

NEUROENDOCRINE CONTROL OF ENERGY HOMEOSTASIS IN NON-MAMMALIAN VERTEBRATES AND INVERTEBRATES

EDITED BY: Suraj Unniappan, Ian Orchard and María Jesús Delgado
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NEUROENDOCRINE CONTROL OF ENERGY HOMEOSTASIS IN NON-MAMMALIAN VERTEBRATES AND INVERTEBRATES

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Editorial: Neuroendocrine Control of Energy Homeostasis in Non-mammalian Vertebrates and Invertebrates

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Keywords: food intake, fish, energy homeostasis, invertebrates, metabolism, hormones

Editorial on the Research Topic

Neuroendocrine Control of Energy Homeostasis in Non-mammalian Vertebrates and Invertebrates

Energy is critical for all physiological processes, including the growth and reproduction of animals, and so the maintenance of energy balance is essential for species survival. Imbalances in energy intake and expenditure lead to abnormal physiology and debilitating diseases, contributing to a growing interest in the study of energy balance. Among the myriad of factors that contribute to energy intake and expenditure, hormones and endogenous factors with hormone-like actions are prime elements. The use of comparative approaches to study hormones regulating metabolism and other physiological functions are very important and impactful. It helps to address species-specific differences in hormones and their action, which are essential in tailoring custom approaches to utilize endocrinology for the benefit of species survival, sustainability, and health. Comparative neuroendocrinology research using fish (aquaculture and food sustainability) and invertebrates (disease and/or vector control) are examples of this. Both mammals and non-mammals, and invertebrates have been used to study the mechanism of endocrine and metabolic diseases. Several hormone-based pharmaceuticals that are commercialized for metabolic and endocrine diseases have its roots in comparative endocrinology research using model organisms. Due to these, we conceived and coordinated the publication of a Research Topic on the “*Neuroendocrine Control of Energy Homeostasis in Non-mammalian Vertebrates and Invertebrates*.” There were 14 original research articles and 4 reviews in this issue, covering a broad spectrum of research across both invertebrates (8 articles) and vertebrates (10 articles). This e-book presents the collection of the above articles that highlight many recent advances in the neuroendocrine regulation of energy homeostasis. The research presented here encompasses cellular, molecular, and organismal level biology, employing a large number of cutting edge tools. These include techniques associated with genetic and molecular biology, biochemistry, behavior, physiology, and pharmacology; the reports are state-of-the art and timely in nature.

Four review articles consider the biology of insulin-like peptides (ILPs) signaling in mosquitoes, neuropeptide F (NPF) in invertebrates, the roles of ghrelin and motilin in regulating gut motility in vertebrates, and the gut microbiota and energy homeostasis in fish. Sharma et al. provide a very comprehensive commentary on the structure, distribution and functions of ILPs in regulating metabolism, growth and reproduction, as well as the vectorial capabilities of mosquitoes by influencing mosquito-pathogen interaction. Overall, this review highlights the significance of ILPs

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in an important disease vector, and points the reader to priorities that researchers should focus on in future research. Fadda et al. summarize the roles of the neuropeptide Y (NPY) family, neuropeptide F (NPF) peptides, in the regulation of energy balance in invertebrates. Their very systematic review provides an excellent overview of NPF history, nomenclature, and signaling, and the roles of NPF in energy homeostasis in an array of invertebrate groups. Meanwhile, Kitazawa and Kaiya enlighten us on the roles of motilin and ghrelin, two very important gut peptides, on gut motility in vertebrates. This very thorough review covers a large number of species from teleost fish to humans, studying the motility effects of these two gut peptides. While both peptides and their receptors are present across most species studied, there appears to be species-specific differences in their roles on gastrointestinal motility. In addition, huge gaps in knowledge still exist when it comes to a full understanding of these two peptides. Butt and Volkoff focus on a very different aspect; the role of gut microbiota on metabolic homeostasis, which remains a current area of prolific research focus in vertebrates. This article discusses the role of gut microbiota in regulating energy balance and energy-utilizing functions including stress, reproduction, immunity, development and growth. It considers the factors affecting gut microbiota, which include age, sex, genetics, life stage, diet and nutrient composition, and how the gut microbiota respond to the use of antibiotics, pre-/pro- and symbiotics. Overall, the knowledge covered under these reviews has implications in vector control, and aquaculture (fish metabolism).

Among the original research articles, six focus on invertebrates. Post et al. report their interesting findings on how ILPs regulate longevity by deactivating glycogen phosphorylase, which is a key player in glycogen storage and gluconeogenesis. Defferari et al. report the first insulin receptor, and showed how conserved the insulin cell signaling mechanisms that contribute to energy homeostasis are in a blood feeding insect, *Rhodnius prolixus*. Kawabe et al. study bombyxin II, an ILP in *Bombyx mori*. Their key findings indicate that bombyxin II injection resulted in an increase in oxygen consumption and an upregulation of an enzyme activator of glycolysis in a tissue specific-manner. Meanwhile, Marco and Gäde present an original article that focuses on the ligand-receptor interactions of five forms of the adipokinetic hormone (AKH) found in the sphingid moth, *Hippotion eson*. They found that there is no signature bioactive core in these peptides, and the conformation provided by the full-length active peptide is ideal for most effective receptor binding. Sharma, Pooraiouby et al. in a second article (original research), furnish details of the of ILPs in black-legged tick, *Ixodes scapularis*. Their initial characterization found ILP sequences, and their tissue-, and life-/feeding-stage-specific expression in this tick, and provided impetus for future research on these important metabolic peptides in ticks. Wahedi et al. add new information on structure-activity relationships of *Aedes aegypti* receptors for insect hormones with gonadotropin releasing hormone-like properties, namely AKH, corazonin (CRZ) and AKH/CRZ-related-peptide. They identified a number of critically-positioned amino acids within these peptides that enable receptor binding.

All these articles contribute significantly to our understanding of cellular and/or organismal metabolic processes and the mechanism by which hormones regulate such processes in a variety of invertebrates.

Among articles that inform us on new research data from vertebrates, most used fish as a model organism. Perelló-Amorós et al. report the nutritional regulation of ghrelin and its receptors in a cultured fish, gilthead sea bream (*Sparus aurata*). They found that the tissue-specific expression of endogenous ghrelin, an orexigen, and its receptors fluctuate during fasting. While Perelló-Amorós et al. provide new insights on an orexigen Blanco et al. pursue nesfatin-1, an anorexigen in another cultured fish, the rainbow trout (*Oncorhynchus mykiss*). They found that injection of nesfatin-1 into the brain resulted in a suppression of feeding, an increase in glucose sensing and in lipogenic enzymes in the brain. Meanwhile, the same treatment reduced gluconeogenic and lipogenic mRNAs, and enhanced enzymes that promote fatty acid oxidation. Chen et al. look into how temperature affects feeding in goldfish (*Carassius auratus*). They found a decrease in feeding with lowering temperatures and corresponding changes in appetite regulatory peptides. Liu et al. focus on the melanocortin 4 receptor (Mc4r) and its role in the growth of medaka (*Oryzias latipes*). While the growth of medaka that had genetically lost Mc4r was significantly suppressed compared to wildtype controls, no changes in puberty were found in the *mc4r* knockout fish. Nakamachi et al. indicate that adenylate cyclase-activating polypeptide (PACAP) and PAC1R are involved in the regulation of feeding in zebrafish (*Danio rerio*), where intracerebroventricular injection of PACAP reduced feeding without affecting locomotor activity. Otero-Rodiño et al. use morphometric analysis to map the glucosensing system within the rainbow trout brain. They found glucokinase expressed in neurons that co-expressed an orexigen, agouti-related peptide, in the rostral hypothalamic neurons of the trout brain. Gómez-Boronat et al. reveal the diurnal changes in n-acyl ethanolamines (NAEs), a group of lipid signaling molecules in goldfish. NAEs showed a daily rhythm in their tissue specific expression, and were significantly elevated post-prandially in the gut. This suggests an anorectic role for NAEs in goldfish. These articles provide novel insights on the hormonal regulation of energy balance in fish.

The sole article on avians reports primary research on the neurosecretory protein GL (NPGL). Shikano et al. reveal that the chronic intracerebroventricular infusion of NPGL resulted in a depot-specific increase in white adipose tissue mass, which indicates a role for this peptide in creating a positive energy balance. They also found an increase in liver mass, suggesting a positive role for centrally administered NPGL on *de novo* lipogenesis in chicken. There were no submissions on studies using mammals.

In conclusion, this e-book provides research data and reviews that furnish opportunities to gather significant new knowledge on hormones and metabolism. From neuroanatomy to cell physiology to whole animal biology, the authors consider several key determinants that influence hormones and their actions. These include sex, age, temperature, locomotion, feeding,

tissue-specific expression, structure of hormones, receptors, mode of administration and species used. The extensive nature of the research articles assisted in the broader scope of this e-book. We tremendously enjoyed working with the authors, reviewers, and editorial staff, whose contributions were immeasurable to the success of this Research Topic and the resulting e-book. As co-editors, we are deeply thankful to everyone involved in making this issue a success. It is our sincere hope that this compendium will remain useful to researchers interested in the neuroendocrinology of energy homeostasis. Finally, we want to thank our readers, who are also the ultimate reviewers of the work presented. Enjoy reading!

AUTHOR CONTRIBUTIONS

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Drosophila Insulin-Like Peptides DILP2 and DILP5 Differentially Stimulate Cell Signaling and Glycogen Phosphorylase to Regulate Longevity

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Insulin and IGF signaling (IIS) is a complex system that controls diverse processes including growth, development, metabolism, stress responses, and aging. *Drosophila melanogaster* IIS is propagated by eight *Drosophila* insulin-like peptides (DILPs), homologs of both mammalian insulin and IGFs, with various spatiotemporal expression patterns and functions. DILPs 1–7 are thought to act through a single *Drosophila* insulin/IGF receptor, InR, but it is unclear how the DILPs thereby mediate a range of physiological phenotypes. We determined the distinct cell signaling effects of DILP2 and DILP5 stimulation upon *Drosophila* S2 cells. DILP2 and DILP5 induced similar transcriptional patterns but differed in signal transduction kinetics. DILP5 induced sustained phosphorylation of Akt, while DILP2 produced acute, transient Akt phosphorylation. Accordingly, we used phosphoproteomic analysis to identify distinct patterns of non-genomic signaling induced by DILP2 and DILP5. Across all treatments and replicates, 5,250 unique phosphopeptides were identified, representing 1,575 proteins. Among these peptides, DILP2, but not DILP5, dephosphorylated Ser15 on glycogen phosphorylase (GlyP), and DILP2, but not DILP5, was subsequently shown to repress enzymatic GlyP activity in S2 cells. The functional consequences of this difference were evaluated in adult *Drosophila dilp* mutants: *dilp2* null adults have elevated GlyP enzymatic activity relative to wild type, while *dilp5* mutants have reduced GlyP activity. In flies with intact insulin genes, *GlyP* overexpression extended lifespan in a Ser15 phosphorylation-dependent manner. In *dilp2* mutants, that are otherwise long-lived, longevity was repressed by expression of phosphonull *GlyP* that is enzymatically inactive. Overall, DILP2, unlike DILP5, signals to affect longevity in part through its control of phosphorylation to deactivate glycogen phosphorylase, a central modulator of glycogen storage and gluconeogenesis.

Keywords: insulin, IGF, *Drosophila* insulin-like peptides, glycogen phosphorylase, glycogen, metabolism, aging, signaling bias

INTRODUCTION

Insulin and insulin-like growth factor signaling (IIS) is an extensive network crucial for development, growth, nutrient sensing, aging, and stress responses (1–3). Dysfunction in IIS contributes to metabolic syndrome, diabetes, and cancer (4, 5), yet genetic modification of IIS can extend lifespan in many animals (6–8). Mammalian insulin and IGF ligands each have respective receptors, although each ligand can activate either receptor, receptors can form hybrid dimers, and the receptors themselves activate similar kinase cascades with multiple redundant components (9). It is currently unknown how IIS ligands use common receptors and pathways to produce different cellular and organism phenotypes such as glucose homeostasis for insulin and development and differentiation for IGF. Here, we use *Drosophila melanogaster* as a model system to understand how various insulin-like peptides [*Drosophila* insulin-like peptides (DILPs)] function through the fly's sole insulin/IGF tyrosine kinase receptor (InR) to mediate specific physiological traits.

In *Drosophila*, ligand-activated InR phosphorylates a single insulin receptor substrate (IRS) *chico*, the homolog of mammalian IRS1–4, to induce the phosphorylation cascade of phosphoinositide-3-kinase (PI3K), phosphoinositide-dependent-kinase-1, and Akt (protein kinase B) (10). This signal transduction culminates to repress the forkhead transcription factor dFOXO, the homolog of mammalian FOXO1, 3a, and 4 (11). Fly IIS and FOXO mutants affect larval growth, adult size, lipid metabolism, stress responses and aging (8, 12, 13). Data suggest that DILPs have distinct spatial and temporal expression patterns and regulate shared and specific functions (13, 14). For instance, embryos express *dilp4*, larvae express *dilp2*, *dilp3*, and *dilp5*, and pupae upregulate *dilp1* and *dilp6* expression (1, 13, 14). In adults, *dilp2* modulates adult lifespan and blood sugar (8, 15), *dilp5* mediates protein metabolism (16), and *dilp3* is suggested to regulate lipid metabolism (17). Given the diversity of these functions, it is poorly understood how specificity can be produced by similar ligands signaling through a common InR receptor.

