invertebrate leptin signaling is beginning. Protein structures have been modeled for leptin, leptin receptor, and endospanins across an extensive evolutionary timescale, but models (although useful) are simply predictions to be tested. Now that we have identified conserved motifs and conserved sites of leptin–leptin receptor interaction, these predictions should be tested with *in vitro* functional assays.

The bird leptin problem has been solved in a genomic sense, but is just initiating physiologically. Now that the bird receptor assays (49) can be used with homologous ligands (hopefully soon), we should learn if birds are truly different among vertebrates in leptin signaling. We assert that understanding the endocrinology of how birds manipulate lipid stores will pay dividends in comparative endocrinology, agriculture, and human disease. Robust data from decades of research demonstrate that many species of birds perform large-scale manipulation of energy stores, and preliminary (but compelling) data indicate that they are doing so either without leptin (or other known adipokines) or by using leptin in a fundamentally different way (e.g., through an uncharacterized pathway). If leptin signaling in birds is truly different, it means that there is another way that vertebrates manipulate energy stores and thus potentially new avenues to pursue that will help us understand human obesity.

Amphibian leptin models are well developed with homologous recombinant leptin and receptor assays (15, 24), but relatively little is known for reptiles. These groups appear to adhere to the lipostat model, while birds and teleost fishes may not. As such, more species diversity in amphibian and reptile leptin studies could be very important in understanding leptin function as an adipostat.

In fishes, we are now past the point where one's fish species of choice can be interpreted as representative of all fishes. Given that bony fishes have been on the planet ~370 MY longer than modern mammals (www.timetree.org), it is not surprising that they may be diverse in their leptin signaling. Phylogenetic analyses make it clear that teleost fishes are diverse in the structures of their leptin, leptin receptor, and *LEPROT* genes, and it is likely that reported differences among species represent true species divergence rather than methodological idiosyncrasies. Although we argue that there is a lack of evidence for adipostat function in fishes, the future may reveal that an "origin(s)" of that status within a fish clade, and we simply need to sample fish diversity more completely (e.g., non-teleost fishes need attention).

If we are to move forward, we must have comparable variables to assess species diversity. As such, reliance on relative qPCR for expression data does not allow quantitative comparisons among species; the community needs well-validated ELISAs (such as that developed for salmonids and tilapia) for multiple species. In this same light, the non-coding regions near leptin and leptin receptor need to be studied with more detail to gain an understanding of how expression is controlled throughout evolution. We need to pursue knockout models in non-mammals for laboratory approaches comparable to those using *ob/ob* and *db/db* mice. Finally, we need to measure leptin signaling responses of unmanipulated animals in the field and take advantage of the tremendous diversity of life histories that are well suited for leptin questions [The Krogh Principle (145)]. In doing so, the comparative community will contribute to understanding of human obesity similar to how *Drosophila* studies contributed to genetics, how shark-rectal gland contributed to kidney function, or how the squid giant axon contributed to neurobiology.

ETHICS STATEMENT

All procedures performed by the authors that used animals were approved by the institution's IACUC.

AUTHOR CONTRIBUTIONS

All authors contributed to drafting and editing of the manuscript. JWP completed all molecular modeling.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00058/full#supplementary-material.

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Leptin Induces Mitosis and Activates the Canonical Wnt/β-Catenin Signaling Pathway in Neurogenic Regions of *Xenopus* Tadpole Brain

*Melissa Cui Bender, Christopher J. Sifuentes and Robert J. Denver**

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

In addition to its well-known role as an adipostat in adult mammals, leptin has diverse physiological and developmental actions in vertebrates. Leptin has been shown to promote development of hypothalamic circuits and to induce mitosis in different brain areas of mammals. We investigated the ontogeny of leptin mRNA, leptin actions on cell proliferation in the brain, and gene expression in the preoptic area/hypothalamus of tadpoles of *Xenopus laevis*. The level of leptin mRNA was low in premetamorphic tadpoles, but increased strongly at the beginning of metamorphosis and peaked at metamorphic climax. This increase in leptin mRNA at the onset of metamorphosis correlated with increased cell proliferation in the neurogenic zones of tadpole brain. We found that intracerebroventricular (i.c.v.) injection of recombinant *Xenopus* leptin (rxLeptin) in premetamorphic tadpoles strongly increased cell proliferation in neurogenic zones throughout the tadpole brain. We conducted gene expression profiling of genes induced at 2 h following i.c.v. injection of rxLeptin. This analysis identified 2,322 genes induced and 1,493 genes repressed by rxLeptin. The most enriched Kyoto Encyclopedia of Genes and Genomes term was the canonical Wnt/β-catenin pathway. Using electroporation-mediated gene transfer into tadpole brain of a reporter vector responsive to the canonical Wnt/β-catenin signaling pathway, we found that i.c.v. rxLeptin injection activated Wnt/β-catenin-dependent transcriptional activity. Our findings show that leptin acts on the premetamorphic tadpole brain to induce cell proliferation, possibly acting *via* the Wnt/β-catenin signaling pathway.

Keywords: leptin, neurogenesis, *Xenopus*, metamorphosis, Wnt/β-catenin

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***Correspondence:**
Robert J. Denver
rdenver@umich.edu

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INTRODUCTION

Leptin is a protein hormone secreted by adipocytes that signals energy stores to the adult brain. The plasma concentration of leptin fluctuates in proportion to fat mass, and leptin acts on feeding control centers in the hypothalamus to suppress food intake and increase whole body metabolism (1). Leptin (*lep*) and its receptor (LepR) are widely expressed, and the hormone has been shown to have pleiotropic actions in physiology and development of diverse vertebrate species (2, 3). The actions of leptin are mediated by the long form of the LepR (LepRb), which engages several intracellular signaling pathways, especially the Janus kinase 2/signal transducer and activator of transcription (JAK2/STAT) (4, 5) and phosphoinositide 3-kinase (PI3K) signaling pathways (6, 7).

Recent findings support that leptin has important roles in neurological development, especially development of leptin-responsive feeding control circuits in the hypothalamus. Leptin-deficient

mice (*ob/ob*) have reduced brain weight, volume, and DNA content that can be restored by injecting leptin (4, 8). Leptin induces mitosis in several brain areas in rodents (8–10), and in tissue culture (11, 12), and this role for leptin is likely related to the brain size defects seen in *ob/ob* mice. The hormone also promotes formation of neuronal projections among hypothalamic nuclei involved with feeding (13–16).

In rodents, serum leptin concentration increases markedly in the neonate, then declines in the juvenile adult (17–19). This "postnatal leptin surge" may play a critical role in the development of the hypothalamic feeding control circuit (13, 15, 16). Cells in the ventricular zone (VZ)/subventricular zone (SVZ) of the third ventricle (3V) of neonates express functional LepR; LepR mRNA expression declines in the VZ/SVZ through development then appears in the arcuate nucleus and ventromedial hypothalamus (18). The LepR is expressed within the VZ of the 3V in embryonic/fetal brain (18, 20) and these neural progenitor/stem cells (NSCs) may be precursors of hypothalamic feeding control and hypophysiotropic neurons of the adult (21).

Genes for *lep* have now been isolated from numerous mammalian species, birds, reptiles, amphibians, and fishes (2, 3, 22). Our earlier findings in *Xenopus* support that the adipostat function of leptin was present in the earliest tetrapods (23, 24). By contrast, a role for leptin in feeding and energy balance in fishes remains unresolved (2, 3). Like mammals, tadpoles of *Xenopus laevis* develop competence to respond to leptin signaling during the postembryonic developmental period of metamorphosis (Melissa Cui Bender and Robert J. Denver, unpublished data). We found that functional LepR is expressed in regions surrounding the 3V of premetamorphic tadpole brain, suggesting that leptin can act within tadpole neurogenic zones. In the current study, we investigated whether leptin can promote mitosis in developing *Xenopus* tadpole brain by administering recombinant *Xenopus* leptin (rxLeptin) to premetamorphic tadpoles by intracerebroventricular (i.c.v.) injection, then we analyzed cells in M phase of the cell cycle using immunohistochemistry (IHC) for phosphorylated histone 3 (pH3). We also conducted a gene expression screen for early (2 h after i.c.v. rxLeptin injection) leptin-induced transcriptional changes in tadpole preoptic area/hypothalamus. This screen identified the canonical Wnt/β-catenin signaling pathway as the major intracellular signaling pathway induced by leptin in premetamorphic tadpole brain. Using electroporation-mediated (EM) gene transfer of a Wnt/β-catenin-responsive reporter plasmid into tadpole brain, we provide additional evidence that leptin activates functional Wnt/β-catenin signaling.

MATERIALS AND METHODS

Animal Care and Use

We obtained *X. laevis* tadpoles from in-house breeding and raised them in dechlorinated tap water maintained at 21–23°C with a 12L:12D photoperiod. Tadpoles were fed frog brittle twice daily (NASCO, Fort Atkinson, WI, USA) and developmental stages were determined using the Nieuwkoop–Faber (NF) staging table (25). We anesthetized NF stage 50 *X. laevis* tadpoles (premetamorphic tadpoles) in a buffered solution of 0.002% benzocaine (Sigma) before administering i.c.v. injection of rxLeptin [produced as described by Crespi and Denver (23)], or plasmid injections for EM gene transfer (described below). For i.c.v. injection, we used a Drummond microinjector to deliver 50–150 nL of solution containing 0.6% saline, rxLeptin (20 ng/g BW) or plasmid DNA, plus 0.01% fast green dye to the area of the 3V as described previously (23, 24, 26). We chose this dose of rxLeptin based on our previously published work that showed that i.c.v. injection caused a dose-dependent suppression of food intake in the Western spadefoot toad, with 20 ng/g BW rxLeptin causing maximal suppression (23). Animals were killed by immersion in 0.1% benzocaine for 2 min before tissue harvest. All procedures involving animals were conducted under an approved animal use protocol (PRO00006809) in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Michigan.

RNA Isolation for Gene Expression Analyses

For developmental analysis of *lep* mRNA, we extracted total RNA from whole animals beginning at NF stage 45. For NF stages 45–54, we pooled three animals per replicate and for NF stages 58–66, one animal per replicate ($n = 5$–6/NF stage). We also dissected and isolated total RNA from the portion of the carcass containing the fat pads (we removed tail and other organs; we refer to this as "adipose tissue"), liver, brain, and gut from tadpoles throughout metamorphosis (NF stages 50, 54, 58, 62, 66) for analysis of *lep* mRNA ($n = 6$/NF stage). For analysis of gene expression by microarray and reverse transcriptase quantitative real-time PCR (RTqPCR), we injected tadpoles i.c.v. with 0.6% saline or rxLeptin (20 ng/g BW), and 2 h later, we killed the animals, removed the brain, and microdissected the middle region of the brain ($n = 6$/treatment; the region of the diencephalon containing the preoptic area and hypothalamus, the neuroendocrine and feeding control regions of the brain) (27). We isolated RNA using the Trizol reagent (Invitrogen) following the manufacturer's instructions.

For RTqPCR analysis of gene expression, we developed SYBR Green assays that spanned exon–exon boundaries for each gene. We treated 1 μg total RNA with 1.5 U RNase-free DNase I (Roche, Indianapolis, IN, USA) to digest contaminating genomic DNA, then reverse-transcribed the RNA into cDNA using 250 ng random hexamers and Superscript II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions [see also Ref. (28, 29)]. Minus RT controls were included. Oligonucleotides used for RTqPCR are given in **Table 1**. We conducted quantitative, real-time PCR using the ABsolute™ Blue qPCR SYBR Green Low Rox Mix (ABgene), and reactions were run on an ABI 7500 Fast qPCR machine. Relative quantities were determined using standard curves generated with pooled cDNAs.

IHC for pH3 and Phosphorylated STAT3 (pSTAT3)

We analyzed the effects of i.c.v. injection of rxLeptin on mitosis in the premetamorphic tadpole brain using IHC for pH3. The serine

TABLE 1 | Sequences of oligonucleotide used to validate microarray results by SYBR Green reverse transcriptase quantitative real-time PCR.

Gene name	Forward	Reverse
ctnnb1	5′ GAATTGGCCACTCGAGCAA 3′	5′ ACCTGGTCCTCGTCATTAAGC 3′
sox8	5′ GGGCAAACTGTGGCGTTTA 3′	5′ CTCAGCCTCCTCCACAAAGG 3′
socs3	5′ AGAACCTACGCATCCAGTGTGA 3′	5′ GGCACTTCGTGGGTCAGTCT 3′
arrb2	5′ TCAGTCAGACAATACGCAGACATC 3′	5′ GCCACCGGGCCACTTGTAC 3′
dab2	5′ CAGCAGCTGCCACTGGAA 3′	5′ ATTGTTGTGCGTGAGAGTTTAC 3′
mad2l1	5′ AAGAACTTGCAACCGTTAAACT 3′	5′ TCACGAACAATGCCGTCTTTC 3′
rpL8	5′ TTTGCTGAAAGAAATGGCTACATC 3′	5′ CACGGCCTGGATCATGGA 3′

10 at the amino terminus of H3 is phosphorylated during late G2 through M phase of the cell cycle and is therefore used to detect dividing cells [for *X. laevis* brain: (30–32)]. We administered two i.c.v. injections of 0.6% saline or rxLeptin (200 ng/g BW) (23) into the region of the 3V of NF stage 50 tadpoles; the second injection was 24 h after the first, and tadpoles were killed 48 h after the first injection (i.e., 24 h after the second injection). We then processed brains for IHC for pH3 following the method of Denver et al. (30). Briefly, we prepared 10 μm thick transverse cryosections through entire tadpole brains and immunostained sections with a rabbit antiserum against human pH3 (1:500; Cat. #0650 EMD Millipore, Billerica, MA, USA). Primary immune complexes were detected using a secondary antibody conjugated with Cy3 (1:500, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). For IHC for pSTAT3, a marker for activated LepR, we administered i.c.v. injections of 0.6% saline or rxLeptin (20 ng/g BW) (24) to NF stage 50 tadpoles and collected brains 1 h later for fixation and sectioning. We conducted IHC for pSTAT3 on *Xenopus* brain as described previously (24) using an antiserum generated against a phosphopeptide AP[pY]LK from mouse STAT3 (Tyr705; 1:200; Cat. # 9131, Cell Signaling), which is 100% conserved with *X. laevis* STAT3 (24, 33). Images of immunostained slides were captured using an Olympus XI-81 microscope, and the total number of pH3 positive cells per brain was counted. Five brains were analyzed per treatment. *Xenopus* neuroanatomy is based on Tuinhof et al. (34) with modifications by Yao et al. (35).

DNA Microarray Analysis of Gene Expression after i.c.v. rxLeptin Injection

We conducted DNA microarray analysis on total RNA isolated from the preoptic area/hypothalamus of NF stage 54 *X. laevis* tadpoles (~0.2 g BW) 2 h after i.c.v. injection of 0.6% saline or rxLeptin (20 ng/g BW). We isolated total RNA from microdissected brain regions of three replicate pools (three brains per pool) per treatment. Samples were hybridized to the Affymetrix GeneChip *Xenopus laevis* Genome 2.0 Array ($n = 3$/treatment) at the Affymetrix and Microarray Core Facility at the University of Michigan. We selected a subset of the induced genes to cover a range of expression values (fold change) for validation by RTqPCR (**Table 1**).

Microarray Data Background Correction, Normalization, and Expression Quantification

We used the Robust Multichip Average method (36) from the Affy package (v1.52.0) (37) to calculate background-corrected, normalized expression values. Genefilter (v1.56.0) (38) was used to remove uninformative probesets (internal control probesets, or probesets with low variance or background level expression values).

Microarray Data Differential Expression Analysis and Annotation

Principal component analysis plots of the normalized, filtered samples showed samples of the same treatment (saline or rxLeptin) clustered together, and that the two treatments clustered separately. We used the limma package (v3.30.2) (39) to apply empirically derived array weights to individual samples, which were then used to calculate differential expression values (40). Annotations for each gene were added using the annotation file supplied by Affymetrix for the *X. laevis* Genome 2.0 Array.

Gene Ontology (GO) and Pathway Analysis

Gene ontology term enrichment analysis was conducted using a $\log_2$ fold change ($\log_2$FC) ranked list from limma, containing genes with a Benjamini and Hochberg (BH)-corrected p-value, or false-discovery rate (FDR) <0.05, as input into clusterProfiler (v3.2.4) (41). This analysis determines which molecular function, biological process, or cellular component GO terms are positively or negatively enriched in rxLeptin-treated samples compared with saline-treated controls, at a p-value < 0.05, while taking into account the magnitude and direction of change. Due to the hierarchical and redundant nature of GO terms, we obtained a summarized list of GO terms using Revigo to remove redundant terms (42).

We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) (43) pathways enrichment analysis using a $\log_2$FC ranked list of all differential gene expression data from limma (FDR < 0.05) as input into clusterProfiler (v3.2.4) (41). An additional KEGG module (highly annotated functional units of a metabolic network) enrichment analysis was conducted using the same $\log_2$FC ranked list as input into clusterProfiler (v3.2.4). Gene expression values for KEGG pathways were plotted on the respective pathways using the pathview package (v1.14.0) (44).

Enriched biological processes GO terms, KEGG pathways, and KEGG modules were displayed using the Enrichment Map application (v2.2.0) (45) in Cytoscape (v3.4.0) (46).

EM Gene Transfer and *In Vivo* Reporter Assay

We used EM gene transfer (30, 47–50) and i.c.v. microinjection of 0.6% saline or rxLeptin (20 ng/g BW) to investigate whether

leptin can activate the Wnt/β-catenin signaling pathway in tadpole brain *in vivo*. We divided NF stage 50 tadpoles into three groups for bipolar electroporation. Tadpoles were electroporated with a DNA mixture containing pRenilla-null (50 ng/μL; for dual-luciferase assay normalization), pCMV-eGFP (500 ng/μL; to visualize transfection efficiency), and one of the following firefly luciferase reporter vectors (each at 1 μg/μL): pGL4.23 empty (negative control), GAS-luciferase (positive control for leptin activity) (23, 24) or pGL4.23-6TCF (reporter of activated canonical Wnt/β-catenin signaling). The pGL4.23-6TCF vector contains six T cell factor (TCF) sites upstream of a minimal promoter driving luciferase expression, and therefore reports activation of the canonical Wnt/β-catenin signaling pathway (gift of Dr. Ken Cadigan). A separate group of tadpoles received pGL4.23-6TCF, pRenilla-null, and pCMV-EGFP plus a constitutively active β-catenin expression vector pcDNA3-S33Y β-catenin (51) (gift of Dr. Eric Fearon) or an empty vector (pCMVneo-empty). Plasmid concentrations for electroporation are based on Yao et al. (52). Twenty-four hours after EM gene transfer we screened tadpoles for strong GFP expression, and then separated them into eight groups: pGL4.23-empty reporter, saline, or rxLeptin; GAS-luciferase, saline, or rxLeptin; pGL4.23-6TCF, saline, or rxLeptin; pGL4.23-6TCF, pCMVneo-empty, or pcDNA3-S33Y β-catenin. Tadpoles receiving i.c.v. injections were given saline or rxLeptin (20 ng/g BW). Two hours after injection, tadpoles were killed and brains harvested for dual-luciferase assay following the protocol described by Yao et al. (52). We had a sample size of eight tadpoles per treatment.

Data and Statistical Analysis

We analyzed data for gene expression by RTqPCR, pH3 cell counts and dual-luciferase assay data by one-way ANOVA or by unpaired Student's *t*-test ($p < 0.05$). Derived values were $\log_{10}$-transformed before statistical analysis. Fisher's least squares difference *post hoc* test was used to separate the means following ANOVA. We used the SYSTAT 13.0 computer program (SPSS Inc.).

RESULTS

Ontogeny of *lep* mRNA in Tadpoles during Metamorphosis, and Effects of rxLeptin on Mitosis in Premetamorphic Tadpole Brain

Whole body *lep* mRNA increased 3.3-fold between stages 48 and 50, which is immediately before the onset of metamorphosis ($p < 0.001$, ANOVA; **Figure 1A**). The mRNA level increased further (7.6-fold) from stages 50 to 54, and remained elevated throughout metamorphosis and in the postmetamorphic frog (NF stage 66). The pattern of changes in *lep* mRNA in adipose tissue during metamorphosis was similar to whole body (**Figure 1B**; $p = 0.002$, ANOVA). By contrast, *lep* mRNA decreased in liver ($p = 0.001$) and was low and unchanged in brain throughout metamorphosis (**Figure 1B**). These findings, and other results from our laboratory support that fat tissue is the major source of *lep* mRNA in the tadpole (Melissa Cui Bender and Robert J. Denver, unpublished data). This developmental pattern of *lep* mRNA paralleled changes in cell proliferation in tadpole brain during metamorphosis (**Figure 2A**) (30). Injection of rxLeptin strongly increased pH3-immunoreactivity (35-fold; $p < 0.0001$; Student's unpaired *t*-test) in cells within the VZ/SVZ throughout the tadpole brain (**Figures 2B,C**). Also, i.c.v. rxLeptin injection (1 h) induced the appearance of pSTAT3 immunoreactivity (pSTAT3-ir) in the VZ/SVZ in the region of the 3V (**Figure 2D**), supporting the expression of functional LepR in these cells. The inset in **Figure 2D** shows a high magnification view of the VZ/SVZ with elongated cells undergoing migration out of the neurogenic zone.

i.c.v. rxLeptin Injection Induces Rapid Transcriptional Responses in Early Premetamorphic (NF Stage 54) Tadpole Brain

To investigate the molecular basis for leptin action on tadpole brain, we conducted microarray analysis on the region of the diencephalon containing the preoptic area/hypothalamus of NF stage 54 tadpoles at 2 h following i.c.v. injection of 0.6% saline or rxLeptin injection (20 ng/g BW). The transformed log ratio (M) and mean average (A) plot (MA plot) in **Figure 3A** shows that the data are normally distributed, with a zero-centered transformed log ratio. Additionally, this plot shows that genes with a significant BH-adjusted *p*-value (FDR < 0.05), plotted in red, are distributed evenly across MA expression levels, indicating an ability to detect differentially expressed genes at any range of expression. This plot also indicates that an absolute log2 fold change (log2FC) as low as ~0.5 can be detected with confidence (FDR < 0.05). This analysis identified 2,322 induced and 1,493 repressed genes (Table S1 in Supplementary Material). The top 20 induced and repressed genes are given in **Tables 2** and **3**, respectively. The microarray dataset has been deposited in the Gene Expression Omnibus archive at the National Center for Biotechnology Information (GEO accession #GSE97243).

Using these differential expression data, we conducted several enrichment analyses, allowing us to classify the global gene regulation changes into several general categories (general biological process regulation, metabolism, cellular components, adhesion, immune signaling, cell signaling, development, cell division and neurogenesis, and others) and highlighting several biological processes or pathways of interest. The enrichment map in **Figure 3B** shows these data: enriched biological process GO terms (*p*-value < 0.15), KEGG pathways (*p*-value < 0.15), and KEGG modules. We validated a subset of the induced genes by RTqPCR (**Figure 4**).

The top ten GO terms involved developmental processes (**Figure 3B**; **Table 4**; Table S2 in Supplementary Material). Noteworthy is the Wnt/β-catenin signaling pathway, which was found by each enrichment analysis (biological process GO, KEGG pathway, KEGG module; **Figure 5**). Also, signaling by two TGFβ family members, bone morphogenetic protein (BMP) and activin, was among the enriched KEGG modules (**Figure 6**; Table S3 in Supplementary Material; tab KEGGmodules.filt). The hedgehog and insulin signaling pathways were also found to be enriched (Figures S1 and S2 in Supplementary Material).

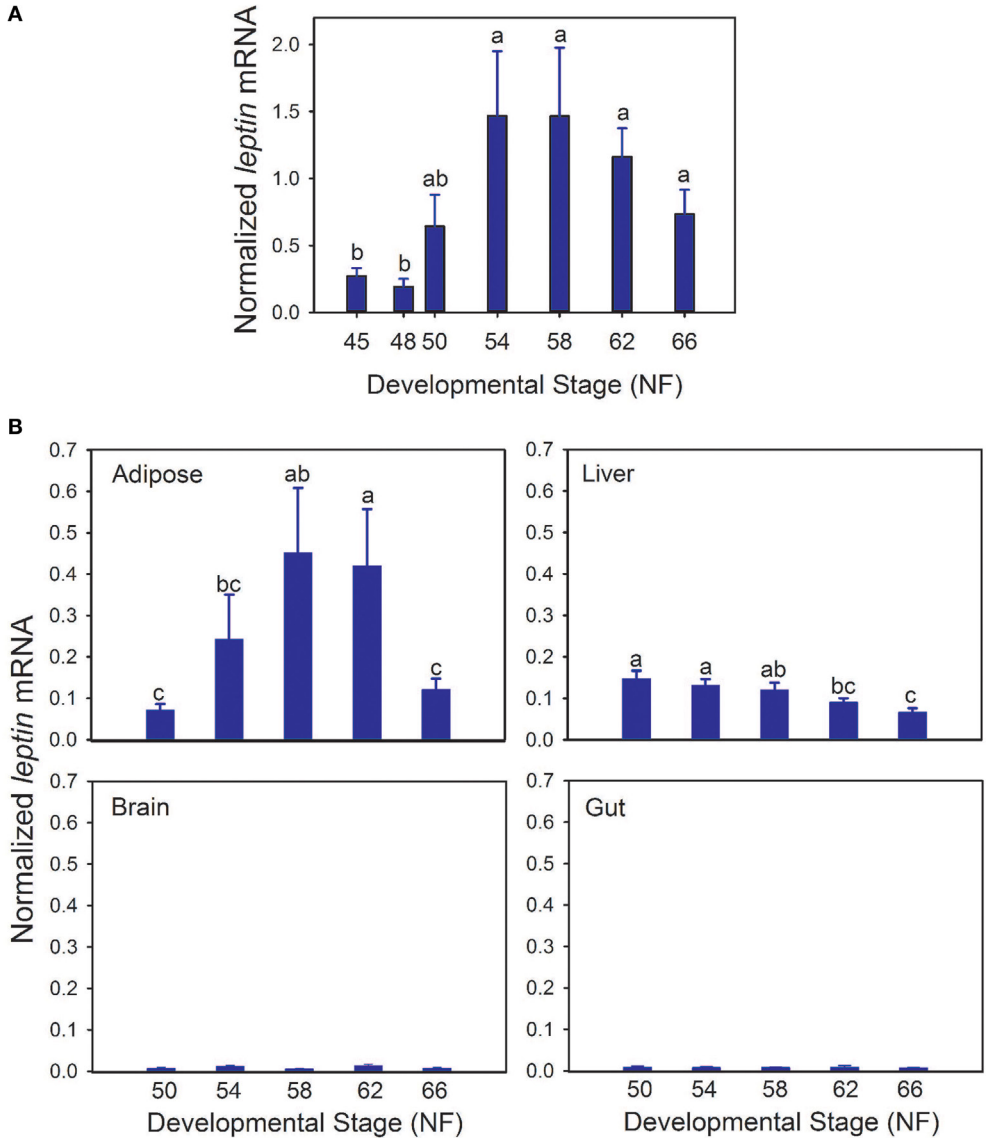

FIGURE 1 | **Transcription of the *lep* gene is activated during tadpole metamorphosis**. **(A)** Whole body *lep* mRNA increases during tadpole metamorphosis. **(B)** Developmental changes in *lep* mRNA in four tadpole tissues during metamorphosis. We analyzed *lep* mRNA by reverse transcriptase quantitative real-time PCR. Note that the scales of the graphs in panel **(B)** are not directly comparable to the graph in panel **(A)** since the samples were analyzed in separate assays using a relative quantification method (see Materials and Methods). Means with the same letter are not significantly different [$p < 0.05$; Fisher's least squares difference test; $n = 5$–6/Nieuwkoop–Faber (NF) stage].

Wnt/β-Catenin Signaling Is Activated in Premetamorphic Tadpole Brain by i.c.v. rxLeptin Injection

To test if leptin can activate the canonical Wnt/β-catenin signaling pathway in tadpole brain *in vivo*, we conducted EM gene transfer with different reporter plasmids, and injected saline or rxLeptin i.c.v. Injection of rxLeptin did not alter luciferase activity in pGL4.23-empty vector-transfected brain, but caused a strong increase (12.9-fold; $p < 0.0001$, unpaired Student's *t*-test) in luciferase driven by the GAS-luciferase vector, which reports pSTAT3 signaling induced by leptin binding to the LepR (**Figure 7**) (23, 24). This confirmed activation of LepR signaling in tadpole brain after i.c.v. rxLeptin injection. We observed a threefold increase in luciferase activity ($p < 0.001$) driven by the pGL4.23-6TCF (Wnt/β-catenin pathway) reporter vector following injection of rxLeptin. Forced expression of constitutively active β-catenin confirmed the activity of the pGL4.23-6TCF reporter (47-fold increase; $p < 0.0001$).

DISCUSSION

Leptin has well-established roles in adult physiology, but its developmental actions are less understood. Here, we show that *lep* expression increases strongly during tadpole metamorphosis

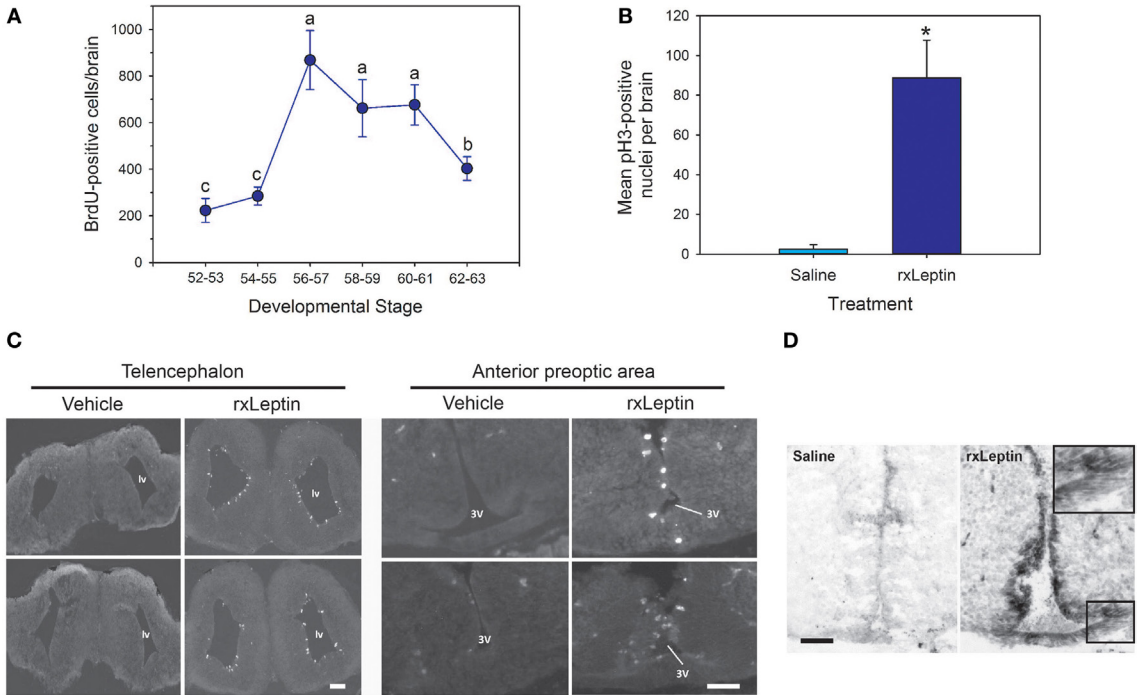

FIGURE 2 | Leptin induces mitosis in premetamorphic tadpole brain. **(A)** Changes in cell proliferation in tadpole brain throughout metamorphosis analyzed by BrdU incorporation [data are modified from Denver et al. (30); reproduced with permission]. **(B)** Quantification of pH3 positive cells in tadpole brain following intracerebroventricular injection of saline or recombinant *Xenopus* leptin (rxLeptin) (200 ng/g BW). Tadpoles were given two injections, the second 24 h after the first, and then they were killed 48 h after the first injection. *Denotes a statistically significant difference ($p < 0.05$; Student's unpaired t-test). **(C)** Images of transverse sections of the region of the telencephalon [lateral ventricle (lv)] and anterior preoptic area [location of neurosecretory neuron cell bodies; third ventricle (3V)] of tadpole brain stained for pH3. Scale bars = 120 μM. **(D)** Induction of pSTAT3 immunoreactivity in cells located in the ventricular zone (VZ)/subventricular zone (SVZ) of premetamorphic (Nieuwkoop–Faber stage 50) tadpole brain by rxLeptin (20 ng/g BW; 1 h). The inset shows a higher magnification view of the VZ/SVZ with elongated cells undergoing migration out of the neurogenic zone.

in parallel with an increase in mitosis in tadpole brain. Also, i.c.v. injection of rxLeptin induced mitosis in neurogenic zones of premetamorphic tadpole brain. Gene expression screening identified genes regulated by leptin signaling in early premetamorphic tadpole brain, and highlighted several signaling pathways (Wnt/β-catenin, TGFβ, hedgehog, and insulin signaling) through which leptin may effect its regulatory role in neurogenesis. We also show that leptin activates Wnt/β-catenin pathway signaling in tadpole brain *in vivo*, as evidenced by activation of a Wnt/β-catenin pathway reporter vector.

Leptin Promotes Neurogenesis in *Xenopus* Tadpole Brain

We observed a strong induction of mitosis in neurogenic zones of the premetamorphic tadpole brain following i.c.v. injection of rxLeptin. The rapid increase in pSTAT3-ir in cells of these regions 1 h after rxLeptin injection supports that they express functional LepR, and therefore that the action of leptin on mitosis is likely to be direct. In rodent embryonic/fetal brain, LepR mRNA is expressed within the VZ/SVZ of the 3V (18, 20). The LepR mRNA level in the VZ/SVZ declines through development, after which time it appears in the arcuate nucleus and ventromedial hypothalamus (18). These LepR mRNA-expressing NSCs may be precursors of hypothalamic feeding control and hypophysiotropic neurons of the adult (21). The LepR is also expressed in the VZ/SVZ (progenitor cell niches) of adult monkey brain (53).