The unique spatiotemporal expression patterns of DILPs may be sufficient to confer their specific phenotypes. An alternative, but not mutually exclusive model proposes that DILPs differentially activate InR to induce distinct cell signaling patterns that communicate specific downstream phenotypes. Studies from mammalian systems support such a signaling bias model: cells engineered to express only the insulin receptor (IR), IGF-1R, or IGF-2R produce distinct signaling and gene expression patterns in response to insulin, IGF-1, or IGF-2 (18, 19). At a biochemical level, Cieniewicz et al. (20) found that the IR was differentially phosphorylated and dephosphorylated on several individual tyrosine residues when cells were treated with insulin, IGF-1 or IGF-2. Furthermore, the IR but not IGF receptors regulates FKHR phosphorylation, GSK-3 inactivation, and glycogen synthesis (21, 22).

Models of ligand–receptor interaction propose that IR ligands with different receptor-binding kinetics can induce distinct downstream signaling (23, 24). Empirically, Sciacca et al. (25) found that the insulin analogs aspart, lispro, and glulisine produce more

sustained Akt phosphorylation than insulin, which generates transient Akt phosphorylation. Analogs such as [B-Asp¹⁰] insulin and [A-His⁸, B-His⁴, B-Glu¹⁰, B-His²⁷] insulin, which have strong receptor-binding affinity, induced sustained receptor phosphorylation and increased mitogenicity (26), and insulin analogs with weaker receptor-binding affinity produced less mitogenic potential (27, 28). Recently, decreased mitogenicity was reported for the insulin analog dicarba where the A6-A11 disulfide bond is replaced with a rigid bridge to reduce flexibility in its receptor interaction (29). Collectively, these data suggest that insulin, IGF, and insulin analogs can differentially bind to receptors to produce distinct signals and generate different downstream phenotypes.

Drosophila insulin-like peptide 2 and DILP5 are two principal hormones made in the insulin-producing cells of the adult *Drosophila* brain (30). They are thought to regulate sugar and protein metabolism respectively (31), and to uniquely respond to dietary sugar and protein (32). We compared cells stimulated *in vitro* with DILP2 or DILP5 to determine their cell signaling outputs measured as Akt and InR phosphorylation, transcript profiles and global patterns of protein phosphorylation. In S2 cells, DILP2 and DILP5 regulate highly similar sets of mRNA transcripts and are equally potent in their ability to stimulate Akt phosphorylation. On the other hand, at a given dose DILP2 and DILP5 differentially modulate the kinetics of Akt phosphorylation, with DILP2 producing acute stimulation and DILP5 generating prolonged signal. Expanding this non-genomic view of biased signaling, we used global phosphoproteomic analysis and found substantial qualitative differences between DILP2 and DILP5. In particular, DILP2 uniquely regulates dephosphorylation of glycogen phosphorylase (GlyP) at Ser15, the residue that represses enzymatic activity in mammals and *Drosophila* (33–35). In concordance, we find that *dilp2* mutant flies have elevated GlyP enzymatic activity, and that overexpression of *GlyP* extended longevity in adults with normal insulin in a GlyP phosphorylation-dependent manner that corresponded with GlyP enzymatic activity. In a complementary way, we infer that the phosphorylation status and enzymatic activity of *GlyP* contribute to the lifespan extension of *dilp2* mutants because expression of phosphonull, inactive *GlyP* partially rescues extended longevity of *dilp2* mutants. Together, these data demonstrate that non-genomic signaling bias at the cellular level plays a role in differential physiological and life history functions of two insulin-like peptides.

MATERIALS AND METHODS

Cell Culture and DILP Stimulation

S2 cells were cultured in Schneider's media (Gibco) with 10% FBS (Gibco) and maintained at 28°C with ambient CO₂. For overnight serum depletion, cells were held for 16 h in Schneider's media without FBS, supplemented with 0.5% BSA and 25 mM HEPES. DILP5 peptide (36) stock at 50 μM and DILP2 peptide stock (37) at 100 μM stimulated overnight depleted cells compared with control depleted cells treated in parallel with an equal volume of 0.001 N HCl solvent (vehicle). The dose and duration of DILP stimulation for each experiment is detailed in the figure legend.

RNA-Seq and Data Analysis

S2 cells at a density of $\sim 1 \times 10^7$ cells per 100 cm cell culture dish were serum depleted overnight and stimulated with 100 nM DILP2, 100 nM DILP5 or vehicle for 1 h. Cells were washed with sterile, cold PBS and resuspended in lysis buffer (NE BioLabs magnetic mRNA isolation kit # S1550S). mRNA was purified and fragmented (Ambion # AM8740), then reverse transcribed to cDNA (Invitrogen SuperScript III # 18080051). DNA was purified by Agencourt Ampure XP beads and quantified by Qubit. DNA was repaired by End-It DNA Repair Kit (Epicenter, # ER0720) then supplemented with Klenow fragment (NE BioLabs # M0212s). Adapters were ligated using the T4 DNA Ligase Quick Ligase Kit (Enzymatics L603-HC-L) and PCR enriched using Phusion DNA polymerase (NE BioLabs # F-531). Libraries were gel purified, selecting DNA from 200 to 500 bp, and gel extracted (Qiagen Qiaquick # 28704). The selected libraries were sequenced on a HiSeq2500 with 50 bp single-end reads. Fastq files were assembled by Tophat and Bowtie, and assemblies were mapped to *D. melanogaster* dm3 assembly. In R, transcript counts and RPKM values were computed by the “easyRNASeq” package, and differential expression was calculated by the “edgeR” package. Transcript abundances by RPKM were trimmed to exclude genes with 0 counts to calculate coefficient of variance and determine the top 10% of genes with most variance. All raw RPKM expression data are listed in Table S1 in Supplementary Material.

Quantitative RT-PCR

Adult flies, serum-depleted S2 cells, and DILP-treated S2 cells at a density of about 1×10^6 cells/ml were lysed in Trizol (Invitrogen) by mechanical force with two 3.2 mm steel beads in a TissueLyser. RNA was Trizol extracted, treated with Turbo DNase (Invitrogen), and quantified on a NanoDrop ND-1000 (Thermo Fisher Scientific Inc.). RNA was reverse transcribed with iScript cDNA synthesis (Bio-Rad Laboratories, Inc.). Quantitative RT-PCR was conducted on an ABI prism 7300 Sequence Detection System (Applied Biosystems) using SYBR green PCR master mix (Applied Biosystems). Relative mRNA levels were calculated relative to RP49 expression by the comparative Ct method. Primer sequences are listed in Table S2 in Supplementary Material.

Western Blots

S2 cells at a density of about 1×10^6 cells/ml were serum-depleted overnight and stimulated with DILP2, DILP5 or vehicle at the indicated concentration for the designated duration. Cells were washed briefly with sterile, cold PBS and resuspended in NP40 lysis buffer (Thermo Scientific # FNN0021) supplemented with 1 mM PMSF, PhosSTOP phosphatase inhibitor cocktail (Roche # 04906837001) and Protease Inhibitor Cocktail (Invitrogen # 78425). Cells were incubated on ice for 30 min, and vortexed every 10 min. Cell lysates were spun down for 10 min at 13K rpm and the supernatant incubated at 70°C with gel loading buffer and reducing reagent. Samples were loaded onto SDS-PAGE gels (Invitrogen NuPAGE # NP0321) and run at 200 V for 45 min. Gels were transferred to nitrocellulose membrane (Whatman # 10401396) and blocked with 5% BSA in TBS-T for

1 h. Membranes were incubated with primary antibody diluted 1:1,000 in 5% BSA in TBS-T overnight at 4°C with gentle rocking. Antibodies for Westerns were purchased from Cell Signaling Technology: *Drosophila* phospho-Akt Ser505 (# 4054); Pan-Akt (# 4691); Pan-phospho-ERK1/2 Thr202/Tyr204 (# 4370); Pan-ERK (# 9102); *Drosophila* phospho-S6K (# 9209); Pan-S6K (# 2708); IGF-R Tyr1131 (# 3021); actin (# 4967), or from Phospho-Solutions: *Drosophila* phospho-Akt Thr342 (# p104-342). Blots were washed for 5 min three times in TBS-T and incubated in horseradish peroxidase conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted 1:5,000 in 1% BSA for 1 h at room temperature. Subsequently, blots were washed for 5 min three times in TBS-T and incubated with ECL reagent (Perkin Elmer # NEL121001EA). Final blots were imaged and volume densitometrically quantified with ImageLab (Bio-Rad).

Phosphopeptides Sample Preparation and Enrichment for LC-MS/MS Analysis

Cell pellets were lysed in urea buffer (8 M urea, 1 mM sodium orthovanadate, 20 mM HEPES, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, pH 8.0, 20 min, 4°C), sonicated and cleared by centrifugation (14,000 g, 15 min, 4°C). Protein concentration was measured (Pierce BCA Protein Assay, Thermo Fisher Scientific, IL, USA) and a total of 100 μ g protein per sample was subjected to trypsin digestion. Tryptic peptides were desalted using C18 Sep-Pak plus cartridges (Waters, Milford, MA, USA) and lyophilized for 48 h to dryness. Phosphopeptides were enriched with Titansphere Phos-TiO tips (GL Sciences, Tokyo Japan) following the manufacturer's protocol with modifications. We first prepared the condition buffers (containing TFA, CH₃CN, and lactic acid) and elution buffers (1% NH₄OH in water and 40% CH₃CN). Then, the condition buffer was added to the Phos-TiO tips (centrifuge at 3,000 g, 22°C). After conditioning, desalted tryptic peptides from Jurkat total lysates (CD3/4 stimulated or unstimulated) were mixed with a synthetic phosphoserine standard (FQpSEEQQTDELQDK, AnaSpec, San Jose, CA, USA) at a ratio of 5 fmol standard: 1 μ g sample. The mixture was loaded onto tips using centrifugation at 1,000 g at 22°C. After loading, the column was washed with condition buffers followed by elution buffers. Acetic acid was used to acidify TiO₂-enriched samples, which were dried almost to completeness. The dried-eluted phosphopeptides were reconstituted in buffer A (0.1 M acetic acid) at a concentration of 1 μ g/ μ l, and 5 μ l was injected for each analysis.

The LC-MS/MS was performed on a fully automated proteomic technology platform (38, 39) with an Agilent 1200 Series Quaternary HPLC system (Agilent Technologies, Santa Clara, CA, USA) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The LC-MS/MS workflow follows Ahsan et al. (40). Peptides were separated through a linear reversed-phase 90 min gradient from 0 to 40% buffer B (0.1 M acetic acid in acetonitrile) at a flow rate of 3 μ l/min through a 3 μ m 20 cm C18 column. Electrospray voltage of 2.0 kV was applied in a split flow configuration, and spectra were

collected using a top-9 data-dependent method. Survey full scan MS spectra (m/z 400–1,800) were acquired at a resolution of 70,000 with an AGC target value of 3×10^6 ions or a maximum ion injection time of 200 ms. The peptide fragmentation was performed *via* higher-energy collision dissociation with the energy set at 28 NCE. The MS/MS spectra were acquired at a resolution of 17,500, with a targeted value of 2×10^4 ions or a maximum integration time of 200 ms. The ion selection abundance threshold was set at 8.0×10^2 with charge state exclusion of unassigned and $z = 1$, or six to eight ions and dynamic exclusion time of 30 s.

Phosphoproteomics Analysis

Peptide spectrum matching of MS/MS spectra of each file was searched against a species-specific databases (UniProt; downloaded 2/1/2015) using MASCOT v. 2.4 (Matrix Science, Ltd., London, UK). A concatenated database containing “target” and “decoy” sequences was used to estimate the false discovery rate (FDR) (41). Msconvert from ProteoWizard (v. 3.0.5047), using default parameters and the MS2Deisotope filter, was used to create peak lists for Mascot. The Mascot database search was performed with the following parameters: trypsin enzyme cleavage specificity, two possible missed cleavages, 10 ppm mass tolerance for precursor ions, 20 mmu mass tolerance for fragment ions. Search parameters permitted variable modification of methionine oxidation (+15.9949 Da) and static modification of carbamidomethylation (+57.0215 Da) on cysteine. To identify the phosphoresidues, we included additional variable modification of phosphorylation (+79.9663 Da) on serine, threonine, and tyrosine residues. The resulting peptide spectrum matches (PSMs) were reduced to sets of unique PSMs by eliminating lower scoring duplicates. To provide high confidence, the Mascot results were filtered for Mowse Score (>20). Peptide assignments from the database search were filtered down to a 1% FDR by a logistic spectral score as previously described (41, 42). To validate the position of the phosphorylation sites, the Ascore algorithm (43) was applied, and the reported phosphorylation site position reflected the top Ascore prediction.