Several lines of evidence from studies in rodents support that leptin controls cell proliferation in developing brain and can also induce neurogenesis in adults. For example, the reduced brain weight and DNA content in *ob/ob* mice is restored by leptin injection (4, 8), and leptin has been found to induce mitosis in rodent brain *in vivo* (8–10), and in tissue culture (11, 12). The large increase in *lep* mRNA in tadpoles during metamorphosis, a postembryonic developmental period that has been compared to the neonatal/early postnatal period in direct developing species like mammals (54), may be similar, both phenomenologically and functionally, to the postnatal leptin surge that occurs in rodents (17–19). It has been hypothesized that this postnatal increase in leptin plays a pivotal role in the development of the hypothalamic feeding control circuitry (13, 15, 16, 55–56). Leptin has also been shown to promote neurogenesis in the hippocampus of adult mammals (57), which may depend on Wnt/β-catenin signaling (58). The extra-hypothalamic actions of leptin on adult neurogenesis and neural plasticity may improve cognition and mood in animal models of depression and anxiety, and circulating leptin concentration is negatively correlated with Alzheimer's disease in humans (59), and promotes neurogenesis in a mouse model

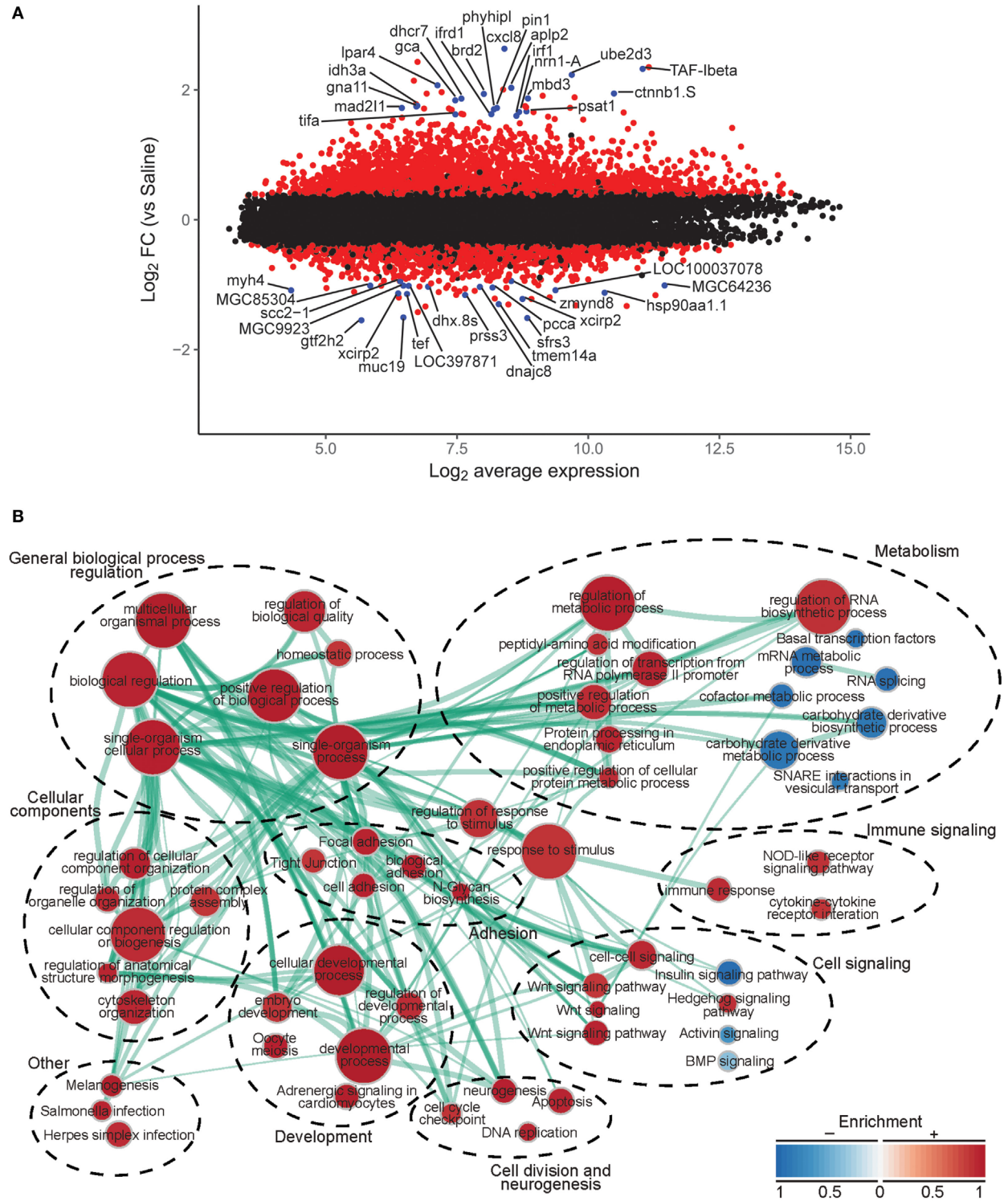

FIGURE 3 | Analysis of microarray data from preoptic area/hypothalamus of saline or recombinant *Xenopus* leptin (rxLeptin)-injected [intracerebroventricular (i.c.v.) 20 ng/g BW; 2 h] Nieuwkoop–Faber stage 54 tadpoles. (A) Transformed log ratio and mean average plot [$\log_2$ fold change ($\log_2$FC) vs. average expression] of saline vs. rxLeptin-injected tadpole mRNA levels (see Materials and Methods). Dots represent genes: red = genes with a false-discovery rate <0.05; blue = top 20 differentially expressed and annotated *Xenopus laevis* genes; black = all other genes. **(B)** Biological processes, pathways, and modules affected by i.c.v. rxLeptin injection. Enrichment map of enriched biological process gene ontology terms (redundancy reduced, $p < 0.05$), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways ($p < 0.05$), and KEGG modules. Circular node color reflects positive (red) or negative (blue) enrichment. Node color intensity reflects the degree of enrichment relative to the most highly enriched in either direction. Lines (green) represent significant genetic information shared between connected nodes (above 50%), and line thickness represents the degree of shared information.

TABLE 2 | Top twenty genes with annotation induced by recombinant *Xenopus* leptin in premetamorphic tadpole brain.

Gene symbol	Gene title	Entrez gene ID	log_2 fold change	Adjusted *p*-value
LOC100036815/cxcl8	Hypothetical protein LOC100036815/interleukin-8	100036815	2.63	0.00001
ctnnb1	Beta-catenin protein	399274	2.32	0.00013
ube2d3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog)	403384	2.23	0.00018
lpar4	Lysophosphatidic acid receptor 4	779407	2.07	0.00018
aplp2	Amyloid beta (A4) precursor-like protein 2	431790	2.03	0.00018
set/TAF-Ibeta1	SET nuclear oncogene///TAF-Ibeta1	379599///399349	1.94	0.00014
brd2	RING3 protein	779057	1.94	0.00028
mbd3	Methyl-CpG binding domain protein 3	398135	1.87	0.00018
dhcr7	7-Dehydrocholesterol reductase	379273	1.87	0.00028
gca	Grancalcin, EF-hand calcium binding protein	444080	1.84	0.00014
idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha	444419	1.75	0.00017
gna11	Guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	380535///779103	1.74	0.00026
pin1	Peptidylprolyl *cis/trans* isomerase, NIMA-interacting 1	503670	1.72	0.00018
mad2l1	MAD2 mitotic arrest deficient-like 1	380433	1.72	0.00074
phyhipl	Phytanoyl-CoA 2-hydroxylase interacting protein-like	447238	1.70	0.00037
psat1	Phosphoserine aminotransferase 1	494700	1.67	0.00013
nrn1-A	Neuritin 1-A	373709	1.66	0.00025
ifrd1/MGC69123	Interferon-related developmental regulator 1	379667///494857	1.63	0.00019
tifa	TRAF-interacting protein with forkhead-associated domain	734184	1.62	0.00026
irf1	Interferon regulatory factor 1	398826	1.60	0.00007

TABLE 3 | Top twenty genes with annotation repressed by recombinant *Xenopus* leptin in premetamorphic tadpole brain.

Gene symbol	Gene title	Entrez gene ID	log_2 fold change	Adjusted *p*-value
gtf2h2/MGC81060	General transcription factor IIH subunit 2	443754	−1.55	0.00027
sfrs3	Splicing factor, arginine serine-rich 3	380152	−1.51	0.00018
muc19	Mucin 19, oligomeric	378670	−1.50	0.00018
tmem14a	Transmembrane protein 14A	444418	−1.30	0.00613
xcirp2	Cold-inducible RNA binding protein 2	379484	−1.22	0.00391
prss3	Protease, serine, 3	447093	−1.16	0.01023
tef	Thyrotrophic embryonic factor	379940	−1.14	0.00018
hsp90aa1.1/MGC82579	Heat shock protein 90 kDa alpha family class A member 1	444024	−1.12	0.00030
myh4	Similar to myosin, heavy polypeptide 4, skeletal muscle	399414	−1.09	0.00145
LOC100037078	Hypothetical protein LOC100037078	100037078	−1.09	0.01290
pcca	Propionyl Coenzyme A carboxylase, alpha polypeptide	734347	−1.04	0.00330
dnajc8	DnaJ (Hsp40) homolog, subfamily C, member 8	447089	−1.03	0.00224
dhx.8S/MGC80994	DEAH-box helicase 8 S homolog	444315	−1.03	0.00145
LOC397871	Larval beta II globin	397871	−1.02	0.00106
MGC85304	MGC85304 protein	447105	−1.02	0.00207
MGC99235	MGC99235 protein	447690	−1.01	0.00053
MGC64236	Hypothetical protein MGC64236	379516	−1.01	0.00292
scc2-1	Scc2-1B	445865	−0.95	0.01224
zmynd8	Zinc finger, MYND-type containing 8	733267	−0.94	0.01584
hmgn1/MGC64236	High mobility group nucleosome binding domain 1	379516	−0.94	0.00244

of Alzheimer's disease (60). Leptin has also been shown to be neuroprotective and induces neurogenesis and angiogenesis after stroke (61–63).

Cell proliferation in tadpole brain during metamorphosis depends on thyroid hormone (30). However, other mitogens such as leptin, activators of the TGFβ pathway, neurotrophins, and insulin-like peptides likely also play important roles in this developmental process. Indeed, there may be synergy between different signaling pathways, some that are affected by nutritional state like leptin and insulin-like peptides, that determine cell expansion and development of neural structures and pathways. Leptin has been found to synergize with thyroid hormone to induce proliferation of chondrocytes in growth plates, and also to promote terminal differentiation (64). Whether similar synergy occurs in the developing brain, which is critically dependent on thyroid hormone for its development, requires further study.

Leptin Activates Canonical Wnt/β-Catenin Signaling in *Xenopus* Tadpole Brain

Using gene expression screening, we discovered sets of genes that are rapidly induced or repressed by leptin signaling in the tadpole preoptic area/hypothalamus. The major pathways regulated by leptin included the Wnt/β-catenin and TGFβ signaling pathways.

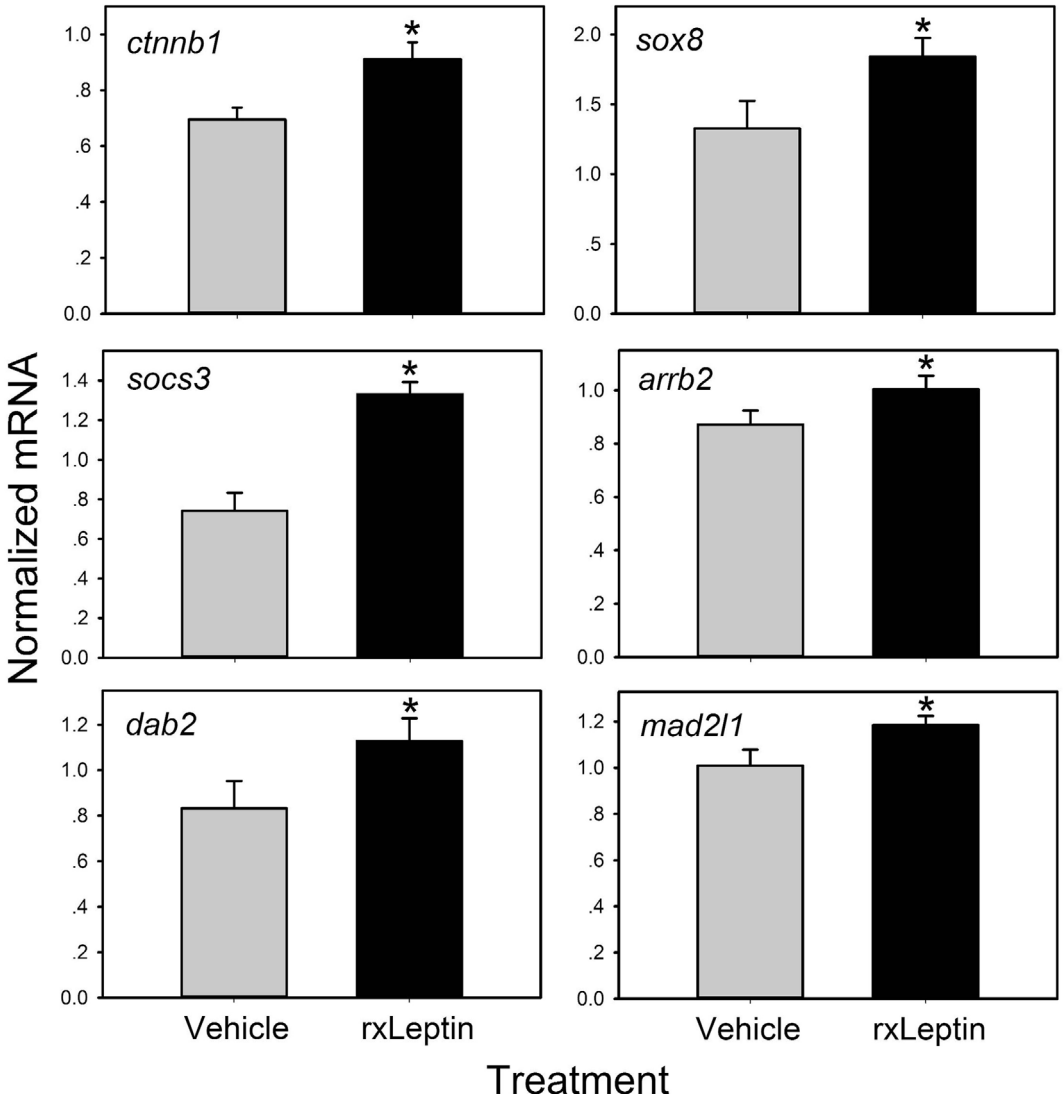

FIGURE 4 | Validation of leptin-induced genes in Nieuwkoop–Faber stage 54 *Xenopus laevis* tadpole preoptic area/hypothalamus by reverse transcriptase quantitative real-time PCR (RTqPCR). Tadpoles received an injection intracerebroventricular of 0.6% saline or recombinant *Xenopus* leptin (rxLeptin) (20 ng/g BW) and were killed 2 h later for tissue harvest for RNA isolation. Gene expression was analyzed by SYBR Green RTqPCR and normalized to the reference gene *rpL8* which was not affected by rxLeptin injection (data not shown). Asterisks indicate statistically significant differences from saline injected controls (*$p < 0.05$; unpaired Student's *t*-test).

TABLE 4 | Top ten gene ontology (GO) terms corresponding to developmental processes.

ID	Description	Set size	Enrichment score	NES	*p*-Value	*p*.adjust	*q*-Values
GO:0044767	Single-organism developmental process	470	0.298665447	1.479435427	0.00101833	0.159221311	0.159221311
GO:0048856	Anatomical structure development	452	0.305758564	1.512476805	0.00101833	0.159221311	0.159221311
GO:0032502	Developmental process	473	0.305340626	1.513106572	0.001019368	0.159221311	0.159221311
GO:0044707	Single-multicellular organism process	433	0.301770432	1.490562662	0.001023541	0.159221311	0.159221311
GO:0032501	Multicellular organismal process	463	0.29984464	1.483930907	0.00102459	0.159221311	0.159221311
GO:0007275	Multicellular organism development	407	0.300744558	1.475210668	0.003089598	0.400102987	0.400102987
GO:0030154	Cell differentiation	203	0.319354548	1.47945519	0.004429679	0.491694352	0.491694352
GO:0009790	Embryo development	84	0.391329957	1.634661027	0.006157635	0.598060345	0.598060345
GO:0048513	Animal organ development	137	0.342563144	1.530163684	0.007900677	0.649743161	0.649743161
GO:0048869	Cellular developmental process	242	0.301733778	1.42896268	0.008583691	0.649743161	0.649743161

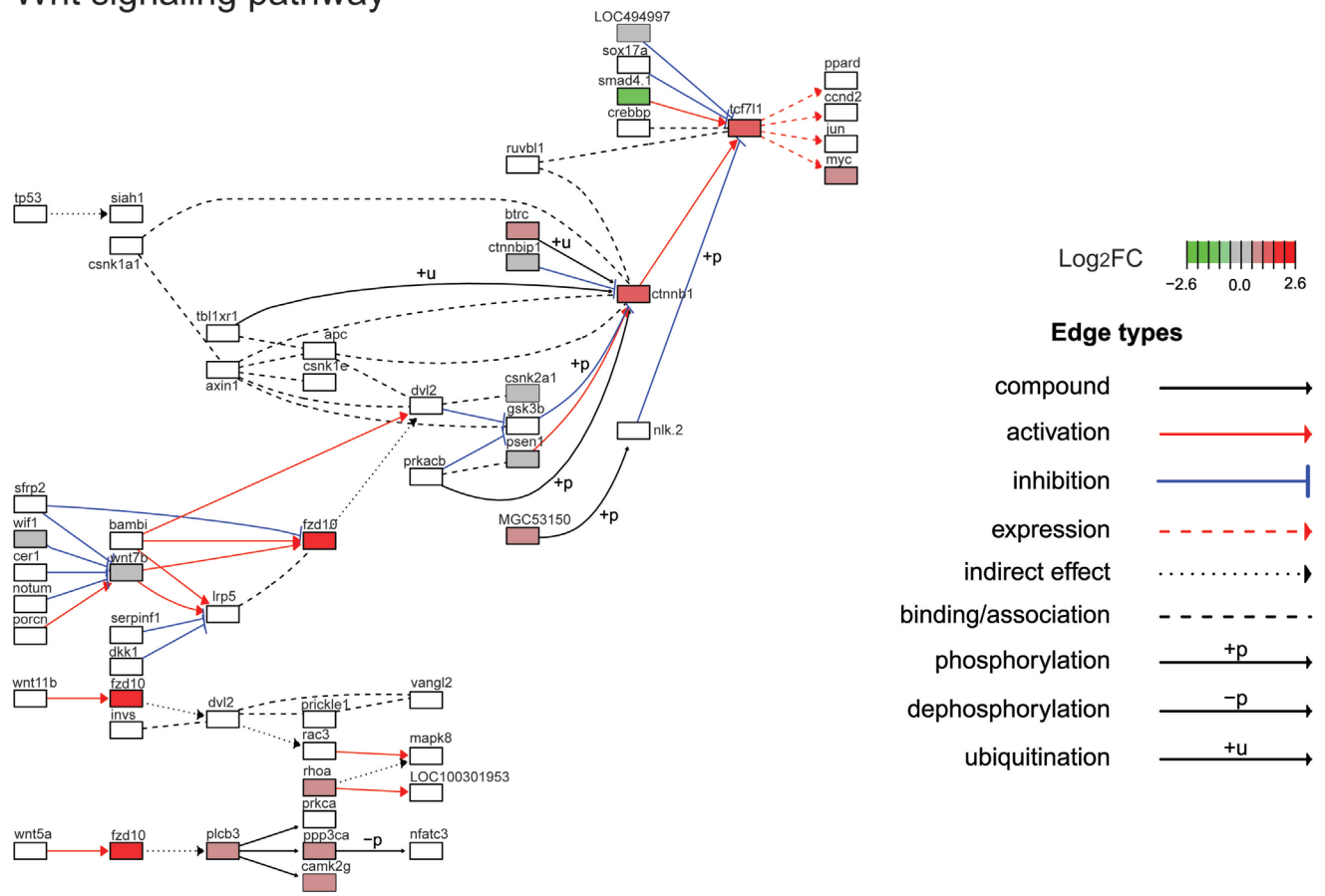

FIGURE 5 | Gene expression changes in the Wnt/β-catenin signaling pathway. Shown is the Kyoto Encyclopedia of Genes and Genomes Wnt/β-catenin signaling pathway with differential gene expression values plotted for genes with false-discovery rate <0.05. The color of each gene represents the direction of regulation (red = positive; gray = no change; green = negative). $\log_2$FC = $\log_2$ fold change. The intensity of the color is the value relative to the most highly regulated gene ($\log_2$FC = 2.6) in the entire dataset.

Notably, we identified several genes involved in the Wnt/β-catenin signaling pathway, including β-catenin, the central component of the canonical Wnt/β-catenin pathway. The Wnt proteins, and the canonical Wnt/β-catenin intracellular signaling pathway, play central roles in development and disease (65), including development of the central nervous system, and are known to promote cell proliferation (66). The Wnt ligands bind to cell surface receptors of the Frizzled (Fz) and low-density lipoprotein-related protein families (67). In canonical Wnt/β-catenin signaling, the absence of Wnt ligand causes cytoplasmic signaling components such as glycogen synthase kinase 3β (GSK3β) to phosphorylate β-catenin, leading to the latter's degradation. Activation of Wnt receptors leads to phosphorylation of GSK3β, allowing β-catenin to accumulate in the cytoplasm. The accumulated β-catenin translocates to the nucleus, where it interacts with lymphoid enhancer-binding factor 1 with lymphoid enhancer-binding factor 1 (also known as TCF) to activate transcription of Wnt/β-catenin target genes (67, 68).

In addition to the Wnt receptor Fzd10 and β-catenin (ctnnb1), one of the TCFs, Tcf7l1 (also known as TCF-3) was induced by leptin in tadpole brain. This transcription factor is a transcriptional repressor (67) and was recently found to promote growth of colorectal cancers (69). *Myc* was also identified in our screen and is a Wnt/β-catenin target gene that functions in mitosis (70). The cyclin-dependent kinase 10 was induced by leptin and is known to be modulated by Wnt/β-catenin signaling (66, 71).

There is mounting evidence from different systems and tissues that leptin induction of mitosis and cell survival depends on Wnt/β-catenin signaling (72, 73). For example, the level of β-catenin in the cytoplasm is maintained low through continuous proteasome-mediated degradation controlled by a protein complex of GSK3/APC/Axin (65). Phosphorylation of GSK3 on serine 9 by activated Wnt/β-catenin signaling leads to destruction of this complex and the accumulation of β-catenin. β-Catenin translocates to the nucleus to regulate gene transcription (65). Leptin acts as a growth factor for different kinds of tumor cells

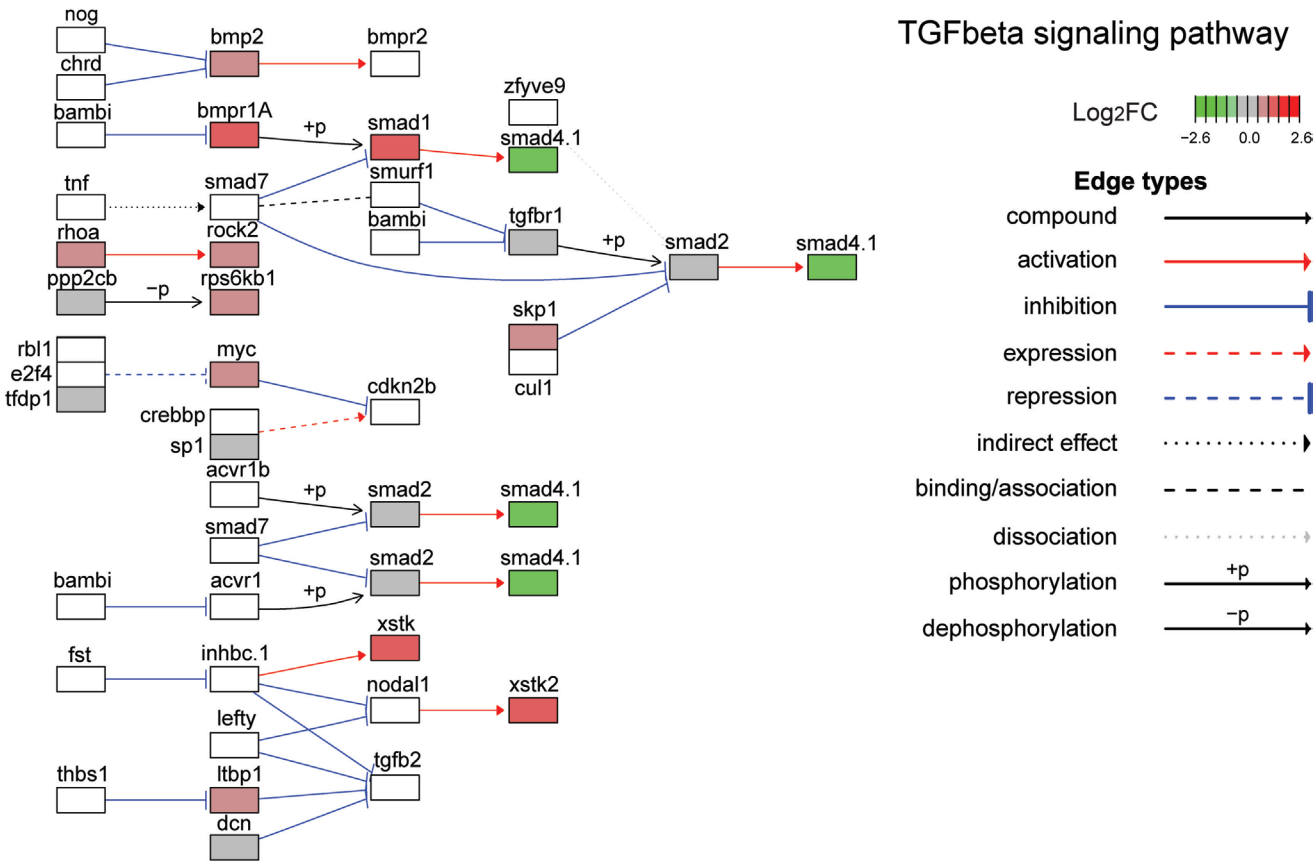

FIGURE 6 | Gene expression changes in the TGFβ signaling pathway. Shown is the Kyoto Encyclopedia of Genes and Genomes TGFβ signaling pathway with differential gene expression values plotted for genes with false discover rate <0.05. The color of each gene represents the direction of regulation (red = positive; gray = no change; green = negative). $log_2FC = log_2$ fold change. The intensity of the color is the value relative to the most highly regulated gene ($log_2FC = 2.6$) in the entire dataset.

(73, 74), causes rapid nuclear translocation of β-catenin, and activates other components of the Wnt/β-catenin pathway (74–76). Leptin-dependent activation of Wnt/β-catenin signaling has also been shown to play a role in leptin action in the adult hypothalamus to modulate glucose homeostasis and energy balance (77–80).

Leptin Modulates TGFβ, Hedgehog, and Insulin Signaling in *Xenopus* Tadpole Brain

After the Wnt/β-catenin pathway, the next most enriched pathway activated by leptin in tadpole brain was that regulated by the TGFβ superfamily of ligands, in particular bone BMP and activin. The TGFβ signaling pathway plays central roles in animal development, including cell proliferation, cell differentiation, and apoptosis (81, 82). Several components of this pathway were modulated by leptin in tadpole brain, including ligands, cell surface receptors, and receptor-activated transcription factors of the SMAD family (see **Figure 6**).

The hedgehog signaling pathway was also found to be activated by leptin (Figure S1 in Supplementary Material). A proposed mechanism of leptin-induced neurogenesis in murine transient amplifying neuroblasts involves hedgehog signaling regulation (83). We also found that leptin modulated components of the insulin signaling pathway (see Figure S2 in Supplementary Material). Interactions between leptin and insulin signaling, and potential therapeutic uses for leptin in normalizing type 2 diabetes have been described recently (84–86). The signal transduction pathways initiated by insulin and leptin are both distinct and overlapping. For example, leptin activates the JAK2–STAT pathway, while insulin activates the mitogen-activated protein kinase pathway. Both insulin and leptin appear to activate the PI3K pathway. Recent findings show that insulin can potentiate leptin signaling (87).

Leptin and Developmental Programming

Early life nutrition (from maternal source—placenta or yolk, or from feeding—larva) can influence adipocyte production of leptin in the fetus (88, 89) or larva (*Xenopus*, Melissa Cui Bender and Robert J. Denver, unpublished data). Alterations in hormone production, influenced by nutrition during critical periods of development can affect the timing of development, and exert lasting effects on the structure and function of hypothalamic

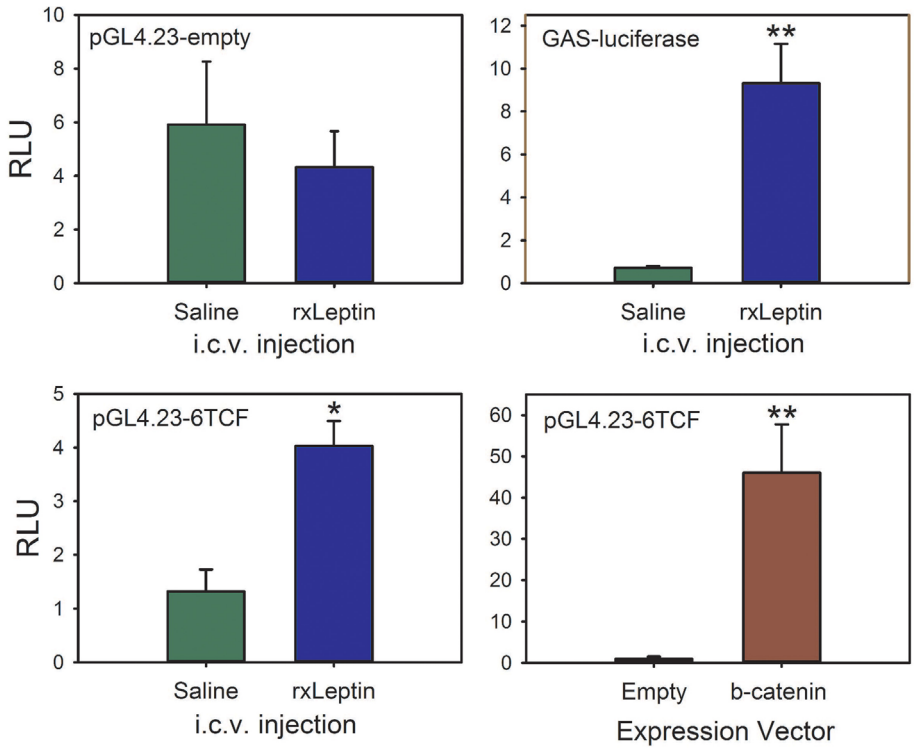

FIGURE 7 | The canonical Wnt/β-catenin signaling pathway is activated by leptin signaling in *Xenopus laevis* tadpole brain. We injected plasmids into the region of the third ventricle of premetamorphic (Nieuwkoop–Faber stage 50) tadpoles and transfected them by biopolar electroporation-mediate gene transfer. Twenty-four hours after transfection, we screened tadpoles for EGFP expression, and then separated them into eight groups. The reporter vector is given at the top left of each panel. Tadpoles received intracerebroventricular (i.c.v.) injections of 0.6% saline or recombinant *Xenopus* leptin (rxLeptin) (20 ng/g BW); a separate group was cotransfected with the pGL4.23-6TCF reporter vector and either empty expression vector (pCMVneo-empty) or a vector that expresses constitutively active β-catenin (pcDNA3-S33Y β-catenin). Two hours after i.c.v. injection, tadpoles were killed and brains harvested for dual-luciferase assay. Asterisks indicate statistically significant differences (*$p < 0.001$, **$p < 0.0001$; $n = 8$ tadpoles/treatment).

feeding and neuroendocrine circuits, a phenomenon termed developmental programming (88–93). There is mounting evidence that early life nutrition is an important determinant for risk of obesity (94), and modulation of leptin signaling during critical developmental periods can have long-term consequences for adult physiology (95–99). In animal models, undernutrition during pregnancy leads to a premature leptin surge, and offspring develop leptin resistance as adults, especially following a high-fat diet (100). A decrease or loss of leptin signaling in early postnatal life impairs development of the feeding circuit, and also predisposes individuals to leptin resistance and obesity as adults (95–99, 101–103).

The cellular and molecular mechanisms by which leptin acts on the developing brain to "program" the hypothalamic feeding control circuit are poorly understood (104). One possible mechanism of action is for circulating leptin in the neonate/tadpole to act within neurogenic zones of the developing brain to induce expansion of a LepR expressing cell population, which ultimately establishes the leptin-responsive network in the hypothalamus and other brain regions. Mammals develop competence to respond to leptin signaling during early postnatal development (15, 16, 105); amphibians develop competence to respond to leptin during early premetamorphosis (Melissa Cui Bender and Robert J. Denver, unpublished data). Functional LepR is detected in the VZ/SVZ during early postnatal development in rodents (18) and premetamorphosis in *Xenopus* (**Figure 2D**). Based on our findings and those in the literature, we hypothesize that leptin acts on the neural progenitor population *via* Wnt/β-catenin signaling to induce mitosis and promote cell survival.