Relative Quantitation of Phosphopeptides

Phosphopeptide abundance was quantified from selected ion chromatograms (SIC) peak areas. Retention time alignment of individual replicate analyses was performed as described by Demirkan et al. (44). Peak areas were calculated from SICs in R 3.0 based on the Scripps Center for Metabolomics' XCMS package (version 1.40.0). This approach performed multiple passes through XCMS' central wavelet transformation algorithm (implemented in the centWave function) over increasingly narrower ranges of peak widths with parameters: mass window of 10 ppm, minimum peak widths ranging from 2 to 20 s, maximum peak width of 80 s, signal to noise threshold of 10 and detection of peak limits *via* descent on the non-transformed data enabled. SIC peak areas were determined for every phosphopeptide identified by MS/MS. In the case of a missing MS/MS for a particular peptide in one replicate, the SIC peak area was calculated according to the peptide's isolated mass, and the retention time was calculated from retention time alignment. A minimum SIC peak area equivalent to the typical spectral noise level of 1,000

was required of all data reported for label-free quantitation. Individual SIC peak areas were normalized to the peak area of the standard phosphopeptide DRVpYHPF that was exogenously spiked before phosphopeptide enrichment and reversed-phase elution into the mass spectrometer. Quantitative analysis was applied to five replicate experiments. To select phosphopeptides that show a statistically significant change in abundance between control and treatment cells, q -values for multiple hypothesis tests were calculated based on p -values from two-tailed unpaired Student's t -tests using the R package QVALUE as described by Storey (2003) and Storey and Tibshirani (45, 46).

Glycogen and Glucose Quantification

Glycogen and glucose were quantified as described by Tennessen et al. (47). Flies were mated for 2 days, and females separated into groups of six flies. Food vials were changed every other day, and at age 8–10 days, flies were briefly anesthetized with light CO₂, collected in a microcentrifuge tube and flash frozen in dry ice. With tubes kept on ice, flies were homogenized in 100 μ l PBS using a motorized plastic pestle. A 10 μ l aliquot was removed for BCA protein quantification, and the remaining 90 μ l was heat treated at 70°C for 10 min. Samples were spun down at 14K rpm for 3 min at 4°C, and the supernatant removed to a new tube. Samples were diluted 1:10, and standard curve dilutions for glucose and glycogen were made by diluting stocks to 160 μ g/ml, making 1:1 serial dilutions for 80, 40, 20, and 10 μ g/ml. 25 μ l of each sample was pipetted to four wells of a clear microplate, and 25 μ l of each glucose or glycogen standard was pipetted to two wells. Amyloglucosidase enzyme (Sigma, # A1602) was diluted 1.5 μ l into 998.5 μ l PBS, and 25 μ l diluted enzyme was pipetted to the glycogen standards and two sample wells. 25 μ l PBS was pipetted to the glucose standards and to the other two sample wells. The plate was wrapped in parafilm and incubated at 37°C for 1 h. 100 μ l Glucose Hexokinase Reagent (Thermo Scientific TR15421) was pipetted to each well, and the plate was incubated at room temperature with gentle rocking for 15 min. The absorbance was read at 340 nm on a SpectraMax M5 platereader using Softmax Pro software. The glycogen concentration was quantified by subtracting the glucose absorbance from the total glycogen + glucose absorbance and normalized to total protein content quantified by BCA.

Glycogen Phosphorylase Activity Assay

Activity of glycogen phosphorylase was adapted and modified from protocols used for mammalian cells (48). Five female flies 7–10 days old were harvested in 150 μ l NP40 lysis buffer with inhibitors (see Western Blots) without centrifugation. 10 μ l lysate was combined with reaction mixture in a 96-well plate on ice. Reaction mixture consisted of 50 mM Na glycerophosphate pH 7.1, 10 mM K₂PO₄, 5 mM MgCl₂, 1 mM DTT, 0.2% glycogen, 0.5 mM NAD⁺, 1.6 U phosphoglucomutase, and 1.6 U glucose-6-phosphate dehydrogenase in a total reaction of 300 μ l. The plate was brought to room temperature (25°C), and fluorescence was measured at excitation 350 nm, emission 470 nm (SpectraMax M5 platereader) for NADH generation. Activity was calculated by determining the fluorescence after

45 min incubation, relative to total protein determined by BCA assay (Thermo Scientific # 23227).

Cloning *Drosophila* GlyP

GlyP cDNA was obtained from the *Drosophila* Genomics Resource Center (stock # LD24485) and cloned into pENTR-TOPO Gateway vector (Thermo Fisher). Mutations S15A and S15D were made using the GENEART Site-Directed Mutagenesis System (Invitrogen A13282). Wild-type, S15A, and S15D GlyP coding sequences were sub-cloned into the *Drosophila* transgene vector pUAST-attB and were submitted to GenetiVision Corporation (Houston, TX, USA) for transgene embryo injection and stock generation.

Fly Husbandry

Flies were reared and maintained at 25°C, 40% relative humidity and 12-h light/dark. Adults were maintained upon agar-based diet with cornmeal (0.8%), sugar (10%), and yeast (2.5%), or upon the same diet supplemented with 200 μ M mifepristone (RU486) or ethanol control. Stocks were backcrossed to w¹¹¹⁸ for at least five generations.

Lifespan Assays

Two- to three-day-old mated female adult flies reared in density-controlled bottles were collected with light CO₂ anesthesia and pooled in 1 l demography cages at a density of 100–125 flies per cage. Three independent cages were used per genotype. Food vials were changed every day for the first 3 weeks, then every 2 days for the remainder of the experiment. Dead flies

were removed and recorded every other day. Survival analysis and Cox Proportional Hazard analysis were conducted in R using the “surv” package and “survdiff” function. To adjust for mortality caused by the RU486 covariate independent of genotype, Gompertz mortality models were fit to control genotypes given RU486 or ethanol vehicle using the “flexsurv” package and “flexsurvreg” function. The mortality estimate decreased by RU486 treatment was applied to Gompertz mortality models for test genotypes given RU486 or ethanol vehicle.

Starvation Assays

Two- to three-day-old mated female adult flies reared in density-controlled bottles were collected with light CO₂ anesthesia into glass vials containing 1% agar solution at a density of about 15 flies per vial. Eight independent vials were used per genotype. Vials were changed every 2–3 days to ensure flies did not desiccate. Dead flies were counted every 8 h. Data analyzed as in Section “Lifespan Assays.”

RESULTS

Drosophila insulin-like peptides 1–8 have varied physiological functions (8, 49, 50) which may arise from specific amino acid sequences and structures that interact with InR in precise ways. When DILP peptide sequences are aligned with that of human insulin and IGF (Figure 1), the B and A chains of the proposed mature pro-hormones show low sequence identity among DILPs and between DILPs, insulin and IGF, except for cysteine residues required for disulfide bonds connecting the

B chain

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DILP1:  -----MVT---PTGSGHQLPPGNHKLGGPALSDAMDV-VCPHG-FNTLP--
DILP2:  -----TLCSEKLNVEVLSM-VCEEY-NPVIHP-
DILP3:  -----TMKLCGRKLPETLSK-LCVYG-FNAMT---
DILP4:  -----RRKMCGEALIQALDV-ICVNG-FT-----
DILP5:  -----ANSLRACGPALMDMLRV-ACPNG-FNSMFA-
DILP6:  -----S---PL---APTEYEQRRMMCSTGLSDVIQK-ICVSG-TVA----
DILP7:  LQHTEEGLEMLFRERSQSDWENVWHQETHSRCRDKLVRLQYW-ACEKD-IYRLT--
DILP8:  -----SFCSLERMKKFAMEACEHL-FQADEGA
IGF     -----GPETLCGAELVDALQF-VCGRGFGFYFNKPT
insulin -----FVNQHLCGSHLVEALYL-VCGERGFFYTPKT

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A chain

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DILP1:  ---HLTGGVYDECCVK-TCSYLELAIYCLPK-----
DILP2:  -----QGIVERCCKK-SCDMKALREYCSVVRN----
DILP3:  -----DGVFDECCLK-SCTMDEVLRICAAPRT---
DILP4:  -----IAHECCKE-GCTYDDILDYCA-----
DILP5:  ---DFRGVVDSCCRK-SCSFSTLRAYCDS-----
DILP6:  ---DLQNVTDLCCKSGGCTYRELLQYCKG-----
DILP7:  -SDGNTPSISNECCTKAGCTWEEYAEEYCPNKRNRHY
DILP8:  DHSSSYNNIPYCCLN-QCEE---EFFC-----
IGF     -----GIVDECCFR-SCDLRRLEMYCAPLKPAS
insulin -----GIVEQCCTS-ICSLYQLENYCN-----

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FIGURE 1 | *Drosophila* insulin-like peptide (DILP) amino acid sequences have a low degree of identity between each other and with human insulin and IGF. DILP B chain and A chain sequences based on predicted cleavage sites. Alignment made using EMBL-EBI Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>).

B and A chains (51, 52). In addition, among most ligands there is a highly conserved tyrosine in the A chain and a leucine in the B chain that are proposed to function in ligand–receptor interactions (36). DILP7 and DILP1 are longer than other DILPs and have very unique sequences relative to DILPs 1–6. Human insulin, IGF, and some DILPs contain one or two phenylalanines at the C-terminal tail of the B chain, although these residues are not found in DILP2, DILP6 and DILP7.

Drosophila insulin-like peptide 2 and DILP5 are similarly expressed and released from brain medial secretory neurons in adult *Drosophila*, yet appear to have distinct roles in glucose and protein metabolism and in aging (8, 16). To understand how these related ligands generate different outcomes *in vivo*, we treated *Drosophila* S2 cells in culture with purified synthetic DILP2 (37) and recombinant DILP5 (36). At doses ranging from 0.1 to 100 nM, DILP2 and DILP5 stimulated comparable increases in Akt phosphorylation at Ser505 (**Figure 2A**), suggesting they have similar potency. Likewise, DILP2 and DILP5 were equally efficient at stimulating InR and Akt phosphorylation in a ligand competition assay (**Figure 2B**). Finally, DILP2 and DILP5 similarly stimulated phosphorylation of Akt at Thr342, S6K at Thr398, and ERK at Thr202/Tyr204, though S6K phosphorylation is slightly stronger after DILP5 stimulation than DILP2 (**Figure 2C**). Together, these data suggest that the synthetic DILP2 and recombinant DILP5 reagents have comparable potency and ability to stimulate S2 cell signaling.

Human insulin and IGF, and *Drosophila* DILPs regulate activity of FOXO transcription factors to affect downstream pathways through control of gene expression (53–55). To determine whether DILP2 and DILP5 stimulate different or similar gene expression profiles, we conducted RNA-Seq from mRNA of S2 cells stimulated with each DILP at 100 nM for 1 h (**Figures 2D,E**; **Figure S1** and **Table S1** in Supplementary Material). Transcript profiles induced by DILP2 and DILP5 were not distinguishable based on hierarchical clustering of the top 2.5% responding genes, while profiles stimulated by DILP2 and DILP5 were clearly distinct from profiles of cells in the absence of DILP (depletion control) (**Figure 2D**). On the other hand, DILP2, DILP5, and control profiles were mutually distinct based on principle component analysis of all RPKM values (**Figure S1B** in Supplementary Material). DILP5 stimulation induces and represses more genes overall than DILP2 stimulation. Of the 2,053 genes regulated by DILP5 and 1,646 genes regulated by DILP2 (differentially expressed relative to unstimulated controls, DE, FDR < 0.05), 1,366 genes were shared between DILP2 and DILP5 (**Figure S1A** in Supplementary Material; **Table 1**). Several of these shared, differentially expressed genes were validated by qRT-PCR from independent biological samples (**Figure 2E**). About 33% of DILP5 targets are unique to DILP5 and approximately 16% of DILP2 targets are unique to DILP2 (**Table 1**; **Figure S1A** in Supplementary Material). Two of the uniquely DILP2-regulated genes, Hex-C and CG33774, were validated by qRT-PCR from independent biological samples (**Figure S1E** in Supplementary Material).

The log₂ fold changes for genes regulated by either DILP were low overall: most were below twofold although some were up to fourfold for DILP2 stimulation and fivefold change for

DILP5 (**Figures S1C,D** in Supplementary Material). Despite modest effect size, we observed low FDR for most differentially expressed genes (**Table 1**). However, only three genes showed statistical significance for differential expression between DILP2 and DILP5 (**Table 1**). Overall, DILP2 and DILP5 appear to have largely similar effects on the transcriptional responses of cells, at least on the time point of 1 h.

In addition to transcription, insulin and IGFs modulate non-genomic cell signaling. In particular, human insulin and IGF ligands can induce specific cell responses by generating different kinetics of phosphorylation upon Akt and the insulin and IGF receptors (20, 25). To explore how DILP2 and DILP5 affect phosphorylation kinetics through a common receptor in *Drosophila*, we studied the time course of Akt phosphorylation in DILP2- and DILP5-stimulated cells by Western blots (**Figure 3A**). DILP2 rapidly and transiently induces pAkt, with a peak at 3 min that quickly recedes to baseline. On the other hand, DILP5 stimulates sustained pAkt for at least 1 h (**Figure 3A**). Such differences could arise from differences in negative feedback that determine signal termination or persistence as receptors are internalized and degraded, maintained in endosomes, or continue to signal from the cell surface (56, 57). To assess InR signaling persistence, we measured InR Tyr1131 phosphorylation over a 1 h time course of DILP2 or DILP5 stimulation. DILP2 and DILP5 stimulate similar, sustained receptor phosphorylation (**Figure 3B**), suggesting that dynamics of receptor activation alone does not account for the differential kinetics of pAkt in DILP2- and DILP5-stimulated cells.