Future fate mapping studies can investigate if cells born in the ependymal layer following leptin injection migrate to and populate the preoptic area and ventral hypothalamus. It will also be interesting to investigate the consequences of aberrant leptin signaling during early postembryonic development on later-life physiology and hypothalamic feeding control centers using the *Xenopus* model system. Leptin signaling in early stage *Xenopus* tadpole brain can be easily activated by i.c.v. injection of rxLeptin. Furthermore, cells in the tadpole VZ/SVZ can be transfected by EM gene transfer with expression plasmids (e.g., pCS2-xLepR) to force LepR expression in stem/progenitor cells, followed by injection of rxLeptin i.c.v. to activate LepR signaling. We predict that forced expression of LepR in progenitor/stem cells in the developing brain will promote proliferation followed by differentiation of these cells into the LepR expressing lineage.

ETHICS STATEMENT

All procedures involving animals were conducted under an approved animal use protocol (PRO00006809) in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Michigan.

AUTHOR CONTRIBUTIONS

MB participated in the design of experiments, conducted the leptin mRNA analysis, immunohistochemistry for pH3, DNA microarray analyses and electroporation-mediated gene transfer, and participated in writing the manuscript. CS did the bioinformatics analyses of the DNA microarray data and participated in writing the manuscript. RD participated in the design of experiments, analysis of the data, and in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00099/full#supplementary-material.

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REVIEW
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Energy Homeostasis in Monotremes

Stewart C. Nicol *

Biological Sciences, University of Tasmania, Hobart, TAS, Australia

In 1803, the French anatomist Étienne Geoffroy Saint-Hilaire decided that the newly described echidna and platypus should be placed in a separate order, the monotremes, intermediate between reptiles and mammals. The first physiological observations showed monotremes had low body temperatures and metabolic rates, and the consensus was that they were at a stage of physiological development intermediate between "higher mammals" and "lower vertebrates." Subsequent studies demonstrated that platypuses and echidnas are capable of close thermoregulation in the cold although less so under hot conditions. Because the short-beaked echidna *Tachyglossus aculeatus*, may show very large daily variations in body temperature, as well as seasonal hibernation, it has been suggested that it may provide a useful model of protoendotherm physiology. Such analysis is complicated by the very significant differences in thermal relations between echidnas from different climates. In all areas female echidnas regulate T_b within 1°C during egg incubation. The lactation period is considered to be the most energetically expensive time for most female mammals but lactating echidnas showed no measurable difference in field metabolic rate from non-lactating females, while the lactation period is more than 200 days for Kangaroo Island echidnas but only 150 days in Tasmania. In areas with mild winters echidnas show reduced activity and shallow torpor in autumn and early winter, but in areas with cold winters echidnas enter true hibernation with T_b falling as low as 4.5°C. Monotremes do not possess brown adipose tissue and maximum rates of rewarming from hibernation in echidnas were only half those of marmots of the same mass. Although echidnas show very large seasonal variations in fat stores associated with hibernation there is no relationship between plasma leptin and adiposity. Leptin levels are lowest during post-reproductive fattening, supporting suggestions that in evolutionary terms the anorectic effects of leptin preceded the adiposity signal. BMR of platypuses is twice that of echidnas although maximum metabolism is similar. High levels of thyroid hormones in platypuses may be driving metabolism limited by low body temperature. Monotremes show a mosaic of plesiomorphic and derived features but can still inform our understanding of the evolution of endothermy.

Keywords: echidna, platypus, hibernation, leptin, thyroid, brown adipose tissue, basoendothermy, evolution of endothermy

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***Correspondence:**
Stewart C. Nicol
s.c.nicol@utas.edu.au

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INTRODUCTION

The monotremes are the least speciose of the major extant mammal groups: there are roughly 5,500 species of eutherian mammal and 350 marsupial species but only five extant monotreme species and these are restricted to Australia and New Guinea: the platypus (*Ornithorhynchus anatinus*; Grant, 2015), the short-beaked echidna (*Tachyglosus aculeatus*), and three species of long-beaked

echidna (*Zaglossus* spp.; Griffiths, 1978; Flannery and Groves, 1998; Nicol, 2015). Unlike all other mammals, which give birth to live young, monotremes lay eggs. Their unusual reproductive biology and various aspects of their anatomy has led to their frequent depiction as primitive mammals, only slightly removed from the "lower vertebrates." The term "lower vertebrates" with all its overtones of the *scalae naturae* or "Great Chain of Being" dating back to the ideas of Aristotle (Mayr, 1982), is normally applied to the fish, amphibians, and reptiles (Bennett, 1978). The major problem of using the terms "higher" and "lower" in describing taxa is that they are closely linked to the idea that humans and their closest relatives are the goal of a progression toward a higher level of complexity (Diogo et al., 2015). This thinking persists when biologists read phylogenetic trees as ladders of progress or assume that species-poor lineages that appear "early branching" are basal (Omland et al., 2008).

Despite previously expressing reservations about the application of the concepts of "highness" and "lowness" to animals (Darwin, 1854), in the Descent of Man Darwin wrote "The Monotremata are plainly allied to the Marsupials; forming a third and still lower division in the great mammalian series" (Darwin, 1871). The term "lower mammals" continued to be used until relatively recently in the comparative physiology literature to refer to monotremes, marsupials, and some placentals, particularly when discussing thermoregulation (Johansen, 1962). The similarly problematic term "primitive" is often still used to describe the extant monotremes (Omland et al., 2008), but while many aspects of their anatomy and physiology are plesiomorphic it does not follow that this is the case in all aspects of monotreme biology. Egg-laying is clearly plesiomorphic, but the brain of monotremes, particularly the tachyglossids, is comparable in size and complexity to that of eutherian carnivores. Even in the post-cranial skeleton, which is often described as primitive, the monotremes demonstrate mosaic evolution, combining primitive with very specialized features, e.g., retaining a shoulder girdle of a therapsid pattern but possessing a pelvis of therian pattern (Crompton and Jenkins, 1973). From the first physiological investigations, discussion of the physiology of the monotremes has been influenced by the presumption of primitivity in all aspects of their biology.

A distinguishing feature of the "higher vertebrates"—mammals and birds—is endothermy, the maintenance of a high and (relatively) constant body temperature by metabolic means (Bennett and Ruben, 1979). This distinction was integral to the classification of animals proposed by Linnæus, who divided animals into six classes: Mammalia, birds, amphibia, fishes, insects, and worms. The mammals and birds he grouped together as having a heart with two auricles and two ventricles, and warm red blood; the amphibia (which included reptiles) and fishes were grouped together as having one auricle and one ventricle, and cold red blood (Kerr, 1792). In 1803, the French anatomist Étienne Geoffroy Saint-Hilaire decided that the newly described echidna and platypus did not fit in the Linnæan groupings and should be placed in a separate order, the monotremes, intermediate between reptiles and mammals (Geoffroy Sàint-Hilaire, 1803). Much of the debate about the status of the monotremes revolved around their mode of reproduction but the consensus was that they were primitive and imperfect mammals and close to reptiles.

The first physiological measurements of monotremes reinforced this view. Body temperatures of both Australian monotremes were measured by the Russian explorer turned Australian biologist, Nicholas Miklouho-Maclay: he found the echidna to have a temperature of 28°C and the platypus 24.8°C (Miklouho-Maclay, 1883, 1884). Sutherland (1896) found an average T_b for echidnas of 29.4°C, and commenting on his own, and Miklouho-Maclay's results, wrote "... the platypus, therefore, at only 24.8° is almost a cold blooded animal. The only other genus of monotremes, the echidna, carries us a step upwards". However, he found "an echidna on a cold morning was a low as low as 22°", while one "in a sack, exposed to fierce midday heat registered 36.6°." He commented "This is an immense range for a mammal, and suggests a reptilian want of capacity for temperature regulation." The first measurements of metabolic rate were made by C. J. Martin. Citing Sutherland's work, Martin wrote "Without doubt ...monotremes and marsupials present a stage of physiological development intermediate between the fairly accurate homoeothermism of the higher mammals, and the rudimentary indications in this direction ...which occur in lower vertebrates." Martin's paper (Martin, 1903) was "an attempt to locate more precisely the position of the monotremes and marsupials in this ascending scale of physiological superiority to the temperature of the environment." As well as measuring rectal temperature, Martin measured metabolic rates by gravimetric estimation of CO_2 production in a range of Australian animals, including a platypus and three echidnas. Martin found a mean rectal temperature for the monotremes of 29.8°C and metabolic rates which are quite close to much more recent measurements (**Figure 1**).

METABOLIC RATE AND BODY TEMPERATURE

Figure 1 shows quite clearly that the monotremes are all characterized by low T_b and metabolic rates, with the platypus having significantly higher basal metabolic rate (BMR) than the echidnas. Depending on the allometric relationship used to calculate the standard eutherian metabolic rates, BMR of the long- and short-beaked echidnas is 25–40% of the corresponding eutherian values, and platypus 70–80% (Dawson et al., 1979; Dawson and Grant, 1980; Capellini et al., 2010; Barker et al., 2016). Can the low metabolic rates of monotremes be attributed to their low T_b? This would be consistent with the metabolic theory of ecology, which claims that the metabolic rate of an organism is a function of its mass and temperature (Gillooly et al., 2001; Brown et al., 2004; Clarke, 2006). Repeated attempts have been made to explain the BMR differences between birds and mammals, and eutherians and marsupials, in terms of differences in T_b (White and Seymour, 2005), and in such comparisons BMR is adjusted to a common T_b using appropriate Q_{10} values. Q_{10} provides a useful way to investigate the mechanisms by which metabolism is supressed in individuals, or within a species, in daily torpor and hibernation (Nicol et al., 1992; Geiser, 2004),

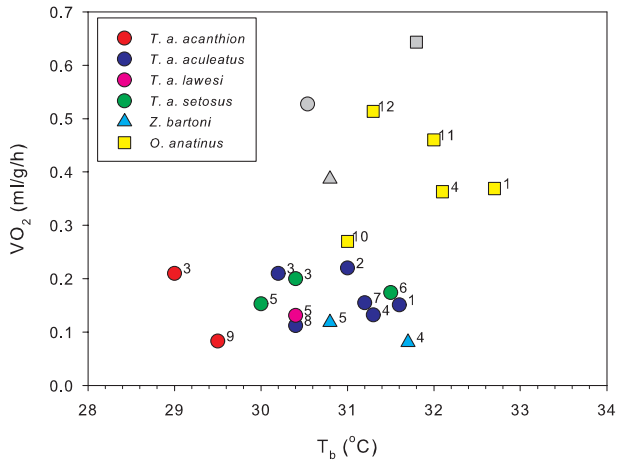

FIGURE 1 | Metabolic rates plotted against body temperature for resting, unrestrained monotremes at thermoneutrality. Colored circles show data from four of the echidna subspecies. Gray symbols are metabolic rates calculated for eutherian mammals of the same mass as the respective monotreme means, using a phylogenetic least-squares model based on data from 543 eutherian mammals (Capellini et al., 2010) plotted against the mean T_b from each monotreme species. The measurements by Martin (1903) (1), are remarkably close to more recent results although his single platypus was only half grown. Of the other platypus data, point 10 with a lower T_b and $\dot{V}O_2$ than the other data, is from a single animal of unspecified mass, whose other physiological responses seem abnormal (Grant and Dawson, 1978), data in point 11 were collected from resting, but not post-absorptive platypuses, and data for point 12 were obtained in the field. The $\dot{V}O_2$ measured for the Western Australian *T. a. acanthion* (9) is much lower than any other for this species. 1, Martin (1903); 2, Schmidt-Nielsen et al. (1966); 3, Augee (1978); 4, Dawson et al. (1979); 5, McNab (1984); 6, Bech et al. (1992); 7, Frappell et al. (1994); 8, Kuchel (2003); 9, Barker et al. (2016); 10, Smyth (1973); 11, Bethge et al. (2001); 12, Frappell (2003).

but using T_b adjustment to allow comparisons between taxa seems a fairly meaningless exercise—it is not really clear what the results of such an adjustment tell us. Monotremes are not just "detuned" eutherian mammals: the average eutherian T_b is lethal for monotremes (Augee, 1976). In the first attempt to apply this temperature "correction" to monotremes, Dawson and Hulbert (1970) found adjusting the BMR of the echidna to 38°C gave a value close to the allometric prediction for eutherian mammals, and more recently Barker et al. (2016) using a Q_{10} with a constant conductance correction, obtained a similar result. However, as pointed out by Dawson et al. (1979) temperature cannot account for the difference between the monotremes: although the mean T_b of resting platypuses is only 1°C higher than the echidnas, BMR is more than twice as high (**Figure 1**).

The BMR of the platypus and echidnas can be partly explained in terms of the evolutionary trade-off hypothesis: the resting metabolic rate of an organism is the result of a trade-off between resting costs and scope for activity, with the precise level being set by lifestyle (Clarke, 2006). Water has a higher thermal conductivity (2.4 × higher) and specific heat (4,000 × higher) than air, leading to higher rates of heat loss in water, and semiaquatic mammals are also relatively inefficient swimmers (Fish, 2000). Because of these energetic disadvantages semiaquatic eutherian species have a higher BMR than similarly sized terrestrial species (Fish, 2000). The platypus has very dense fur which retains a high insulative value in water, and a number of vascular adaptations which reduce heat loss, but even so at water temperatures below 20°C heat loss of resting platypuses is double that in air at the same temperature (Grant and Dawson, 1978; Bethge et al., 2001). Metabolic rate further increases during foraging activity (Fish, 2000; Bethge et al., 2001), but even when foraging at water temperatures very close to freezing, platypuses maintain their body temperature within the normal range (Grigg et al., 1992), which means that heat loss is being matched by increased heat production. Even in the coldest water platypuses forage on average about 12 h/day (Bethge et al., 2009), and these sustained high levels of energy expenditure have selected for a higher BMR than the terrestrial echidna.

BMR is also influenced by phylogeny (Capellini et al., 2010; Clarke et al., 2010), as is T_b (Clarke and Rothery, 2008; Lovegrove, 2012). McNab (1992, 2008) demonstrated that BMR is strongly correlated with diet, and that ant- and termite-eating mammals have a low BMR as well as low T_b (McNab, 1984). A more recent analysis has shown that this well accepted relationship between diet and BMR vanishes when T_b is included in the model (Clarke et al., 2010), suggesting that the underlying relationship is between diet and T_b, with BMR responding through its dependence on T_b. A subsequent analysis of diet and T_b patterns in mammals and birds confirmed this strong relationship between T_b and diet, with predators of invertebrates having the lowest T_b (Clarke and O'Connor, 2014). All the monotremes feed nearly exclusively on invertebrates, although platypuses may occasionally take small fish (Nicol, 2013). Thus, the low T_b and BMR of the monotremes can be considered to be the result of their phylogeny and respective ecological niches, although it could be argued that phylogenetic constraints on T_b and metabolism may have restricted possible ecological niches. Herbivores consuming grass or leaves have a T_b about 2.6°C higher than carnivores taking invertebrate prey (Clarke and Rothery, 2008), and the monotreme line may not have been able to achieve or tolerate higher T_b that would have been necessary to occupy other niches. The operative temperature of active short-beaked echidnas and platypuses is about 32°C and the maximum T_b recorded in active platypuses is about 34.6°C (Grigg et al., 1992) and in echidnas about 35°C (Brice et al., 2002; Nicol and Andersen, 2002; Nicol et al., 2004) but in both species T_b very rarely exceeds 34°C.

Ninety percentage of oxygen consumption at BMR occurs in the mitochondria (Rolfe and Brown, 1997), and while the metabolic differences between reptile and mammal are reflected in differences in total mitochondrial membrane surface area (Else and Hulbert, 1985a), no difference in mitochondrial surface area was seen between the echidna and other mammals (Else and Hulbert, 1985b). Extensive studies on mitochondrial function led Hulbert and Else (2005) to propose the membrane pacemaker theory of metabolism: metabolic rate is determined by the activities of membrane-bound proteins that are either directly or indirectly associated with the energy-consuming processes of cells; the activities of membrane-bound proteins such as transporters, channels, and receptors are influenced by different

membrane environments; and the composition of membranes (such as changes in fatty acid or acyl composition) and concomitant changes in membrane properties is the common underlying factor underpinning change in the metabolic rate of animal. However, mitochondrial proton leak is greater in marsupials than in eutherians, although marsupials have lower BMRs, and thus the differences between mammalian taxa do not seem to be explained by mitochondrial proton permeability (Polymeropoulos et al., 2011).

BMR is generally believed to be an indicator of metabolic capacity (White and Seymour, 2005) and although there has been debate about how the metabolic constraints on life history variables relate to BMR (Johnson et al., 2001; Mueller and Diamond, 2001), in placental mammals energy expenditure on reproduction is positively correlated with energy expended on maintenance. Thus, high-maintenance species harvest more energy and expend more on reproduction than low-maintenance species (McNab, 2002) while a low BMR optimizes longevity. The maximum lifespan of the short-beaked echidna is at least 50 years (Hulbert et al., 2008), while female platypuses have a lifespan of up to 21 years (Grant, 2004). An eastern long-beaked echidna at Taronga Zoo was at least 53 when she died. There are no direct measurements of field metabolic rates (FMR) of platypuses, but FMR of short-beaked echidnas measured using the doubly labeled water method was 2.7 times the BMR (Green et al., 1992; Schmid et al., 2003). $\dot{V}O_2$ max for echidnas estimated from treadmill exercise (Edmeades and Baudinette, 1975) and from maximal rewarming rates from hibernation (Nicol and Andersen, 2008), is ∼1.44 ml O_2 g^{-1} h^{-1}, 9 times the BMR, but only 28% of the value predicted for wild eutherian mammals of the same mass (Taylor et al., 1981). The highest metabolic rate recorded for platypuses is 1.9 O_2 g^{-1} h^{-1} when walking on a treadmill (Bethge et al., 2001), 4.2 times BMR, while the highest metabolic rates recorded while foraging in cold water are only 3.2 times BMR (Grant and Dawson, 1978; Bethge et al., 2001). If the reported maximum metabolic rates are corrected to mass independent values using a mass exponent of 0.67 (White and Seymour, 2005) these become 0.36 ml O_2 $kg^{-0.67}$ min^{-1} for the playpus and 0.35 ml O_2 $kg^{-0.67}$ min^{-1} for the echidna.

Platypuses occur in permanent freshwater environments in the Australian east, from Cooktown in north Queensland (15°S) to Tasmania (43°S) (Nicol, 2013; Grant, 2015). At Cooktown freshwater river temperatures may reach 31°C (Howley, 2012) and in Tasmania platypuses forage in water at nearly 0°C (Bethge et al., 2003). Mean mass of female platypuses from a north Queensland population was 0.75 ± 0.08 kg and from Tasmania 1.21 ± 0.13 kg (Nicol, 2013). This would normally be considered to be an example of Bergmann's Rule; the platypuses from the colder areas will be bigger to limit heat loss, but it may be better to look at it from the inverse view: platypuses in the warmer areas will be smaller to maximize heat loss. This would be consistent with the heat dissipation limit theory—an upper boundary on total energy expenditure is imposed by the maximal capacity to dissipate body heat and therefore avoid the detrimental consequences of hyperthermia (Speakman and Król, 2010), which will occur at lower ambient temperatures in an endotherm with a low T_b. Platypuses have a modest ability to sweat (Augee, 1976; Grant and Dawson, 1978) and thus can lose heat when air temperature exceeds T_b, but during active swimming, when heat production increases by four times over basal (Bethge et al., 2001), the only means of dissipating metabolic heat is by conduction to water. Much smaller body size at the northernmost part of its range is consistent with high water temperatures being an important factor in limiting platypus distribution. Cold water does not appear to be so limiting for platypuses. They have a number of adaptations that minimize heat loss when foraging in cold water: the fur has a high insulation value, higher than that of the polar bear and beaver, and vascular structures in the skin and hind limbs which greatly decrease heat loss (Grant and Dawson, 1978). In a Tasmanian highland lake, platypuses foraged on average 11.9 h/day in summer, and 13.2 h/day in winter when water temperatures frequently approached 0°C (Bethge et al., 2003, 2009). There have been no equivalent studies on platypus in the northern part of their range.

Despite the dramatic differences in adult size between north and south, platypuses from all parts of their range are considered to belong to the same species, although mitochondrial DNA shows two major clades: one from mainland Australia and the other from Tasmania/King Island (Gongora et al., 2012). Echidnas occur from sea level to 1,800 m altitude, and in all parts of Australia, as well as eastern New Guinea, but significant differences in appearance between geographic populations, particularly in the hairiness of the pelage, have resulted in their division into five subspecies (Griffiths, 1978; Augee et al., 2006; Nicol, 2015), although these have not been validated genetically. The most widespread sub-species *T. a. acanthion* which occurs throughout the arid zone in all mainland states and the Northern Territory has long spines and very sparse bristly fur. *T. a. aculeatus*, the sub-species from which the echidnas were first described (Shaw, 1792) occupies the coastal temperate zones in south-east Queensland, New South Wales, Victoria and South Australia. The Tasmanian and Flinders Island subspecies (*T. a. setosus*) has soft thick fur which may completely hide the spines, and was initially believed to be a separate species from mainland echidnas (Nicol, 2015). On temperate Kangaroo Island, the sub-species (*T. a. multiaculeatus*) has very long fine pelage obscured by long, thin spines. The northern sub-species, (*T. a. lawesii*) has long stout spines and thick fur and was first described from New Guinea, but Griffiths (1978) suggests that echidnas from tropical northern Australia also belong to this subspecies.

Augee (1978) found that the conductance of the *T. a. acanthion* was 1.7 times that for *T. a. setosus*. **Figure 1** shows metabolic rates and T_b measurements for all named subspecies. The two points for *T. a. acanthion* were derived from two geographically distant populations, central Queensland (3) and south-west Western Australia (9), but they both show a very low T_b at thermoneutrality, although the metabolic rates are very different. When ambient temperature was reduced from 20°C to 5°C over 58 days, T_b of *T. a. acanthion* dropped to 23°C (Augee, 1978). Generally, echidnas from the warmer parts of Australia seem to have more variable T_b when active than echidnas from cooler climates. **Figure 2** shows T_b records over 10 days in November from echidnas in Tasmania (a) and south-east Queensland (b). November is the time of maximum foraging and

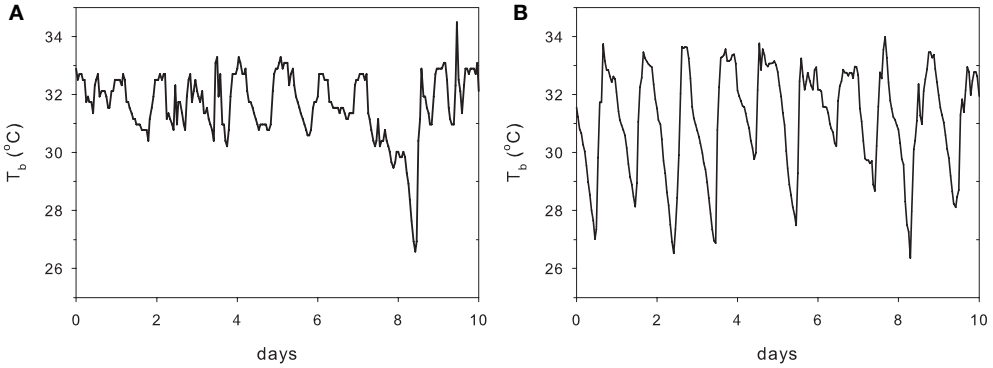

FIGURE 2 | Body temperature of two adult male echidnas over 10 days in November at (A) Lovely Banks in Tasmania and **(B)** Stanthorpe in south-eastern Queensland (redrawn from Grigg et al., 2004). Long term average November air temperatures at Lovely Banks: min 7.4°C, max 19.7°C; Stanthorpe min 12°C, max 25.3°C. T_b of the Tasmanian echidna drops below 30°C once in these 10 days, whereas in the Queensland echidna this occurs nearly every day. Normal daily variation in the Tasmanian echidna: 3°C, Queensland 6°C.

weight gain for males following the mating period (Kuchel, 2003; Nicol and Morrow, 2012). Echidnas in both locations show daily variations in T_b related to activity, rather than T_a (Grigg et al., 2004; Nicol et al., 2004), but in Tasmania T_b only drops below 30°C following several days of inactivity, whereas at the warmer site the pattern resembles daily torpor. For Tasmanian females the daily T_b range of non-lactating individuals in November was 3.1 ± 0.7°C, similar to the males, but lactating females had a significantly greater daily variability (4.8 ± 1.0°C) (Schmid et al., 2003).

Heterothermia appears to be one means whereby echidnas can survive in hot environments: an animal with low initial T_b takes longer to reach dangerous levels. Despite this it has been difficult to reconcile the ability of echidnas to survive for many hours at ambient temperatures exceeding T_b (Brice et al., 2002) with their apparent inability to use evaporative cooling (Augee, 1976), but a recent study shows echidnas have some capacity to increase evaporative water loss (Barker et al., 2016).

HIBERNATION

Many birds and mammals temporarily abandon homeothermic endothermy during times of cold exposure, food shortage or drought, and use the energy minimizing strategies of daily torpor and hibernation (Ruf and Geiser, 2015). The short-beaked echidna is the only one of the monotremes to use these strategies, but the different geographic sub-species vary in their use of torpor and hibernation. Echidnas in all parts of their range show a reduction in activity at about the same time of the year (Nicol and Andersen, 1996; Morrow et al., 2009), but whether they are able to show extended periods of hibernation appears at least partly to depend on the environmental temperature.

At Stanthorpe in Queensland hibernation occurred in 9 out of 15 echidna-years of recording (Kuchel, 2003), whereas at Lovely Banks in Tasmania all echidnas hibernated every year (Nicol and Andersen, 2002). The greater variability in active T_b and use of hibernation mean that in echidnas in warm climates it is difficult to distinguish between torpor and non-torpor (Kuchel and Grigg, 2003). Kangaroo Island echidnas showed reduced activity from April to August, but this varied greatly between individuals, and within individuals from year to year (Rismiller and McKelvey, 1996). Some Kangaroo Island echidnas showed several bouts of hibernation, with T_b profiles similar to "classical hibernators" and a minimum T_b of 11.8°C, while other echidnas in the same area did not hibernate. In the cooler climates of Tasmania and the Australian Alps (Beard et al., 1992) the hibernation period is very distinct. **Figure 3** shows a male Tasmanian echidna entering hibernation at the warmest time of the year. When they have built up sufficient fat reserves, echidnas reduce their activity (Sprent et al., 2012), and dig into the soil, and T_b falls until it is with 0.5–1.0°C of substrate temperature (Nicol and Andersen, 2002; **Figure 4**). The factors that determine the equilibrium T_b can be seen by rearranging the familiar Scholander-Irving model (Nicol et al., 2008):

$$T_b = T_a + \frac{\dot{V}O_2}{C} \quad (1)$$

i.e., T_b falls to a temperature dependent on ambient temperature plus an amount determined by the ratio of hibernating metabolic rate to conductance. This relationship only holds for thermoconforming animals above the lower set point (Geiser, 2001; Nicol and Andersen, 2008). If T_b drops below the set point, most hibernators increase heat production, which is energetically expensive (Geiser, 2004). Echidnas arouse and move to a warmer area which also represents an energetic penalty and increases the chance of predation. Metabolic rate in hibernating echidnas is about 12% of the normal resting value, and at low T_b is relatively independent of T_b (Nicol and Andersen, 1993), while the conductance during cooling is the same as in cold exposed non-hibernating echidnas (McNab, 1984; Nicol and Andersen, 2007a). The minimum T_b recorded from a hibernating echidna is 4.5°C (Nicol et al., 2008), which seems to be the lower set point. Because cooling takes several days (**Figure 4**), daily torpor with a stable torpid T_b is clearly not an option for echidnas.

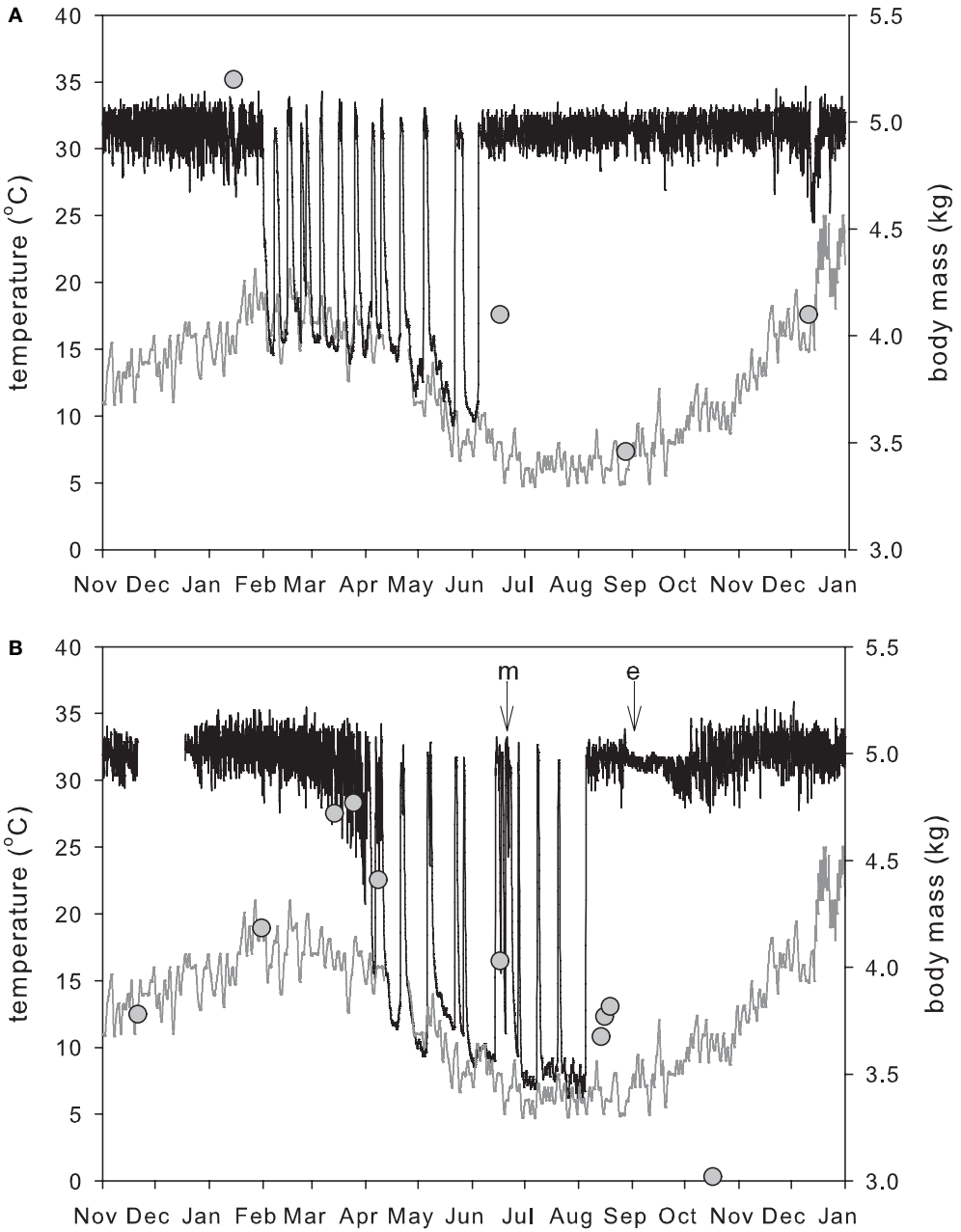

FIGURE 3 | Annual pattern of body temperature and mass in a reproductively active male (A) and female **(B)** in Tasmania in the same year. Black line: body temperature; gray line: soil temperature at 20 cm measured at a Bureau of Meteorology station 2 km from the field site; circles: body mass. The male entered hibernation in summer (Feb 1) after building up fat reserves in spring and early summer. The female reached maximum mass and entered hibernation much later (April 4). As in other deep hibernators, hibernation is broken by periodic arousals, although, unlike most other hibernators, echidnas may move to another location during these euthermic periods (Nicol et al., 2011). The male ended hibernation in early winter (June 4) and was found mating with the female on June 17 (m on panel **B**). The pregnant female then re-entered hibernation, and her final arousal from hibernation was on August 5. Shortly after this she entered a nursery burrow and laid an egg (e). Incubation of the egg takes 10–11 days, during which T_b remains very stable (Beard et al., 1992; Nicol and Andersen, 2006) and in Tasmania the female typically stays in the burrow with the young for 23–48 days before leaving it in a plugged burrow while she forages (Morrow and Nicol, 2012). When she first emerges from the nursery burrow her body mass is at its lowest. Males reach their minimum mass at the end of the mating period.

As in other hibernators (Geiser et al., 1990), hibernation bout length increases as T_b falls (Nicol and Andersen, 2000), and echidnas seem unable to maintain a prolonged hibernation bout when T_b is above 17°C. In the record shown in **Figure 3**, there were several days of very cold nights, cooling the soil, and allowing the male to enter hibernation on February 1. Grigg et al. (2004) have described this behavior as using cold as a resource, i.e., taking advantage of the cold to cool down and

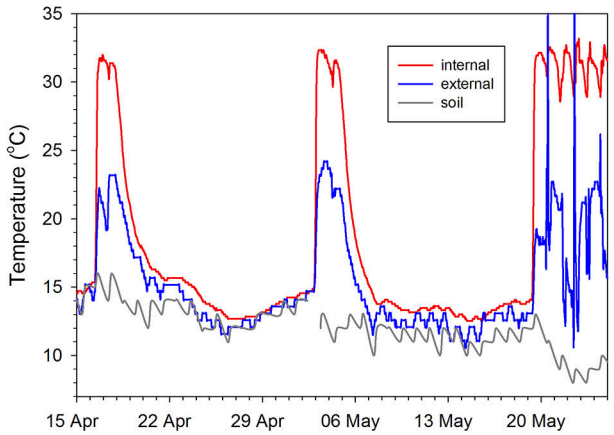

FIGURE 4 | Relationship between internal, external, and soil temperatures for a male echidna during two periodic arousals and final emergence from hibernation. Internal temperature (T_b) was measured by an implanted datalogger (Onset Computer Corporation, Stowaway Tidbit) in the peritoneal cavity, external temperature from a logger (Thermochron iButtons, DS1922L; Maxim/Dallas Semiconductor, TX, USA) attached to a tracking transmitter, which was glued to the spines. Soil temperature was measured at 20 cm depth at a Bureau of Meteorology station 2 km from the field site. Redrawn from Nicol et al. (2008).

enter torpor, thus saving energy. The female, which had not accumulated sufficient fat reserves, did not enter hibernation, but showed only a brief fall in T_b to 28°C. Echidnas show behavioral thermoregulation during hibernation; early in the hibernation season echidnas prefer to hibernate in cool areas, while during the coldest months they may move to warmer hibernacula, giving a preferred hibernating T_b in the range 7–9°C (Nicol and Andersen, 2007a). This is well above the minimum recorded T_b and apparent lower set point of 4.5°C, but may represent a balance between maximizing bout length and thus reducing energetically expensive periodic arousals, and maintaining a safety margin to reduce cold induced arousals. Unlike most hibernators, echidnas do not dig or construct a hibernaculum. Instead they may use existing rabbit or wombat burrows, hollow logs, or dig under tree stumps or into grass tussocks, piles of bark or leaves (Wilkinson et al., 1998) or simply burrow into the substrate, and are often more exposed to ambient conditions.