Insulin and IGF stimulate phosphorylation on proteins besides Akt, including GSK3 β , MAPK, and mTOR (58, 59). To more fully identify differences that are potentially induced by DILP2 and DILP5 signaling, we conducted an unbiased phosphoproteomic analysis in S2 cells treated with DILP2 or DILP5 at 100 nM for 3, 10, or 30 min (**Table S3** in Supplementary Material). Among all samples and replicates, we detected 5,250 unique phosphosites corresponding to 1,575 proteins. **Table 2** lists phosphosites significantly changed by DILP2 or DILP5 at individual time points compared with control. Phosphosites altered by DILP2 stimulation but not by DILP5 included Cindr (Ser452), CG6454 (Thr952), and Supervillin (Ser437) (**Table 2**; **Figure 3F**). Few phosphosites were significantly altered based on rigorous criteria (FDR < 0.05), likely because technical variation between biological replicates was high (**Figure 3C**). Nonetheless, principle component analysis reveals that combined time points of DILP2 and DILP5 phosphoproteomes separate from each other and relative to control cells in three dimensions. The first three principle components accounted for approximately 86.21% of the variance in the data. Time points at 3 min were more similar to control samples (data not shown); however, across all time points, DILP2 and DILP5 produce distinct global phosphorylation patterns (**Figure 3C**, MANOVA $p = 0.005$).

To further resolve potential differences between the DILP2- and DILP5-stimulated phosphoproteomes, we calculated the inflection point for all phosphopeptide fold changes in each condition as previously described (44, 60). The inflection points assigned conservative fold-change thresholds as criteria for

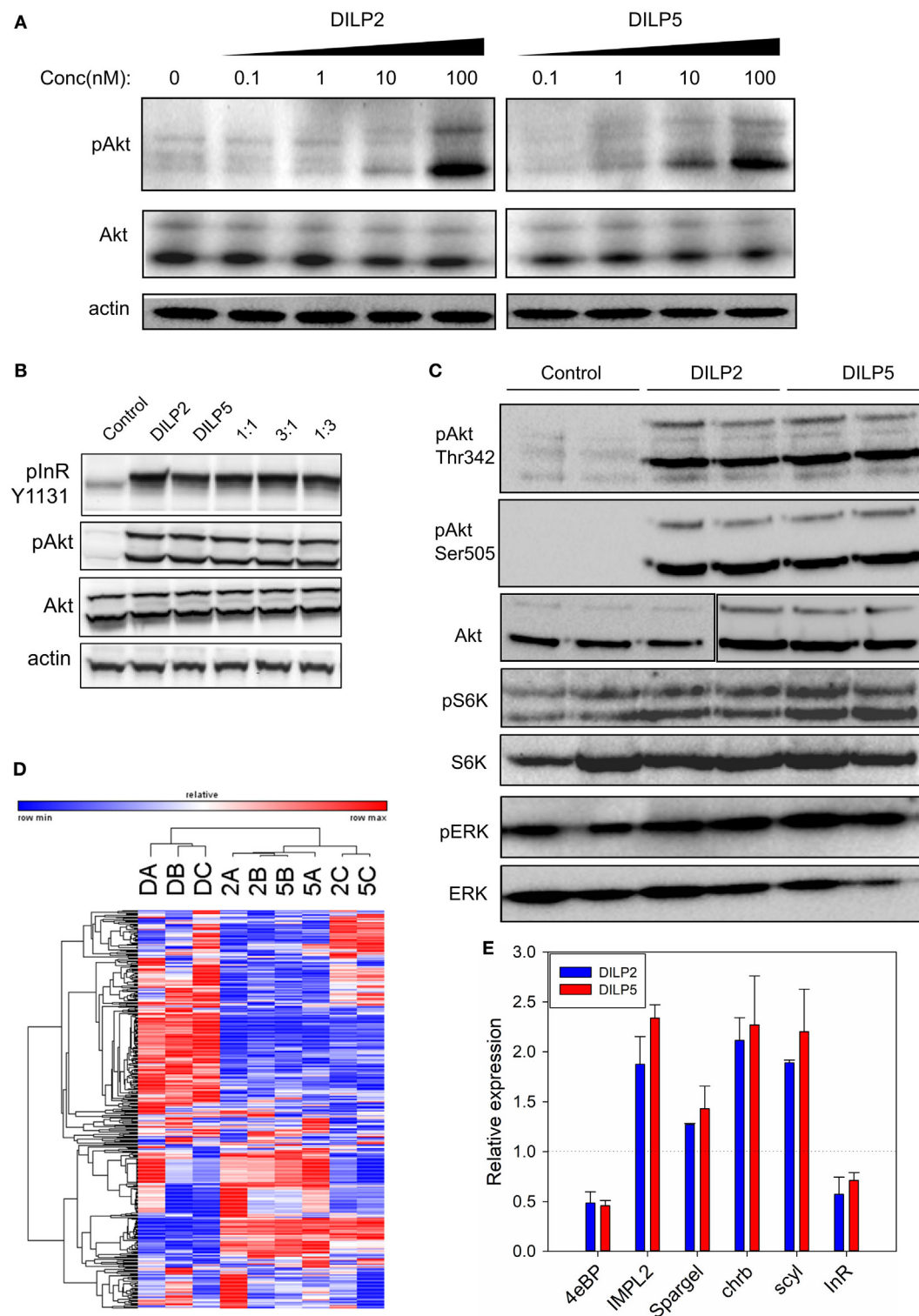


FIGURE 2 | *Drosophila* insulin-like peptide (DILP) 2 and DILP5 induce similar signaling in S2 cells. **(A)** DILP2 and DILP5 stimulate Akt phosphorylation with comparable potency. S2 cells were stimulated with DILP2 or DILP5 for 5 min at the specified concentrations. **(B)** DILP2 and DILP5 activate InR and Akt the same in competition assays, inducing similar phosphorylation each alone, with equal parts each ligand or, with an excess of one ligand. S2 cells stimulated for 5 min with DILP2 or DILP5 at 100 nM, with 50 nM DILP2 and 50 nM DILP5 ("1:1"), with 75 nM DILP2 and 25 nM DILP5 ("1:3"), and with 25 nM DILP2 and 75 nM DILP5 ("3:1"). **(C)** DILP2 and DILP5 similarly stimulate pAkt, pS6K, and pERK at 100 nM for 5 min. **(D)** DILP2 and DILP5 at 100 nM for 1 h induce similar gene expression profiles. Heatmap represents the transcript RPKM values with the top 2.5% coefficient of variation between samples: DILP2 ("2"), DILP5 ("5"), or control serum-depletion ("D"). **(E)** DILP2 and DILP5 at 100 nM for 1 h induce similar changes in gene expression measured by qPCR.

TABLE 1 | DE genes stimulated by *Drosophila* insulin-like peptide (DILP) 2 and DILP5: top 10 with statistical significance.

Comparison	Flybase ID	Gene	log2 Fold change	False discovery rate
DILP2 vs control	FBgn0036165	chrh	1.327375	4.38E-160
	FBgn0001257	ImpL2	1.267441	5.04E-158
	FBgn0261625	CG42708	1.172664	1.45E-144
	FBgn0041094	scyl	1.175456	7.91E-140
	FBgn0033391	CG8026	1.120938	3.63E-131
	FBgn0052103	CG32103	1.044493	2.74E-127
	FBgn0035542	CG11347	-1.15499	4.46E-109
	FBgn0001332	L	-1.20772	1.72E-108
	FBgn0023407	B4	0.815275	3.01E-96
	FBgn0015903	apt	1.088881	4.05E-91
DILP5 vs control	FBgn0036165	chrh	1.355795	9.66E-179
	FBgn0261625	CG42708	1.258132	7.79E-166
	FBgn0033391	CG8026	1.158735	5.35E-123
	FBgn0035542	CG11347	-1.21851	2.25E-118
	FBgn0052103	CG32103	1.037735	8.81E-114
	FBgn0015903	apt	1.175589	1.35E-112
	FBgn0041094	scyl	1.106372	4.55E-108
	FBgn0001257	ImpL2	1.340038	1.49E-103
	FBgn0001332	L	-1.26188	2.36E-98
	FBgn0259176	bun	-0.81646	1.84E-90
Unique to DILP2 vs control	FBgn0004893	bowl	-0.19777	0.003551
	FBgn0020440	Fak56D	-0.186	0.003782
	FBgn0038013	CG10038	-0.22335	0.008633
	FBgn0001149	GstD1	-0.22084	0.000232
	FBgn0034440	CG10073	-0.41936	0.019479
	FBgn0035710	SP1173	0.155922	0.014907
	FBgn0024177	zpg	0.24921	0.008772
	FBgn0002526	LanA	0.105225	0.0386
	FBgn0022699	D19B	-0.14368	0.033286
	FBgn0005672	spi	-0.12572	0.034756
Unique to DILP5 vs control	FBgn0032785	CG10026	-0.50202	0.016946
	FBgn0004635	rho	-0.2252	0.032889
	FBgn0037492	CG10050	0.427592	0.003207
	FBgn0038032	CG10096	-0.79401	0.008805
	FBgn0038033	CG10097	-0.78267	0.010873
	FBgn0003742	tra2	0.163187	0.039883
	FBgn0010638	Sec61beta	0.176851	0.021728
	FBgn0032799	CG10166	0.161421	0.015904
	FBgn0028554	xl6	0.157449	0.007431
	FBgn0037443	CG1021	0.141883	0.022384
DILP2 vs DILP5	FBgn0033865	CG6220	-0.40876	3.27E-06
	FBgn0053774	CG33774	-0.73463	0.001238
	FBgn0044510	mRpS5	-0.25787	0.044839

altered phosphosites (Figure S2 and Table S4 in Supplementary Material). Over the time course, DILP5 largely increases phosphorylation at the detected phosphosites, while DILP2 equally increases or decreases phosphorylation (Figure 3D). In addition, the majority phosphosites changed by DILP2 were unique to one time point, but many of the phosphosites affected by DILP5 were observed across two or three time points (Figure 3E), consistent with the patterns of pAkt kinetics we observed by Western blot.

Among several phosphorylation events unique to DILP2 or DILP5 by inflection point analysis (Figure S2 and Table S4 in Supplementary Material), GlyP (Ser15) was greatly decreased in

abundance by DILP2 but not by DILP5 (Figure 3F). Glycogen phosphorylase (GlyP) is notable as a conserved glycogen catabolic enzyme and the rate-limiting step in glycogenolysis (35, 48, 61, 62), while glycogen storage is emerging as a potential mediator of aging in several model systems (63, 64). The activity of GlyP is regulated through several mechanisms, including phosphorylation of Ser14 in mammalian phosphorylase (65), the analogous residue to *Drosophila* GlyP Ser15. Phosphorylation of Ser14 activates GlyP, and mammalian insulin represses this posttranslational modification (65). Our result shows that DILP2, but not DILP5, leads to dephosphorylation of GlyP at Ser15, suggesting that DILP2 specifically inactivates this central enzyme of glucose metabolism.

We conducted metabolic studies to verify that DILP2 specifically modulates glycogen metabolism and GlyP activity. First, DILP2 stimulation decreased GlyP enzymatic activity in S2 cells while DILP5 stimulation did not (Figure 4A). As well, measured from whole adults, GlyP activity was elevated in *dilp2* mutants but not in *dilp5* mutants (Figure 4D). Second, while *dilp2* and *dilp5* mutants showed reduced total glycogen content compared with wild-type flies (Figure 4B), *dilp5* mutants also have less total glucose (Figure 4C), suggesting that *dilp2* and *dilp5* mutants differentially modulate glycogen turnover. DILP2 appears to repress GlyP activity, and mutation of *dilp2* permits catabolism of glycogen to maintain glucose titers. DILP5 does not directly modulate GlyP activity, and mutation of *dilp5* must affect glycogen and glucose levels through an alternative metabolic pathway.

Unique among *Drosophila* IIS ligands, mutation of *dilp2* is sufficient to extend longevity, and reduced *dilp2* expression is consistently observed in various IIS manipulations that slow aging (8, 15, 53). Here, we report that reduced *dilp2* activates GlyP. Notably, chronological lifespan is reduced in yeast mutant for glycogen phosphorylase (64), and lifespan is extended in *Caenorhabditis elegans* deficient in glycogen synthesis (63). Therefore, we tested whether *GlyP* overexpression extends *Drosophila* lifespan. Wild-type *GlyP*, *GlyP* phosphonull (S15A, inactive enzyme), and *GlyP* phosphomimetic (S15D, constitutively active) were overexpressed using a systemic, RU486-inducible driver to express transgenes exclusively in adults and to provide exact coisogenic controls. Wild-type *GlyP* (Figure 4F) and constitutively active *GlyP* (S15D) (Figure 4H) extended lifespan. Expression of inactive *GlyP* (S15A) (Figure 4G) had no effect on survival. These data suggest that *Drosophila* lifespan is modulated by Ser15 phosphorylation of *GlyP* in response to DILP2. As well, many long-lived IIS mutants are resistant to starvation, and we found that expression of wild-type *GlyP* similarly improved starvation survival (Figure 4I). Starvation survival was not improved by phosphonull *GlyP* (Figure 4J), while phosphomimetic *GlyP* reduced resistance to starvation (Figure 4K). As noted, a recent study in *C. elegans* revealed that decreased glycogen increased longevity (63). Here, *Drosophila* longevity is increased by expressing wild-type or constitutively active *GlyP*, where each decreases total glycogen. Flies that express a phosphonull S15A *GlyP* have normal lifespan and increased total glycogen (Figure 4E).