There is no evidence of hibernation or daily torpor in the long-beaked echidna or the platypus. Captive long-beaked echidnas showed a daily variation of T_b of 2–4°C, with a modal T_b of 31°C, when short-beaked echidnas in the same pen showed both torpor and hibernation (Grigg et al., 2003). Free-ranging platypuses in the southern Alps monitored maintained a T_b close to 32°C throughout the winter (32.1 ± 0.8°C, range 29.2–34.6°C) (Grigg et al., 1992).

Recently Nowack et al. (2016) have demonstrated an increased use of torpor by short-beaked echidnas after fire, and argue that torpor may be an important contributor to survival during natural disasters. Turbill et al. (2011) have shown that hibernation is associated with higher rates of overwinter and annual survival than non-hibernators. This higher survival appears to be due not only to avoidance of sub-optimal environmental conditions (which could include wildfire), but to reduced predation. This further demonstrates that the benefits of torpor extend beyond energy conservation in cold climates (Geiser and Brigham, 2012). The increased survival of hibernating species is linked with the coevolution of traits indicative of slow life histories (Turbill et al., 2011). The limited amount of data from long-beaked echidnas (non-hibernators) suggest very similar life history traits to short-beaked echidnas (hibernators), but as the evolution of slow life histories appears to be related to survival, rather than hibernation *per se* (Turbill et al., 2011) this suggests high rates of survival in long-beaked echidnas. Unfortunately, the echidna fossil record is very sparse and incomplete so that echidna origins are the subject of considerable debate (Phillips et al., 2009; Musser, 2013; Simon, 2013). Short-beaked echidnas appear in the fossil record in the Pleistocene, before which long-beaked echidnas predominated (Musser, 2013), and it seems likely that the slow life history of short-beaked echidnas has not co-evolved with torpor and hibernation, but was a pre-existing tachyglossid trait, which facilitated the expression of torpor and hibernation in this species.

REPRODUCTION AND ENERGETICS

The unusual timing of hibernation in echidnas is clearly related to reproduction: in Tasmanian echidnas, in which hibernation appears obligatory, the time from hatching of the young to weaning is about 150 days, at which time the young weighs about 1.5 kg (Morrow and Nicol, 2012). This relatively slow growth rate of the young and an apparent increase in heterothermy by the mother means that daily energy expenditure of females in mid-lactation was not measurably higher than of non-lactating females at the same time (Schmid et al., 2003). As in other seasonal breeders, male echidnas show testicular involution after the breeding season, presumably as an energy saving measure (Griffiths, 1978; Morrow et al., 2016). In order for the young to be weaned before the females enter hibernation, mating must occur in winter but the very large size of the testes (about 1% of body mass at the beginning of breeding) and the low metabolic rate means that, unlike all other hibernators, testicular recrudescence in Tasmanian echidnas occurs before entry into hibernation (Morrow et al., 2016). The very high competition between males for females selects for early arousal by males, which then seek out females, which are still hibernating (**Figure 3**). Morrow et al. (2015) found that all females that mated prior to July 27 re-entered hibernation, including females that were pregnant. Five of these were monitored; four re-entered hibernation for relatively short periods (3–13 days) but one hibernated for 50 days, showing 4 periodic arousals. Pregnant females that reentered torpor did so no more than 5 days after fertilization, when the embryo would probably be no later than the blastocyst stage (Werneburg and Sánchez-Villagra, 2011; Ashwell, 2013b), and it appears that there is no significant development of the embryo during torpor, as the gestation period is extended by a day for every day in torpor (Nicol and Morrow, 2012). In some respects this is similar to embryonic diapause in marsupials

and some eutherian mammals (Lopes et al., 2004), although it is controlled by temperature rather than hormonally. Hibernation during pregnancy is quite unusual; torpor and reproduction have been widely viewed as mutually exclusive but torpor during pregnancy has now been observed in monotreme, marsupial, and eutherian mammals (McAllan and Geiser, 2014). In the majority of species these torpor bouts are daily events lasting a few hours, rather than the extended deep hibernation seen in some female echidnas, however Willis et al. (2006) recorded deep multiday torpor (i.e., hibernation) of up to 5.6 days in pregnant hoary bats (*Lasiurus cinereus*), which enabled parturition to be delayed in unfavourable weather. Similarly, female echidnas that are mated very early, thus benefitting from mating with the fittest males, re-enter hibernation, delaying egg-laying until conditions are more favourable, and ensuring that that maximum growth rate of the young coincides with the period of greatest ecosystem productivity (Nicol and Morrow, 2012; Morrow et al., 2015).

An earlier observation in which a pregnant Kangaroo Island echidna entered torpor only 2 days before egg-laying (Geiser and Seymour, 1989) appears to be different from the hibernation in early pregnancy observed in Tasmanian echidnas. 16 days after capture this female was found to be torpid with a T_b of 21°C, but T_b and activity had returned to normal 6 h later. An egg shell was found in the cage 2 days later. There is no indication whether the young died accidentally after the egg was laid, or had not survived to this stage. We have 60 records of T_b from pregnant Tasmanian echidnas but none of these show any indication of late stage torpor, although several entered torpor after losing the egg or the young. Because the Kangaroo Island echidna young did not survive, it is not clear whether what happened was a stress response of a captive animal, or normal physiological behavior in a sub-species which shows numerous differences from eastern echidnas.

Throughout Australia echidna mating occurs at approximately the same time (June–September), although it appears to be slightly earlier in more southern populations (Morrow et al., 2009). This is in contrast to platypuses, which do not hibernate, and in which breeding begins earlier in more northern populations (Nicol, 2013). Data are only available from a small number of Australian locations, but it appears that in more northern parts of eastern Australia echidna lactation durations are similar to those in Tasmania (Beard et al., 1992; Beard and Grigg, 2000), whereas on Kangaroo Island (South Australia) (Rismiller and McKelvey, 2003) and in Western Australia (Abensperg-Traun, 1989) young are weaned at 204–210 days, although at similar body mass to eastern echidnas. This could be another manifestation of differences in energetics between the geographic sub-species.

A particularly interesting feature of echidna reproduction and thermoregulation is shown in the T_b record following egg-laying in **Figure 3**. While the mother is in the nursery burrow T_b is remarkably constant, particularly during the first 10–11 days, which is the egg-incubation period (Nicol and Andersen, 2006; Morrow and Nicol, 2012), where the range is about 1.2°C. This pattern was first observed in echidnas in the Australian Alps (Beard et al., 1992) and subsequently in echidnas in south-east Queensland (Beard and Grigg, 2000).

THYROID HORMONES

As noted above, perhaps the major distinction between birds and mammals and "lower vertebrates" is that all birds and mammals are endothermic, even when inactive. The contribution of the monotremes to our understanding of the evolution of endothermy is discussed later in this review, but whatever the selective process, the acquisition of endothermy appears to be closely linked to thyroid hormones (Little and Seebacher, 2014). The elevated metabolism associated with endothermy in mammals is produced by leaky cell membranes, and thyroid hormones play a key role in regulating metabolic rate by increasing leakiness and thus increasing cellular ATP turnover (Hulbert, 2000). In mammals there is a stoichiometric relationship between oxygen consumption and consumption of thyroid hormones (Tomasi, 1991).

Hulbert (2000) has compiled a comprehensive listing of concentrations of thyroid hormones in vertebrate plasma and I have drawn heavily on his review in this section. Birds and eutherian mammals have much higher circulating levels of thyroid hormones—principally 3′,5′,3,5-l-tetraiodothyronine (thyroxine, T4)—than "lower vertebrates". In adult reptiles, total plasma thyroxine (TT4) ranges from 1 to 14.5 nmol L^{-1}, while in birds (apart from ostriches, which have low values) TT4 is in the range of 15.9–34 nmol L^{-1}. In small to medium sized eutherian mammals TT4 is typically is in the range 20–80 nmol L^{-1} (Hulbert, 2000), and TT4 of active echidnas is 15 nmol L^{-1} (Hulbert and Augee, 1982; Nicol et al., 2000), at the low end of the normal range for eutherian mammals and consistent with a low metabolic rate. The only significantly lower TT4 for an adult small mammal comes from a poikilothermic rodent, the naked mole-rat (*Heterocephalus glaber*), with a TT4 of 5 nmolL^{-1}, which increases to 7 nmol L^{-1} during cold exposure (Buffenstein et al., 2001).

By contrast with the values for echidnas, TT4 in adult platypuses is high (64 nmol L^{-1}) (Hulbert and Grant, 1983), at the upper end of the range for eutherian mammals, and presumably associated with a BMR that is two and a half times that of echidnas. TT4 levels in platypuses did not vary significantly with season, and similarly in active echidnas there was no difference between summer and winter values, but plasma levels of all thyroid hormones in echidnas fell significantly during hibernation (Nicol et al., 2000; **Table 1**). This is different from what has been observed in other hibernators. Eutherian hibernators show the lowest levels of thyroid hormones pre-hibernation (Hulbert and Hudson, 1976; Young, 1984; Kwiecinski et al., 1991; Damassa et al., 1995; Tomasi and Stribling, 1996), whereas in echidnas thyroid hormone levels trend down during the pre-hibernation period and reach their lowest during the hibernation period.

In eutherian hibernators thyroid hormone levels, although starting low, rise progressively during hibernation. In ground squirrels TT4, FT4, TT3, and FT3 are higher during hibernation than in active animals (Magnus and Henderson, 1988a,b). In woodchucks (Young et al., 1979) found TT4 and FT4 to be highest in early spring and lowest in summer and autumn, while TT3 and FT3 were highest during hibernation. In black

TABLE 1 | Plasma thyroid hormone levels in active and hibernating (T_b 5–12°C) echidnas.

Assay	Active	Hibernating
TT4 (nmol L^{-1})	15.2 ± 1.1 (23)	7.47 ± 0.95 (8)
FT4 (pmol L^{-1})	20.2 ± 1.5 (23)	10.7 ± 1.9 (8)
TT3 (nmol L^{-1})	1.64 ± 0.05 (22)	1.09 ± 0.06 (8)
FT3 (pmol L^{-1})	4.61 ± 0.23 (23)	2.76 ± 0.14 (8)

Values are shown as mean ± SEM. Sample sizes are shown in parentheses.

bears all four hormone levels decreased prior to hibernation; free hormones remained low during hibernation but the total levels recovered (Tomasi and Stribling, 1996). In male little brown bats (*Myotis lucifugus*), there is a 5-fold increase in TT4 during the course of hibernation (Damassa et al., 1995) and in females the increase is 8-fold (Kwiecinski et al., 1991).

As thyroid hormones are considered to have a major role in regulating metabolic rate, the first findings of increased levels of thyroid hormones in hibernating rodents were unexpected. Blood levels of thyroid hormones reflect the amount bound to proteins and the balance between the rates of secretion and utilization. Elevated total hormone levels during hibernation have been attributed to greatly reduced clearance rates (Demeneix and Henderson, 1978) and increased levels of binding proteins (Magnus and Henderson, 1988a). Most thyroid hormone circulating in the blood is bound to plasma proteins (99.97% of T4 and 99.7% of T3 in humans; 99.86 and 99.72%, respectively, in euthermic echidnas). In large eutherian mammals, some bats, and many marsupial species, three plasma proteins are involved in this transport: albumin, which in humans binds about 15–20% of T4 and T3; transthyretin (TTR) which binds 10–15% of T4 and T3; and thyroxine binding globulin (TBG), which in humans binds about 70% of T4 and T3 (Mendel, 1989). Adult monotremes possess only two thyroid hormone binding plasma proteins: albumin and a post-albumin globulin (E-TBP); TTR has not been detected in plasma from short-beaked echidnas (*Tachyglossus aculeatus*) either when active or hibernating, from long-beaked echidnas, (*Zaglossus bartoni*), or platypuses (Richardson et al., 1994; Richardson, 2009). Using electrophoresis followed by autoradiography Richardson et al. (1994) found the band caused by binding of radioactive thyroxine to protein in the post-albumin region was less intense in plasma from a hibernating echidna than in plasma from a non-hibernating echidna, indicating a reduction in E-TBP levels during hibernation. By contrast in the bat *M. lucifugus*, although TT4 rises during the course of hibernation, TBG remains at basal levels (Damassa et al., 1995). The differences in patterns of seasonal variation in hormone levels between the echidna and other hibernators may well be related to differences in characteristics and levels of these binding proteins.

BRAIN AND ENERGETICS

Thyroid hormones are essential for nervous system and brain growth and development (Hulbert, 2000), but the brain is separated from the rest of the body by the "blood-brain barrier" which restricts the movement of large molecules (Saunders et al., 1999). Transport of thyroid hormones from the blood to the brain is dependent on TTR, the only thyroid hormone transporting protein made in the brain. TTR is synthesized in the choroid plexus and secreted exclusively into the CSF, transporting thyroid hormones from the blood into the brain and throughout the CSF (Richardson, 2009). Transthyretin synthesis in the choroid plexus is believed to have begun at the stage of the stem reptiles, about 320 Ma, which developed the first traces of a cerebral neocortex (Richardson, 2009), and is synthesized by the choroid plexus of the echidna, the only monotreme in which it has been investigated (Richardson et al., 1994).

The platypus and the echidnas both have large, highly encephalized brains with a six-layered isocortex (neocortex) like all therian mammals, but the two monotreme groups have very different cortical morphology (Ashwell, 2013a). In the platypus the isocortex is lissencephalic (smooth) and thick, while in the echidnas it is gyrencephalic (folded) and thin. The olfactory bulb of the echidna is also gyrified (Ashwell, 2013c).

Brain tissue is energetically expensive and during rest it uses nearly an order of magnitude more energy per unit weight than most other somatic tissues (Mink et al., 1981). Analysis of microanatomical features that reflect metabolic activity of the cerebral cortex (capillary volume fraction, and mitochondrial density) suggest that the echidna cerebral cortex has similar levels of metabolic activity to eutherian mammals (Hassiotis et al., 2005). Assuming this, the energy usage of monotreme brains can be estimated from brain mass, using the equations from Hofman (1983), and then adjusting brain metabolic rates from the T_b of placental mammals (38°C) to the monotreme value of 32°C. From this the percentage of basal oxygen consumption used by the brain would be about 5.8% for the platypus, 8.5% for the short-beaked echidna, and 9.5% for the long-beaked echidna (Nicol, 2013). Most mammals lie in the range from 2–8% (mean value for 240 mammals is 4.6%) with only primates and cetaceans having values above 8% (Hofman, 1983). However, these estimates depend on several assumptions about the scaling of brain metabolism, and a more direct analysis is provided by simply plotting brain mass as a function of BMR (**Figure 5**). This graph demonstrates that the echidnas have very large brains relative to their metabolic rate, comparable to the primates.

The expensive tissue hypothesis states that an increase in brain size must be accommodated by an increase in total metabolic rate or by a reduction of the demands of the other expensive organs, such as heart, liver, kidney, and gastrointestinal tract (Aiello and Wheeler, 1995). Thus, it is argued that the relatively large brain sizes of humans and other primates could not have been achieved without a shift to a high-quality diet, allowing a reduction in gut size. It is doubly puzzling then that the short-beaked echidna has a brain of similar size to that of a similar sized eutherian carnivore but a metabolic rate only 30% of the eutherian prediction and has a diet of extremely low energy density and digestibility (Sprent and Nicol, 2016). Echidnas have brain size to BMR relationships similar to those of primates, suggesting that there must be very considerable fitness benefits for the echidnas to maintain such large brains, i.e., the cognitive benefits must outweigh the metabolic costs (Isler and van Schaik, 2006).