Our data suggest that mutation of *dilp2* modulates lifespan in part through non-genomic regulation of *GlyP*. To directly

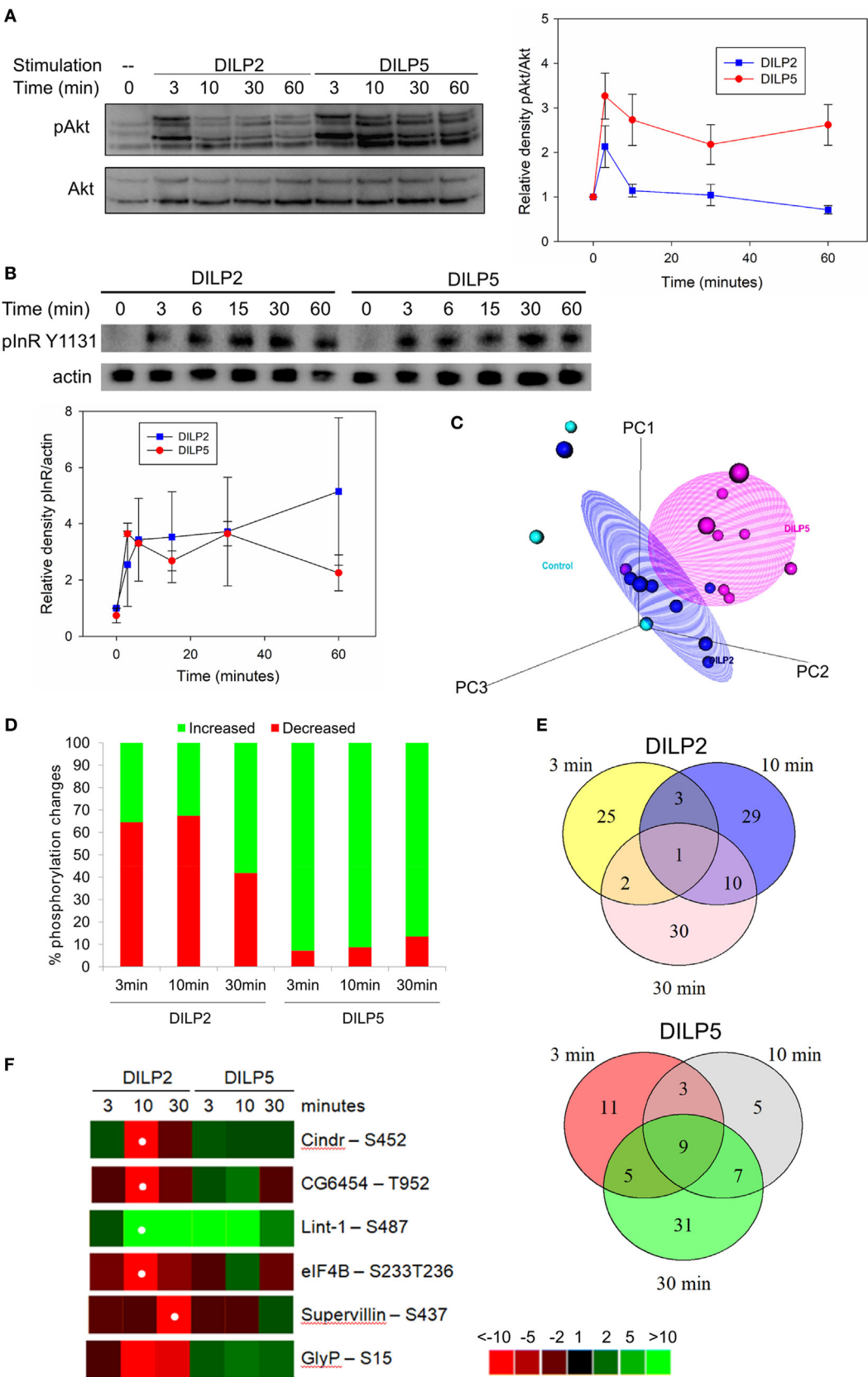


FIGURE 3 | Continued

FIGURE 3 | *Drosophila* insulin-like peptide (DILP) 2 and DILP5 stimulate distinct patterns of protein phosphorylation. **(A)** DILP2 and DILP5 at 100 nM stimulate distinct kinetics of phosphorylation upon Akt across 1 h. Representative blot (left) and quantification (right) of band densitometry analyzed in ImageLab (Bio-Rad), two-way ANOVA DILP2 vs DILP5 $p < 0.001$, *post hoc* $p < 0.05$ for $t = 3$ min, 10, 30, and 60 min. **(B)** DILP2 and DILP5 at 100 nM stimulate plnR to similar extents over 1 h. Representative blot (top) and quantification (bottom) two-way ANOVA $p = 0.482$. **(C)** PCA plot for phosphoproteomic data distinguishes among control, DILP2 stimulation, and DILP5 stimulation. **(D)** Phosphosites increased or decreased by DILP2 or DILP5 at 100 nM for the designated time points (cutoffs to call phosphosites inferred from data-driven fold-change threshold, see Figure S2 in Supplementary Material). **(E)** Venn diagram of phosphopeptides changed by DILP2 (top) and by DILP5 (bottom) across time. There are fewer overlapping phosphopeptides among DILP2 stimulation time points than among DILP5 stimulation time points. **(F)** Heatmap of phosphorylation events regulated by DILP2 and not by DILP5. White dot indicates false discovery rate (FDR) < 0.05 after adjustment for multiple comparison. Glycogen phosphorylase (GlyP) Ser15 FDR = 0.1.

TABLE 2 | Significantly altered phosphosites stimulated by *Drosophila* insulin-like peptide (DILP) 2 and DILP5.

Treatment	Protein	Phosphosite	Ratio	False discovery rate
DILP2, 10 min	slender lobes	Ser780	0.0249	0.0479
	daughter of sevenless	Thr515 Thr518	34.1037	0.0479
	sosondowah	Ser179	0.0218	0.0479
	germinal center kinase III	Ser454	0.0342	0.0479
	elF4B	Ser233 Thr236	0.0014	0.0479
	CG6454	Thr952	0.0358	0.0479
	CG3680	Ser424	0.0107	0.0479
	CG1908	Ser487	197.1951	0.0479
	BtbVII	Thr243 Ser245	640.7984	0.0479
	Cindr	Ser452	0.0049	0.0479
DILP2, 30 min	CG32306	Ser437	0.0147	0.0479
	East	Ser467	0.0615	0.0479
	MAP3K/MLK	Ser582	6.9046	0.0479
DILP5, 3 min	TRAM	Ser359	5.8001	0.0479
	Pez	Ser760 Ser763	24.6908	0.0479
DILP5, 30 min	sterile 20-like kinase	Ser357	302.1428	0.0479

test this hypothesis, we conducted genetic epistasis analysis. With a systemic, RU486-inducible Gal4 driver, we expressed *GlyP* transgenes in long-lived *dilp2* mutant adults. With addition of RU486, we induced variants of *GlyP* to determine if any modulate the longevity benefit of the *dilp2* mutation. RU486 treatment slightly extended lifespan in controls harboring UAS-*GlyP* transgenes while lacking Gal4 transgenes. To identify mortality effects dependent on *GlyP* independent of side effects from RU486, life table data were fit to Gompertz survival models by accelerated failure analysis. RU significantly affected the scale parameter (λ , frailty) but not the shape parameter (γ , slope). To account for this effect on mortality in the experimental genotypes, we subtracted the estimated scale coefficient associated with RU alone from the scale parameter estimated in Gal4/UAS genotypes given RU. Using these adjusted scale parameters and the estimated shape parameters, we recalculated life tables, survival plots and inferences on mortality. After adjusting for side effects of RU alone, survival does not differ between *dilp2* mutant controls (no RU486) and *dilp2* mutants overexpressing wild-type (**Figure 5A**); S15D (**Figure 5C**) *GlyP* (with RU486). As predicted if *dilp2* affects lifespan through control of *GlyP*, longevity is decreased in *dilp2* mutants when we overexpress S15A *GlyP* (**Figure 5B**). Noting that wild-type and constitutively

active *GlyP* extend longevity in adults with wild-type *dilp2*, our genetic interaction analysis verifies that *dilp2* and *GlyP* modulate longevity through a common pathway: *GlyP* activation and phosphorylation at Ser15 is required for mutants of *dilp2* to extend lifespan.

DISCUSSION

Insulin/IGF signaling variously affects animal cells, tissues, and systemic phenotypes through the action of related ligands, similar receptors and shared downstream components (9, 66). Insulin/IGF signaling is notably complex in *Drosophila* where there are seven insulin-like ligands that interact with a single tyrosine kinase insulin-like receptor, and one relaxin-like ligand associated with a G-protein-coupled receptor (10, 49, 67). Here, we studied how related insulin-like ligands, represented by DILP2 and DILP5, produce distinct phenotypes while signaling through a common tyrosine kinase receptor. The functions of several DILPs have been described by genetic and cellular studies. DILPs have distinct gene expression patterns across development, life stage and tissues [reviewed in Ref. (31)]. The spatiotemporal diversity of DILP expression provides one potential mechanism by which these ligands differentially control phenotypes despite sharing a common receptor. Nevertheless, some DILPs are expressed at the same time and place, such as DILP2, DILP3, and DILP5 in adult IPCs (30), or DILP4 and DILP7 in embryos (1). It seems likely, therefore, that insulin/IGF receptors themselves can differentially signal in response to specific ligands.

Insulin ligand sequence and structure may confer this proposed signaling bias at InR. Primary amino acid sequence varies among DILPs. Some insight on that variation can be gained by noting how DILPs differ from human insulin and IGF. DILPs and insulin/IGF differ at residues thought to be required for the ligands to bind the receptor (36, 68) at insulin binding site 1: Ser B9, Val B10, Tyr B16, and Asn A21; at insulin binding site 2: His B10, Thr A8, and Ile A10. Insulin His B10 is especially interesting because this residue is substituted with Asp to create the fast-acting synthetic insulin analog X10, and because it plays a role in the storage of insulin as hexamers in β -cells (27). The B chain of DILP5, insulin, and IGF contains C-terminal phenylalanines and large aromatic residues, but DILP2 lacks these residues. The phenylalanines and large aromatic residues function in insulin dimerization, negative cooperativity and receptor interaction (69), and their mutation produces diabetes (at human insulin Phe 24 and Phe25, the “insulin Los Angeles” and “insulin Chicago” syndromes) (70, 71). Overall, DILP2 and DILP5 vary in many amino acid residues that could potentially

affect receptor binding, and thus differentially modulate receptor conformation and auto-activation. Independent of receptor interactions, DILPs bind circulating factors including acid-labile

subunit (dALS), secreted decoy of InR, and IMP-L2 (72–74) which might differentially affect DILP bioavailability and signaling output.

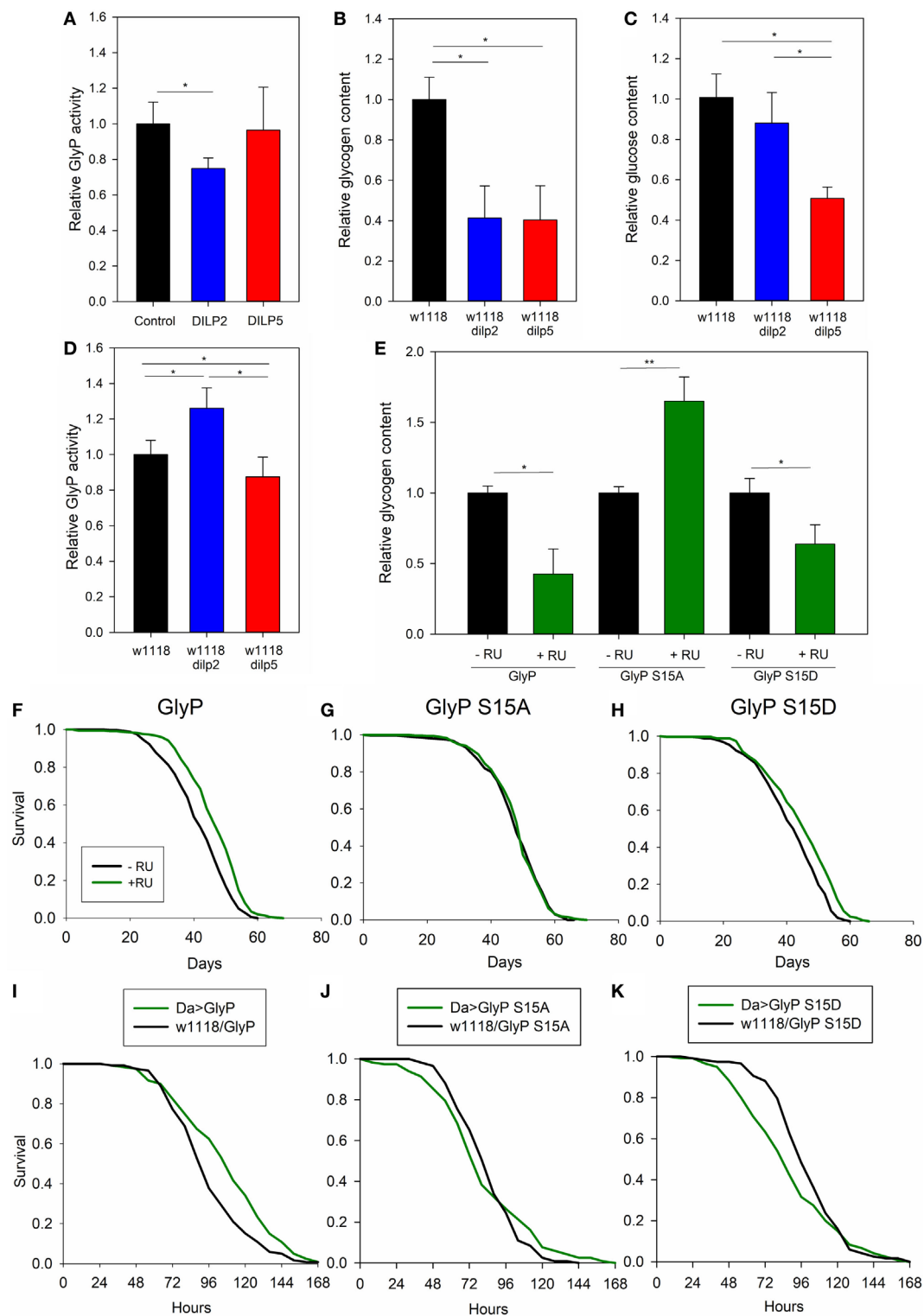


FIGURE 4 | Continued

FIGURE 4 | *Drosophila* insulin-like peptide (DILP) 2 and DILP5 differentially regulate glycogen phosphorylase (GlyP) to modulate lifespan and physiology. **(A)** DILP2 represses GlyP activity in S2 cells relative to cells in control conditions or those stimulated by DILP5. Serum-depleted S2 cells treated with 100 nM DILP or vehicle for 15 min. ANOVA $p = 0.032$, $n = 6$. **(B,C)** *dilp2* and *dilp5* mutants have decreased glycogen content, and *dilp5* mutants have decreased glucose content, relative to wild type. Each replicate from six female flies, age 10 days post-eclosion and 8–10 biological replicates. Glycogen **(B)** ANOVA $p < 0.001$ and glucose **(C)** ANOVA $p < 0.001$. **(D)** *dilp2* mutants have increased GlyP activity relative to wild type; *dilp5* mutants have decreased activity. Each replicate from 6 female flies, age 10 days post-eclosion, 11 biological replicates, ANOVA, $p < 0.001$. **(E)** Whole animal glycogen is affected by overexpressing GlyP with the DaGS-Gal4 driver as a function of Ser15. Each replicate, six female flies, age 10 days post-eclosion, four to five biological replicates. **(F–H)** Systemic GlyP overexpression extends longevity in a Ser15 dependent manner. DaGS-Gal4 (RU486 inducible) drove: **(F)** wild-type GlyP (Cox hazard analysis, $\chi^2 = 46.5$, $p < 0.0001$), **(G)** phosphonull GlyP S15A (Cox hazard analysis, $\chi^2 = 0.1$, $p = 0.75$), **(H)** phosphomimetic GlyP S15D (Cox hazard analysis, $\chi^2 = 30.5$, $p < 0.0001$). Each genotype cohort $n = 348$ –366 adults. **(I–K)** Systemic GlyP overexpression regulates starvation resistance in a Ser15 dependent manner. Da-Gal4 drives: **(I)** GlyP (Cox hazard analysis, $\chi^2 = 10.3$, $p = 0.001$), **(J)** phosphonull GlyP S15A (Cox hazard analysis, $\chi^2 = 0.2$, $p = 0.68$), **(K)** phosphomimetic GlyP S15D (Cox hazard analysis, $\chi^2 = 4$, $p = 0.05$). Each genotype cohort $n = 115$ –120 adults.

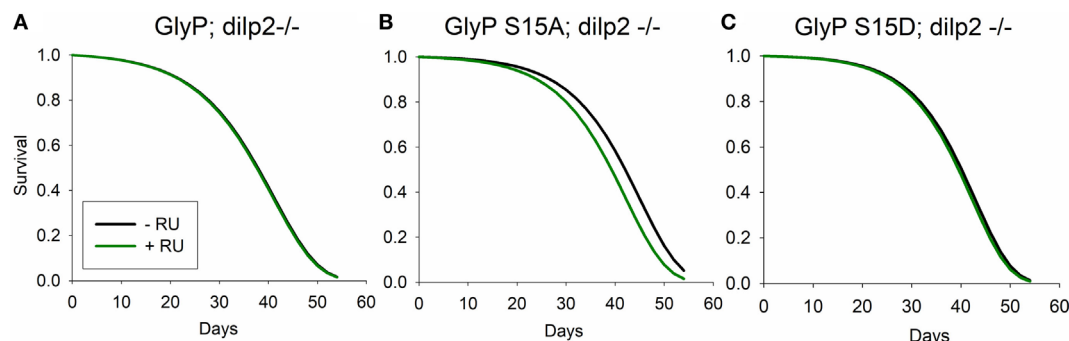


FIGURE 5 | Glycogen phosphorylase (GlyP) phosphorylation is required for longevity conferred by mutation of *dilp2*. Survival calculated from life tables based on estimated Gompertz mortality parameters for UAS-GlyP expression in a *dilp2* mutant background with transgenes driven by systemic DaGS-Gal4. Gompertz mortality scale (l, frailty) parameters of transgene expression genotypes were adjusted for the impact of RU alone upon mortality estimated from control genotypes. **(A)** Overexpression of wild-type GlyP does not extend longevity (Cox hazard analysis, $\chi^2 = 0.1$, $p = 0.76$), **(B)** expression of GlyP S15A decreases lifespan (Cox hazard analysis, $\chi^2 = 24.2$, $p < 0.0001$), and **(C)** expression of GlyP S15D does not extend longevity (Cox hazard analysis, $\chi^2 = 1.2$, $p = 0.28$). Each genotype cohort $n = 351$ –361 adults.

Using synthetic DILP2 and recombinant DILP5, we studied how these related ligands affect cell signaling in a controlled, simplified system: *in vitro* stimulation of *Drosophila* S2 cells. Our preparations of DILP2 and DILP5 ligand have equal potency measured by their ability to induce pAkt in a dose-dependent manner. IIS ligands regulate many cellular functions through control of FOXO transcription factors, so we sought to differentiate these ligands by their potentially unique profiles of induced and repressed mRNA. Based on RNA-Seq and quantitative RT-PCR, DILP2 and DILP5 produce strikingly similar changes in gene expression, where differences are marked by the quantity of transcripts, not their identity. FOXO thus appears to be similarly regulated by DILP2 and DILP5. Mammalian insulin and IGFs provide a precedent for this outcome, where these functionally distinct yet similar hormones produce remarkably concordant transcriptional profiles (18, 19). Naturally, insulin and IGF also operate within cells through non-genomic pathways such as repressing glycogen synthesis and GSK3 β , inducing glucose transport through GLUT4 and altering protein translation (58). To study such alternatives, we compared how DILP2 and DILP5 affect the kinetics and patterns of protein phosphorylation.

We began with Western analysis of InR and Akt phosphorylation across a time course, using time points previously studied in mammalian insulin/IGF signaling and in *Drosophila* cells

stimulated with human insulin (58, 59). DILP2 and DILP5 stimulate prolonged phosphorylation of InR across the assay period, as seen in rat glial cells when IGF-1 simulates IGF-1R (75, 76). On the other hand, DILP2 induces transient Akt phosphorylation that peaks at 3 min, while DILP5 stimulates prolonged, elevated Akt phosphorylation over the tested 60-min time course. Transient versus prolonged kinetics of Akt phosphorylation resembles the differences seen in mammalian tyrosine kinase receptor signaling where ligand-specific receptor-binding kinetics produce “shout” versus “whisper” transduction dynamics (56) with respective associated metabolic and mitogenic potential (26, 27). In mammalian cell model systems, various insulin and insulin analogs likewise can stimulate transient or sustained dynamics of Akt phosphorylation (25).

The dynamics of IR activation has been modeled based on receptor-binding kinetics induced by specific ligands (23, 24). DILP2 and DILP5 may affect receptor off-rates in distinct due to differences in amino acid sequences that induce unique conformational changes. Conformational changes in receptor extracellular and intracellular domains may subsequently alter receptor auto-activation, trafficking, and turnover (77). Models of mammalian receptor tyrosine kinases propose that different signals may result from trafficking of the receptor when it is ubiquitinated and degraded, recycled to the cell surface, or continuously activated in endosomes (56, 57, 78). Each mechanism may impact how the insulin ligands affect receptor

interactions with substrate and adaptor proteins. *Drosophila* has a single IRS *chico*, the homolog of mammalian IRS1–4. In our phosphoproteome analysis, DILP2 and DILP5 similarly phosphorylated Chico Tyr470 and Ser471 (see Table S3 in Supplementary Material), suggesting that these sites do not mediate the distinct impact of the ligands on pAkt dynamics. Many additional adaptor proteins are known for mammalian IIS ligands (79) and several are described for *Drosophila* including Dock, Shc, Ink, and daughter of sevenless (DOS), the homolog of mammalian Gab1 (80). Interestingly, we found that DILP2 increased phosphorylation of the DOS peptide at Thr515 and Thr518 to a greater extent than DILP5. DOS/Gab1 is a scaffold for receptor tyrosine kinase signaling that integrates sevenless signaling, PI3K, and MAPK (81). Future studies may determine if DOS mediates how DILP2 and DILP5 differentially affect the kinetics of pAkt.

Most phosphopeptides affected by DILP stimulation are downstream of the immediate InR substrates. Notably, DILP2, but not DILP5, stimulated dephosphorylation of Cindr, the *Drosophila* homolog of human CIN85, which is associated with endocytosis and clathrin in mammalian cells (82). Moreover, DILP2 dephosphorylated CG6454, a likely homolog of human C2CD5 that interacts with clathrin to regulate GLUT4 translocation in adipocytes (83, 84). Interestingly, inhibition of clathrin-mediated endocytosis can alter insulin-stimulated PI3K activity and Shc and MAPK phosphorylation (78). Thus, DILP2 in contrast to DILP5 may uniquely regulate dynamics of InR endocytosis, GLUT4 membrane translocation, and cytoskeletal structures.

Among other stimulated proteins, glycogen phosphorylase (GlyP) is dephosphorylated by DILP2 but not by DILP5. To evaluate if DILP2 affects GlyP function in S2 cells, we applied a GlyP enzymatic activity assay for insects, adapting techniques from mammalian biology (48). DILP2 treatment decreases GlyP activity, as predicted from the phosphoproteomic data where DILP2 stimulation dephosphorylated GlyP at Ser15. DILP5 stimulation does not alter GlyP activity, in accordance with the phosphoproteomic data where DILP5 did not alter GlyP Ser15. We also validated that DILP2 uniquely regulates GlyP activity *in vivo*. Adult *dilp2* mutant flies, which are long-lived, have elevated GlyP enzymatic activity relative to wild-type flies, while activity is slightly decreased in *dilp5* mutants. Since DILP5 alone does not reduce GlyP Ser15 phosphorylation, the *dilp5* mutation may decrease GlyP activity indirectly because loss of *dilp5* increases *dilp2* expression (data not shown).

dilp2 mutants could in part extend lifespan by its non-genomic control of this glycogen catabolism enzyme, given that DILP2 regulates GlyP Ser15 phosphorylation and that *dilp2* mutants have increased lifespan and GlyP activity. We find that overexpression of GlyP in all tissues modestly (12%) but significantly extends lifespan. However, Bai et al. previously found that depletion of GlyP by RNAi in all tissues was sufficient to extend lifespan (53), while in yeast, loss of GlyP shortens chronological lifespan (63). Although GlyP modulates glycogenolysis in many tissues, it is unknown whether glycogen levels may affect aging. Longevity is not correlated with stored glycogen among yeast mutants lacking various genes involved in glucose and glycogen metabolism (64). Human cultured muscle cells overexpressing glycogen phosphorylase had decreased glycogen stores, but also display improved metabolic homeostasis

through increased glycogen turnover, elevated lipid storage, and enhanced glucose uptake (85). Thus, GlyP overexpression may enhance overall metabolic homeostasis.

Aside from the impact of GlyP on stored glycogen, Favre et al. suggested that *GlyP* may regulate aging through AMP-activated protein kinase (AMPK) and oxidative stress resistance (64). The β -subunit of AMPK has a glycogen-binding domain that acts as a glycogen sensor. Allosteric regulation of this domain affects AMPK phosphorylation and subsequent localization and activity (86). Here, we found increased phosphorylation of the AMPK β -subunit (see Table S4 in Supplementary Material), by DILP2 but not by DILP5 suggesting that activation of fly GlyP may affect aging through secondary effects of altered glycogen flux, rather than simply through glycogenolysis and glucose production. Notably, Gusarov et al. recently demonstrated in *C. elegans* that glycogen modulates longevity by modulating AMPK, superoxide dismutase and oxidative stress resistance, while glycogen phosphorylase deficient worms are short-lived (63). In overwintering insects, glycogen is stored in preparation for diapause and is broken down by glycogen phosphorylase during diapause, a life history state associated with slow to negligible aging (87, 88). Remarkably, GlyP expression is increased during *Drosophila* diapause (89). Therefore, GlyP is situated at the center of a network that regulates energetics, metabolic homeostasis and stress responses that associate with persistence and longevity.