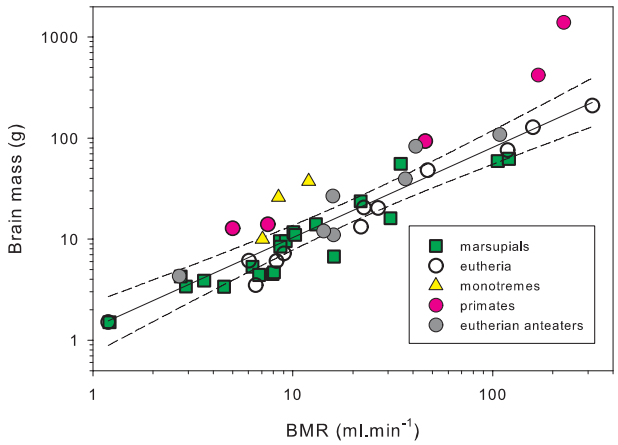

FIGURE 5 | Brain mass as a function of basal metabolic rate in mammals. Both BMR and brain mass scale with body mass, and the relationship between brain mass and BMR illustrates the relative investment of energy in the brain. The regression line and 99% confidence limits have been fitted to the data for 12 non-primate non-myrmecophagous terrestrial placental mammals from 11 families and 9 orders (Afrosoricida, Artiodactyla, Carnivora, Chiroptera, Eulipotyphla, Hyracoidea, Lagomorpha, and Scandentia). The two echidnas (*Tachyglossus aculeatus* and *Zaglossus* sp.), and the primates (*Perodictus potto*, *Nycticebus coucang, Macaca mulatta*, *Pan troglodytes*, *Homo sapiens*) lie well above the upper confidence limit. The giant anteater (*Myrmecophaga tridactyla*), the hairy-nosed wombat (*Lasiorhinus latifrons*), and tamandua (*Tamandua tetradactyla*) also lie above the upper confidence limit. Circles: placental mammals, data from McNab and Eisenberg (1989); squares: marsupials, data from Ashwell (2008), Weisbecker and Goswami (2010). Triangles: monotremes, data from McNab and Eisenberg (1989), Nicol and Andersen (2007b). In order of increasing brain mass they are platypus, short-beaked echidna, long-beaked echidna. Solid shading: primates; gray shading: myrmecophages; open symbols: other terrestrial mammals. Body masses have been restricted to a range of 250 to 70,000 g to avoid any effects associated with very low or very high body masses. Metabolic rates were recalculated for the body mass used for brain mass measurement assuming a within-species exponent for metabolic rate of body mass$^{0.70}$ (Kozlowski and Konarzewski, 2005; White and Seymour, 2005; Sieg et al., 2009). To minimize errors associated with this correction, data sets have been restricted to cases where body masses for BMR measurement and body masses used for brain weight were within 30% of each other. Under these circumstances, any errors in BMR correction are trivial. Redrawn from Nicol (2013).

The fitness benefits must be considerable for short-beaked echidnas, because the species seems to be specialized to minimize energy expenditure, and many aspects of their ecology and behavior are correlated with small brain size in other mammals. Insectivorous eutherian mammals have smaller brains than carnivores and omnivores (Gittleman, 1986), possibly because a larger brain may be necessary to handle a resource that requires more complex foraging strategies and within primates, larger brain size appears linked to monitoring food sources that vary in space and time (Clutton-Brock and Harvey, 1980). The echidna is the only mammal known to have a gyrified olfactory bulb, probably to expand the number of synaptic glomeruli available for the analysis of the odorant repertoire (Ashwell, 2013c). A total of 186 compounds potentially used in olfactory communication by echidnas have been identified in exudates from the cloaca and base of the spur, including volatile carboxylic acids, aldehydes, ketones, fatty acids, methyl esters, ethyl esters, terpenes, nitrogen- and sulfur-containing compounds, alcohols, and aromatics (Harris et al., 2012). Long chain and very long chain monounsaturated fatty acids, sterols, and sterol esters were identified as the major constituents of solid exudates, some of which have not previously been described from any animal skin gland. There are differences in volatile and non-volatile odorant composition between sexes and individuals but there is no single pheromone—echidnas process a complex suite of chemical signals providing a range of information (Harris et al., 2014, 2016). Echidnas deposit feces in latrines (Sprent et al., 2006), and chemical signals from these are likely to be an important means of communication in echidna populations. Processing this complex olfactory information may have been important in the selection process leading to a high investment in the echidna brain. Platypuses have cervical scent glands on both sides of the neck which produce a musky odor and secretions increase during the breeding season (Grant, 2015), but the olfactory bulb is smaller and unfolded, consistent with olfactory communication being less important in this semiaquatic monotreme.

Large brain size in mammals is also associated with longevity and González-Lagos et al. (2010) suggest that because large brains allow flexible behavioral responses to unusual, novel or complex socioecological challenges they will facilitate a longer reproductive life span. This underlines the need for more behavioral studies of echidnas in their natural habitat across their range (Nicol, 2013). It may be significant that both relative brain size and longevity are greater in the echidna than the platypus. Longevity is also correlated with a low basal metabolic rate (Hofman, 1983; White and Seymour, 2004) and it may be difficult to unravel the causal relationships between metabolic rate, brain size and longevity.

LEPTIN AND ENERGETICS

In eutherian mammals, the peptide hormone leptin has a key role in the regulation of fat reserves. Leptin is synthesized and secreted primarily by adipose tissue, and an increase in adiposity in eutherian mammals is normally associated with a corresponding increase in the synthesis and secretion of leptin by adipocytes, resulting in increased circulating leptin concentrations (Denver et al., 2011). Leptin binds to leptin-specific receptors in the hypothalamus, regulating the production of a range of orexigenic and anorexigenic neuropeptides, and resulting in a decrease in food intake, an increase in metabolic rate, and consequently a loss of adipose tissue (Denver et al., 2011; Florant and Healy, 2012). Although it is frequently claimed that leptin is an adipostat in mammals this been demonstrated only for eutherian mammals; it is not true for the short-beaked echidna (Sprent et al., 2012), and although pharmacological doses of leptin inhibit daily torpor in the marsupial *Sminthopsis macroura* (Geiser et al., 1998) the relationship between adiposity and endogenous plasma leptin has not been investigated in marsupial mammals.

Leptin othologs have now been described for all the major classes of vertebrate (Londraville et al., 2014; Prokop et al., 2014), and the Lep gene has been identified in the genome sequence of

the platypus (Denver et al., 2011). The interaction between leptin and the leptin receptor is conserved in terrestrial vertebrates, and in mammals both leptin and its receptor are highly conserved with few variations (Prokop et al., 2012). Sprent et al. (2012) hypothesized that in the echidna as in eutherian hibernators, there would be a strong relationship between adiposity and plasma leptin for most of the year which would change during pre-hibernatory fattening. Instead they found a weak negative relationship between adiposity and plasma leptin. The highest leptin levels were found in both sexes during hibernation and in females during the mating period. As female echidnas return to hibernation after mating, even when pregnant, unless they are further disturbed by males (Harris and Nicol, 2014; Morrow et al., 2015), the high leptin in mating females is most probably also related to hibernation. The lowest leptin levels were recorded from males during the post-reproductive period, when they forage maximally and show their greatest increase in mass (Nicol and Morrow, 2012). Generally high leptin concentrations in echidnas occur during periods when animals show minimal activity, have low body temperatures and do not feed. These results on the echidna are consistent with studies on a variety of non-mammalian vertebrates which have led to the consensus of an ancient role of leptin in regulating food intake and metabolism (Denver et al., 2011; Sprent et al., 2012).

Sprent et al. (2012) suggested that the adipostatic role for leptin in eutherian mammals evolved along with BAT-based thermogenesis. In eutherian mammals, leptin increases brown fat (BAT) activation, decreasing metabolic efficiency and increasing heat production, and burning fat stores (Cannon and Nedergaard, 2004). Heat production in BAT results from the activation of mitochondrial uncoupling protein-1 (UCP1), which leads increased proton leak, rather than ATP production. There is no evidence for BAT thermogenesis in marsupials, and no evidence for BAT in monotremes (Oelkrug et al., 2015). Molecular phylogeny of UCP1 demonstrates that the monotreme and marsupial UCP1 gene is more closely related to that of ectothermic rather than eutherian orthologs, suggesting that monotremes and marsupials may have never evolved a thermogenic competent UCP1 (Oelkrug et al., 2015). The success of eutherian species radiation and niche expansion has been linked to BAT based thermogenesis (Cannon and Nedergaard, 2004; Oelkrug et al., 2015), although Cannon and Nedergaard (2004) clearly overstate the case when they claim that brown fat derived heat is essential for arousal from hibernation in mammals: it may be essential for eutherian hibernators but echidnas and a number of marsupial hibernators (Geiser and Körtner, 2010), with no BAT, arouse from hibernation quite successfully. Augee and Ealey (1968) reported rewarming rates of echidnas to be lower than for other hibernators. Geiser and Baudinette (1990) demonstrated that in mammals rewarming rates were inversely related to body mass but did not find any difference between the rates of rewarming for marsupials and eutherian mammals. The largest marsupial that shows deep hibernation is the mountain pygmy possum (*Burramys parvus*), which weighs less than 70 g (Ruf and Geiser, 2015), which means there were no marsupials of equivalent size to the echidna and marmot to include in this comparison. In large hibernators rewarming follows a sigmoidal trajectory (Nicol et al., 2009) and rewarming rate varies with T_b. **Figure 6** shows peak rewarming rates for marmots, which have significant amounts of BAT, and for echidnas of the same body mass. Not only are rewarming rates of echidnas much lower than those of marmots, but the relationships between T_b and rewarming rate are very different: marmots at low T_b have higher maximal rewarming rates than marmots at higher T_b, while echidnas with a lower T_b have lower peak rewarming rates than warmer echidnas. BAT appears to offer a far superior mechanism for rewarming from very low T_b.

MONOTREMES AND THE EVOLUTION OF ENDOTHERMY

There have been numerous models proposed for evolution of endothermy; of these three have received recent attention because they are physiologically testable in modern mammals such as monotremes (Lovegrove, 2012). The aerobic scope hypothesis supposes that the evolution of endothermy was driven by selection for enhanced aerobic capacity to support sustained locomotor activity. In the original formulation of this Bennett and Ruben (1979) argued that a warmer body and endothermy were secondary consequences of selection for enhanced aerobic scope. Clarke and Pörtner (2010) have proposed a modification of the aerobic scope hypothesis in which the increase in aerobic scope was achieved through an increase in T_b. The parental care model proposes that endothermy may have arisen as a

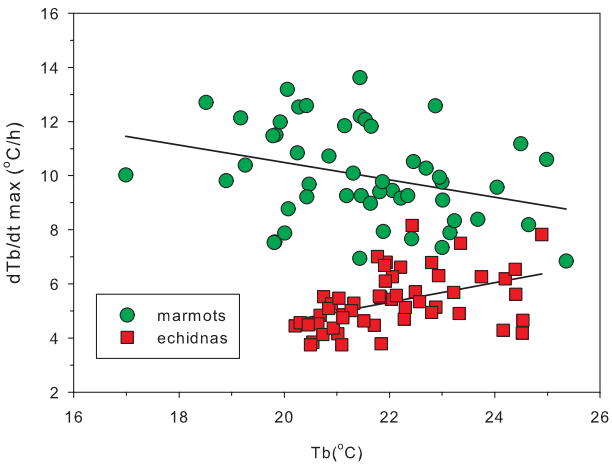

FIGURE 6 | Peak rates of rewarming from hibernation in marmots (*Marmota marmota*) and echidnas (*Tachyglossus aculeatus*) as a function of body temperature. Peak rewarming rates were calculated from the first derivative of a sigmoid curve fitted to the rewarming data, and each point represents one rewarming. Because rewarming rate is inversely related to body mass (Geiser and Baudinette, 1990) the figure compares data from hibernators of the same mass (10 marmots, mass 3.39 ± 0.46 kg, mean ± *SD* and 10 echidnas 3.71 ± 0.75 kg). In marmots, which possess BAT, the highest rewarming rates occur at lower T_b than in echidnas, although active T_b in marmots is 38°C and in echidnas 32°C. The contrasting relationships between peak warming rates and T_b reflect a difference between BAT and muscle thermogenesis. Redrawn from Nicol et al. (2009).

consequence of selection for parental care, because endothermy allows a parent to control incubation temperature, facilitating embryonic development. Higher temperatures speed embryonic development but the costs of this extra thermogenesis would have selected for an increase in aerobic capacity (Farmer, 2000; Farmer and Losos, 2003). The assimilation capacity model (Koteja, 2000) is a variation on the parental care model. It argues that the evolution of endothermy in birds and mammals was driven by two factors: (i) a selection for intense post-hatching parental care, particularly feeding offspring, and (ii) the high cost of maintaining the increased capacity of the visceral organs necessary to support high rates of total daily energy expenditures. What can the monotremes bring to this debate? Although it is now understood that the monotremes do not represent an intermediate step on the way to true endothermy they may still provide some insights into its evolution.

Grigg et al. (2004) suggest that short-beaked echidnas have some of the attributes of a protoendotherm: they are relaxed about maintaining a stable T_b, with large daily cycles of T_b (2–5°C) associated with activity, they show a continuum from daily torpor to long-term torpor or hibernation, which is interrupted by periodic arousals, and they may abandon their normal daily pattern with short periods of torpor at any time of the year. Grigg et al. (2004) stress that this does not mean that the short-beaked echidna displays a primitive or inadequate thermoregulatory ability. Rather they suggest it has retained a plesiomorphic condition to tolerate low T_b. Lovegrove (2012) uses the term protoendotherm to describe mammals such as the echidna which he suggests have retained Cretaceous basoendothermy. His plesiomorphic-apomorphic endothermy (PAE) model suggests that Cretaceous mammals may not have maintained a constant T_b throughout the year and daily torpor and hibernation in certain extant stem tropical mammals is a plesiomorphic condition. Heterothermy in protoendotherms might be considered to be the non-adaptive plesiomorphic state, and periodic normothermy, for example during breeding, as is seen in the echidna, is the adaptive state. In this model, highly seasonal, well-regulated adaptive hibernation in high latitude mammals is a derived state of heterothermy (Lovegrove, 2012).

Leaving aside the difficulty in explaining how torpor in birds and mammals could be derived from the most recent common amniote ancestor, an ectotherm which lived 325 million years ago (Shedlock and Edwards, 2009), it is reasonable to assume that early mammals had a low and variable T_b, and that the low T_b of monotremes (31–32°C), the lowest of any of the mammalian orders (Clarke and O'Connor, 2014), reflects an ancestral condition. However, torpor and hibernation in echidnas is extremely variable between geographic sub-species. In warm climates there seems to be a protoendotherm-like continuum from daily torpor to hibernation, while in cooler areas the expression of hibernation is indistinguishable from Lovegrove's apoendothermic highly seasonal, well-regulated adaptive hibernation (Lovegrove, 2012).

Echidnas do provide some useful insight into the relative merits of the parental care and assimilation capacity models. Very close regulation of maternal T_b during egg-incubation (Beard et al., 1992; Nicol and Andersen, 2006) is consistent with the parental care model as the maintenance of a high and constant temperature must be energetically expensive. The 10–11 day period of egg-incubation corresponds with the period of organogenesis and neurulation in echidnas (Werneburg and Sánchez-Villagra, 2011). These developmental processes are particularly temperature sensitive (Andrews, 2004), and in reptiles and birds embryonic development is very sensitive to variations in temperature as well as the absolute temperature (Shine, 2005; Du and Shine, 2015). As decreased mortality early in life results in a larger gain in Darwinian fitness than can be achieved by a comparable decrease of mortality at an older age (Stearns, 1992), there would be very strong selection for higher energy expenditure during egg-incubation. By contrast, the fact that the metabolic rate of lactating female echidnas is not measurably higher that of non-lactating females, suggests that post-hatching energy expenditure may not necessarily be as strong a selection force as is suggested by the assimilation-capacity model (Koteja, 2000).

The monotremes also provide some support for the proposal that the increase in aerobic scope in endotherms was achieved through an increase in T_b (Clarke and Pörtner, 2010). The very similar maximum metabolic rates of the echidna and platypus, despite their differences in BMR, points to a T_b limitation on metabolic capacity. The more energetically expensive lifestyle and higher BMR of the platypus is associated with thyroid hormone levels which exceed those of the majority of eutherian species. Is the monotreme mitochondrial machinery idling at a much higher rate in the platypus but with still the same temperature limited maximal output as echidnas?

The egg-laying mode of reproduction of the monotremes led early researchers to perceive them as living fossils whose physiology will give insights into the physiology of early mammals. Extant monotremes however are highly specialized, and aspects of their physiology are likely to be strongly affected by their ecological niche. They also have a large brain which accounts for about 9% of resting metabolism, which is certainly not a primitive trait. Monotremes show a mosaic of derived and plesiomorphic features in their embryology and development (Werneburg and Sánchez-Villagra, 2011), adult anatomy (Crompton and Jenkins, 1973) and genome (Warren et al., 2008), reinforcing the picture that mammalian evolution is not a story of linear progress starting with monotremes, passing through marsupials and reaching placentals (Werneburg and Sánchez-Villagra, 2011). This mosaic also clearly extends to energetics and thermoregulation.

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