While mammalian glycogen phosphorylase can be activated by glucagon and protein kinase A (PKA) (90), how insulin inversely represses glycogen phosphorylase is not completely understood. Mammalian insulin is proposed to suppress glycogen phosphorylase by activating protein phosphatase 1 (PP1) to dephosphorylate GlyP Ser15 [reviewed in Ref. (90)]. As well, AMP allosterically enhances glycogen phosphorylase activity while glucose represses its activity. When glucose binds to glycogen phosphorylase, the enzyme allows PP1 to dephosphorylate Ser15 (90). Finally, insulin decreases cellular cAMP levels, which represses PKA. Repressed PKA limits phosphorylase kinase activity that otherwise phosphorylates Ser15 on glycogen phosphorylase. DILP2 may modulate *Drosophila* GlyP through each of these mechanisms whereas DILP5 should not. Based on this contrast, we note that the unique control of Cindr and CG6454 phosphorylation by DILP2 indicates potential roles for glucose uptake in cells as possible mechanisms by which DILP2 regulates GlyP dephosphorylation. This conclusion is consistent with the association of *dilp2* with glucose metabolism compared with the association of *dilp5* with protein metabolism (8, 30).

Studies in mammalian insulin/IGF signaling strive to fully understand how these similar ligands can activate varied signaling pathways through similar receptors to produce different phenotypes (19, 79). Furthermore, in humans, insulin and IGF can activate one another's receptors, as well as IR/IGFR hybrid receptors (9). Consequently, insulin analogs for diabetes treatment can increase mitogenicity and risk of cancer, while manipulating IGF in the treatment of cancer can disrupt glucose homeostasis (91). We demonstrate that DILP2 and DILP5 regulate many parallel cell signaling events, but also uniquely control particular cellular processes to affect distinct organismal physiology. Remarkably, DILP2 uniquely represses GlyP activity, and activation of this enzyme is required for *dilp2* mutants to fully extend longevity.

Overall, we demonstrate that two related insulin-like ligands have the capacity to regulate unique traits through a single receptor. We propose that *Drosophila* DILPs bind InR with different kinetics to cause distinct conformational changes in the receptor and effector proteins, and this subsequently produces different signaling output. An explicit comparison of DILP2 and DILP5 receptor-binding kinetics and structures bound to InR will advance how we understand the mechanisms of insulin signaling bias.

DATA AVAILABILITY STATEMENT

The RNA-Seq datasets generated and analyzed in this study can be found in the Gene Expression Omnibus submission GSE111560. The phosphoproteomic datasets generated and analyzed in this study have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository in the dataset submission PXD009240.

AUTHOR CONTRIBUTIONS

SP and MT designed the experiments, interpreted results, and wrote the manuscript; SP and GK conducted the experiments; SP analyzed the data; MT, JW, and PM conceived the research; all the authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fendo.2018.00245/full#supplementary-material>.

FIGURE S1 | DILP2 and DILP5 induce similar gene expression by RNA-Seq. **(A)** Venn Diagram of differentially expressed genes by DILP2 or DILP5 stimulation. **(B)** PCA plot reveals separation of DILP stimulation from controls and slight separation of DILP2 from DILP5. Volcano plots reveal that DILP2 **(C)** and DILP5 **(D)** both stimulate gene expression changes with strong significance but relatively small log2 fold changes. **(E)** Two differentially expressed genes unique to DILP2 stimulation from RNA-Seq were verified by q-RT-PCR from separate biological samples, $n = 3$, $*p < 0.05$, $**p < 0.01$.

FIGURE S2 | DILP2 and DILP5 stimulate changes in phosphopeptide abundance by phosphoproteomics. **(A–F)** Volcano plots for phosphopeptides identified in DILP2 **(A–C)** or DILP5 **(D–F)** stimulation at the indicated time point. All conditions reveal large log2 fold change values (Log2(FC)) with small significance (Log(Q value)). **(G–L)** Inflection points were calculated from all phosphopeptide log2 fold changes for each stimulation condition. Dotted lines represent the inflection point y-values selected as cut-offs for fold-change thresholds.

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Ghrelin and Its Receptors in Gilthead Sea Bream: Nutritional Regulation

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Ghrelin is involved in the regulation of growth in vertebrates through controlling different functions, such as feed intake, metabolism, intestinal activity or growth hormone (Gh) secretion. The aim of this work was to identify the sequences of *preproghrelin* and Ghrelin receptors (*ghsrs*), and to study their responses to different nutritional conditions in gilthead sea bream (*Sparus aurata*) juveniles. The structure and phylogeny of *S. aurata* *preproghrelin* was analyzed, and a tissue screening was performed. The effects of 21 days of fasting and 2, 5, 24 h, and 7 days of refeeding on plasma levels of Ghrelin, Gh and Igf-1, and the gene expression of *preproghrelin*, *ghsrs* and members of the Gh/Igf-1 system were determined in key tissues. *preproghrelin* and the receptors are well conserved, being expressed mainly in stomach, and in the pituitary and brain, respectively. Twenty-one days of fasting resulted in a decrease in growth while Ghrelin plasma levels were elevated to decrease at 5 h post-prandial when pituitary *ghsrs* expression was minimum. Gh in plasma increased during fasting and slowly fell upon refeeding, while plasma Igf-1 showed an inverse profile. Pituitary *gh* expression augmented during fasting reaching maximum levels at 1 day post-feeding while liver *igf-1* expression and that of its splice variants decreased to lowest levels. Liver Gh receptors expression was down-regulated during fasting and recovered after refeeding. This study demonstrates the important role of Ghrelin during fasting, its acute down-regulation in the post-prandial stage and its interaction with pituitary *Ghsrs* and Gh/Igf-1 axis.

Keywords: ghrelin, GHSR1a, growth hormone, IGF-1, fasting and refeeding

INTRODUCTION

Ghrelin is a peptide hormone secreted mainly by the stomach in vertebrates, but also detected in many other tissues (e.g., intestine, heart, pancreas, and especially pituitary and brain). Ghrelin is synthesized as Preproghrelin, and the mature peptide varies between 12 and 28 amino acids, depending on species and form of Ghrelin, but it shows high sequence homology across vertebrates, including fish (1). Since its discovery, Ghrelin has been involved in many physiological processes like the regulation of feed intake, adiposity, growth, energy and glucose metabolism, intestinal

motility and digestive enzymes activity, among others (2). The first characterization of Ghrelin in a fish species, the goldfish (*Carassius auratus*), was done by Unniappan et al. (3). Later, Kaiya et al. (4) reviewed its function in non-mammalian vertebrates and recently, different publications have investigated its role in other fish species (5–12), but very little is known about this hormone in gilthead sea bream (*Sparus aurata*) (13).

Ghrelin functions through binding to its receptors, which are also known as the growth hormone secretagogue receptors (Ghsrs). The Ghsrs are a family of transmembrane G-protein coupled receptors, and the Ghsr1a isoform, discovered a few years before Ghrelin (14), is known as the active form. An alternative splice variant named Ghsr1b, was also described by the same authors, but its structure lacks two transmembrane domains leading to the impossibility of this isoform to initiate intracellular signaling. Since the discovery of these two receptors, the Ghsrs family has been widely studied and other numerous isoforms (splice variants and paralogues) have been described in vertebrates (15, 16).

ghsrs mRNA is found in many tissues, including brain, stomach, intestine, and especially pituitary gland. The high expression levels detected in the pituitary in vertebrates confirms the role of Ghsrs in the regulation of growth hormone (Gh) production (17). Gh is one of the key elements of the Gh/insulin-like growth factor-1 (Igf-1) axis, which is the main regulator of growth in vertebrates. Depending on factors such as nutritional state, Gh can directly stimulate anabolic or catabolic processes by binding to the Gh receptors (Ghsrs). Moreover, systemic Gh mainly acts in the liver, where it stimulates the production of Igf-1. This growth factor in turn acts in many peripheral tissues stimulating growth-related processes (18). Thus, most of the physiological peripheral roles of Ghrelin appear to be mediated indirectly by the modulation of Gh release (19). In addition, Ghrelin has been described to have an important role in the hypothalamus in mammals, where it acts on different *ghsrs*-expressing cell populations, leading to enhanced expression and release of orexigenic neuropeptides like neuropeptide Y and Agouti-related peptide, hence stimulating appetite in most vertebrates, including diverse fish species (20). Moreover, it has been recently reported, at least in mammals, that Ghrelin acts over the hypothalamic Gh releasing hormone neurons (21). Although there is a controversy on how the different forms of Ghrelin (acylated and unacylated) cross the blood brain barrier to exert this role (22), adding another complex level of regulation.

Fish are capable to resist long fasting periods (23) and the Gh/Igf-1 system, displays interesting changes to adjust metabolism and growth to nutrient supply. Ghrelin in its double role as a hunger hormone and Gh secretion regulator should play an important role in fasting and refeeding physiology, although these aspects are poorly known in fish, especially in gilthead sea bream (13, 24).

The objective of the present work was to identify and characterize Ghrelin and its receptors by analyzing sequences, phylogeny and gene expression through a tissue screening, and to study their responsiveness upon fasting and refeeding in relation with the Gh/Igf-1 axis in gilthead sea bream juveniles.

MATERIALS AND METHODS

Fish Maintenance and Distribution

Gilthead sea bream juveniles (initial body weight 50 ± 3 g; length 15.3 ± 0.68 cm) were obtained from a commercial hatchery (Piscimar, Borriana, Spain) and reared in the facilities of the Faculty of Biology. Forty-two fish were randomly distributed in six 200 L seawater tanks (7 fish/tank) and some extra fish for tissue screening were kept in another 200 L tank. Fish were kept in a seawater recirculation system at a constant temperature of $23 \pm 1^\circ\text{C}$ and at 12 h light/12 h dark photoperiod through the whole experiment. During the acclimation period (2 weeks), fish were fed to apparent satiety twice a day with a commercial diet (Optibream, Skretting, Burgos, Spain). This study was carried out in accordance with the recommendations of the EU, Spanish and Catalan Government-established norms and procedures. The protocol was approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 110/17 and DAAM 9488).

Experimental Design

After acclimation, a period of 21 days of fasting and 7 days of refeeding was designed, according to previous experience (25). During the refeeding period, fish were fed once a day to apparent satiety. Samplings were made at the beginning and end of the fasting period (−21 and 0 days, respectively), and at 2, 5, 24 h and 7 days upon refeeding. The −21 days, 24 h and 7 days samplings were made just before feeding, representing one day fasting. The day 0 sampling was done at the same time of the day, and fish were fed right after to start the refeeding. In each sampling, 6 fish were first anesthetized with MS-222 (0.08 g/L), and once blood was extracted, were sacrificed by an overdose of MS-222 (>0.1 g/L). Then, brain, pituitary, liver and stomach were dissected and stored in liquid nitrogen. Before sacrifice, body weight, body length (standard), and liver and viscera weight were measured to calculate distinct biometric indexes: condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI).

Additionally, 3 fish were sacrificed and sampled for 17 distinct tissues and/or organs. RNA was obtained from tissue samples (30–100 mg) or from the whole pituitary gland and brain with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) and reverse transcribed following the procedures previously described (26). Briefly, 1 μg of RNA was treated with DNase I (Life Technologies, Alcobendas, Spain) following the manufacturer's instructions to remove genomic DNA. After DNase treatment, retrotranscription was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain) for 10 min at 25°C , 60 min at 50°C and 5 min at 85°C . Samples were immediately stored at -20°C for further analysis.

Preproghrelin and *ghsrs* Characterization

Primers for the amplification of the complete coding sequences of *preproghrelin*, *ghsr1a* and *ghsr1b* were designed using Primer3Plus software (27) with the nucleotide sequences obtained from the Nucleotide Database.

of gilthead sea bream at <http://www.nutrigrp-iats.org/seabreamdb> (28, 29)]. The three sequences are deposited in GenBank (NCBI) under accession numbers: MG570187 for *preproghrelin*; MG570188 for *ghsr1a*, and MG570189 for *ghsr1b*. Sequences specificity was confirmed by PCR amplification of transcribed RNA samples from the tissue screening that were run on an agarose gel for size verification.

A multiple Preproghrelin sequence alignment was performed using the default settings of the MAFFT tool online (server) version (<http://mafft.cbrc.jp/alignment/server/>). The phylogeny was inferred using the JTT + G + I model substitution method and an unrooted tree was constructed using the MEGA X software with a bootstrapping value of 1,000. Previously, using the same software, a test was performed to determine which substitution model was the best for our data (data not shown). Unequivocal identity of *ghsr1a* and *ghsr1b* was verified by Blast and BlastX searches, as well as by transmembrane domain analysis by means of TMHMM transmembrane helices prediction program (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>).

Ghrelin, Gh and Igf-1 Plasma Levels

Plasma levels of acylated Ghrelin were measured using the Ghrelin N- radioimmunoassay (RIA) protocol originally described by Hosoda et al. (30) and modified by Jönsson et al. (7) with the exception that plasma was not extracted, just quickly centrifuged (1,000 rpm, 1 min) before pipetting to the RIA tubes, and iodinated human Ghrelin (NEX388010UC, PerkinElmer, USA) was applied as tracer. Anti-rat Ghrelin [1-11] antisera, which specifically recognizes the conserved n-octanoylated Ser3 epitope on Ghrelin, was used at a final dilution of 1:500000 (gift from Dr. Hiroshi Hosoda, Japan). Standard was made using synthetic rainbow trout acylated Ghrelin (Peptide institute, Japan).

All samples were assayed in duplicate and included in one assay. The Ghrelin RIA was validated for gilthead sea bream, and the slopes of the standard curve and of a serial dilution of plasma samples were parallel (Supplementary Figure 1). Plasma levels of Gh and Igf-1 were measured by corresponding RIAs, as previously described (31, 32).

Gene Expression

The mRNA transcript levels were examined by quantitative real-time PCR (qPCR) according to the requirements of *MIQE guidelines* (33) in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). All reactions were performed in the conditions previously described (26). The primers used are listed in Table 1. To amplify the two *ghsrs* the forward primer was designed in a common region, and the reverse primer for *ghsr1a* in a region overlapping exon 1 and 2, and for *ghsr1b* in a region including the differential nucleotides at the end of translation and the 3'-UTR. In addition, elongation factor 1 alpha (*ef1a*), ribosomal protein S18 (*rps18*) and *b-actin* (only in brain) were analyzed and served as reference genes in order to calculate the relative expression of the target genes (34). Both, reference genes stability and relative expression calculation were determined with the Bio-Rad CFX Manager Software (v2.1).

Statistical Analyses

Data was analyzed using IBM SPSS Statistics 22 and are showed as mean $\pm$ standard error of the mean (SEM). Normality and homogeneity of variances were tested with Shapiro-Wilk Test and Levene's, respectively. When data did not follow a normal distribution or did not have homoscedasticity, it was converted by logarithm transformation. Differences among groups were tested by one-way analysis of variance (ANOVA) followed by Tukey HSD or LSD, as *post-hoc* tests. In case of no homoscedasticity, the non-parametric Kruskal-Wallis test was used with the Dunnett's T3 as *post-hoc*. The confidence interval for all analyses was set at 5%.

RESULTS

Preproghrelin and Ghsrs Characterization

Translation of *preproghrelin* nucleotide sequence (907 nucleotide in length) resulted in a 107 amino acid sequence that presented 97% identity with that of another sparid, the blackhead sea bream (*Acanthopagrus schlegelii*), as the most significant result in a BlastX search. The predicted sequence of gilthead sea bream Preproghrelin contained the conserved N-terminal signal peptide (26 amino acids), that yields Proghrelin after cleavage. In the Proghrelin region, the sequence contained the characteristic Ser3 residue, which is the octanoylation target, as well as the GlyArg amidation and cleavage site to obtain the N-terminal mature Ghrelin (20 amino acids) and the C-terminal Proghrelin peptide (Figure 1A).

The nucleotide sequences of *ghsr1a* and *ghsr1b* (1708 and 1793 nucleotide in length, respectively) encoded for 384 (Ghsr1a) and 292 (Ghsr1b) amino acids sequences that shared a 98% of identity with their respective orthologs in the blackhead sea bream (35). In the same way, the TMHMM transmembrane helices program predicted the presence of the characteristic seven transmembrane domains in Ghsr1a, whereas Ghsr1b did not retain the last two due to alternative gene splicing (Figure 1B).

The phylogenetic analysis of the Preproghrelin amino acid sequence is shown in Figure 2. The unrooted tree highlights the conservation of this protein in vertebrates, although it presents clusters that separate the different vertebrate classes and fish orders. Results of the *preproghrelin* and *ghsrs* gene expression screening are shown in Figures 3A,B, respectively. *preproghrelin* was mainly expressed in stomach, but weak expression was also detected in many other tissues (i.e., spleen and head kidney). Regarding the receptors, brain, pituitary and liver were the tissues with highest expression of both, *ghsr1a* and *ghsr1b*, although low levels of expression were also found in many other. In pituitary and brain, the expression levels of *ghsr1a* were very similar, but the expression of *ghsr1b* was higher in liver. Thus, in pituitary and brain the most abundant isoform was *ghsr1a* while in liver was *ghsr1b*.

Fasting and Refeeding Experiment Growth and Morphometric Parameters

Morphometric parameters results are shown in Figures 4A–D. Mean body weight (which was not significantly affected) and CF presented a similar pattern along the fasting/refeeding

TABLE 1 | Sequences, melting temperatures (Tm) and GenBank accession numbers of the primers used for qPCR.

Primer list (<i>Sparus aurata</i>)			
Gene	Sequence (5'-3')	Tm (°C)	Accession Number
<i>ef1a</i>	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	60	AF184170
<i>rps18</i>	F: GGGTGTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	60	AM490061.1
<i>b-actin</i>	F: TCCTGCGGAATCCATGAGA R: GACGTCGCACTTCATGATGCT	60	X89920
<i>preproghrelin</i>	F: CCCGTCACAAAACCTCAGAAC R: TTCAAAGGGGGCGCTTATTG	60	MG570187
<i>ghsr1a</i>	F: GTCGGCGGCTGTGGCAAAGA R: GGCCAACACCACCACCAAC	60	MG570188
<i>ghsr1b</i>	F: CGCACACGCATAACTTTGTC R: GAGGAGGATGAGCAGGTGAA	60	MG570189
<i>gh</i>	F: GCCCCATCGACAAGCACG R: GAGTCTACATTTGCCACCGTCAG	60	FJ827496
<i>ghr-1</i>	F: ACCTGTCAGCCACCACATGA R: TCGTGCAGATCTGGGTCGTA	60	AF438176
<i>ghr-2</i>	F: GAGTGAACCCGGCCTGACAG R: GCGGTGGTATCTGATTCATGGT	60	AY573601
<i>igf-1a</i>	F: AGGACAGCACAGCAGCCAGACAAGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	AY996779
<i>igf-1ab</i>	F: AGTCATTATCCTTCAAGGAAGTCATCC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	EF688015
<i>igf-1abc</i>	F: ACAGAATGTAGGGACGGAGCGAATGGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	60	EF688016
<i>igfbp1a</i>	F: AGTGCGAGTCCTCTCTGGAT R: TCTCTTTAAGGGCACTCGGC	60	KM522771
<i>igfbp2a</i>	F: CGGGCTGCTGCTGACATACG R: GTCCCGTCGCACCTCATTTG	60	AF377998
<i>igfbp4</i>	F: TCCACAAACCAGAGAAGCAA R: GGGTATGGGATTGTGAAGA	60	F5T95CD 02JMZ9K
<i>igfbp5b</i>	F: TTTCTCTCTCGGTGTGC R: TCAAGTATCGGCTCCAG	60	AM963285
<i>igf-rb</i>	F: GCTAATGCGAATGTGTTGG R: CGTCCTTTATGCTGCTGATG	55	KT156847

experiment, decreasing after fasting and slightly increasing afterwards, partially recovering at day 7. Regarding HSI, a significant decrease was observed after fasting, but was significantly increased at day 7 post-refeeding. At 2 h post-prandial the stomach was clearly full, but no food was found in the intestine, whereas at 5 h the stomach had emptied almost all its food content. Thus, VSI was significantly lower after the fasting period. With refeeding, it increased at 2 and 5 h, but at 1 and 7 days the VSI values returned to baseline levels.

Plasma Ghrelin, Gh and Igf-1

Ghrelin, Gh, and Igf-1 plasma concentrations are presented in **Figures 5A–C**. Plasma Ghrelin showed maximum levels after fasting and at 2 h post-prandial, and a significant dip at 5 h, but then returned to high levels after 1 and 7 days. However, it should be taken into account that those samples, as well as the

one before the whole fasting period, were taken after a 24 h fast, which appears to be a potential stimulus for Ghrelin secretion. Circulating Gh increased significantly with fasting. Then, there was no acute post-prandial change but a gradual decrease upon refeeding returning to basal after 7 days. Plasma Igf-1 levels had an inverse pattern to that of Gh; showing significantly lower values after the 21 days fasting period compared to day 0 and then returning to basal levels at 7 days post-refeeding.

Gene Expression

Preproghrelin and ghsrs

Stomach *preproghrelin* gene expression (**Figure 6A**) did not show any change after fasting, but a significant difference was observed after 1 day of refeeding. In the brain, *preproghrelin* gene expression was much lower than in the stomach (**Figure 3A**);

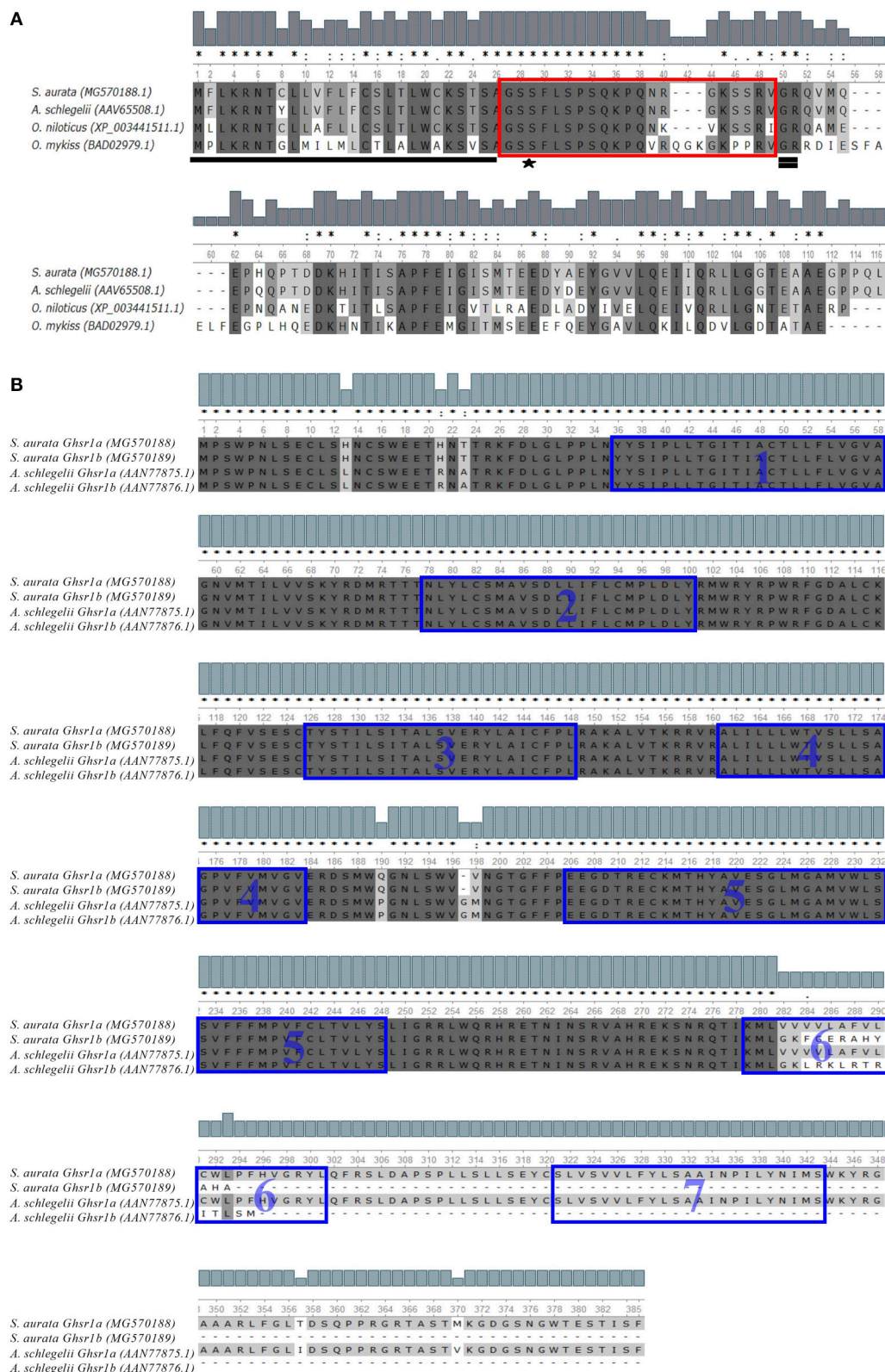


FIGURE 1 | (A) Multiple alignment of the Preproghrelin amino acid sequences corresponding to members of sparidae perciformes (*S. aurata* and *A. schlegelii*), cichlidae perciforme (*O. niloticus*) and salmoniformes (*O. mykiss*). From N- to C-terminal, the signal peptide (underlined), the mature Ghrelin (red boxed) and C-terminal Proghrelin peptide (rest of the sequence) are highlighted. Moreover, the acylation target Ser3 residue (starred) and the GlyArg amidation and cleavage signal

(Continued)

FIGURE 1 | (double underlined) are identified and conserved. **(B)** Amino acid alignment of the translated sequences of *S. aurata* Ghslr1a and Ghslr1b with their respective orthologs of the sparidae perciforme (*A. schlegelii*). Predicted transmembrane domains are blue boxed. Percentage of identity is indicated in grey scale. “.” indicates positions which have a single, fully conserved residue; “:” indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix and “.” indicates conservation between groups of weakly similar properties - scoring ≤ 0.5 in the Gonnet PAM 250 matrix.

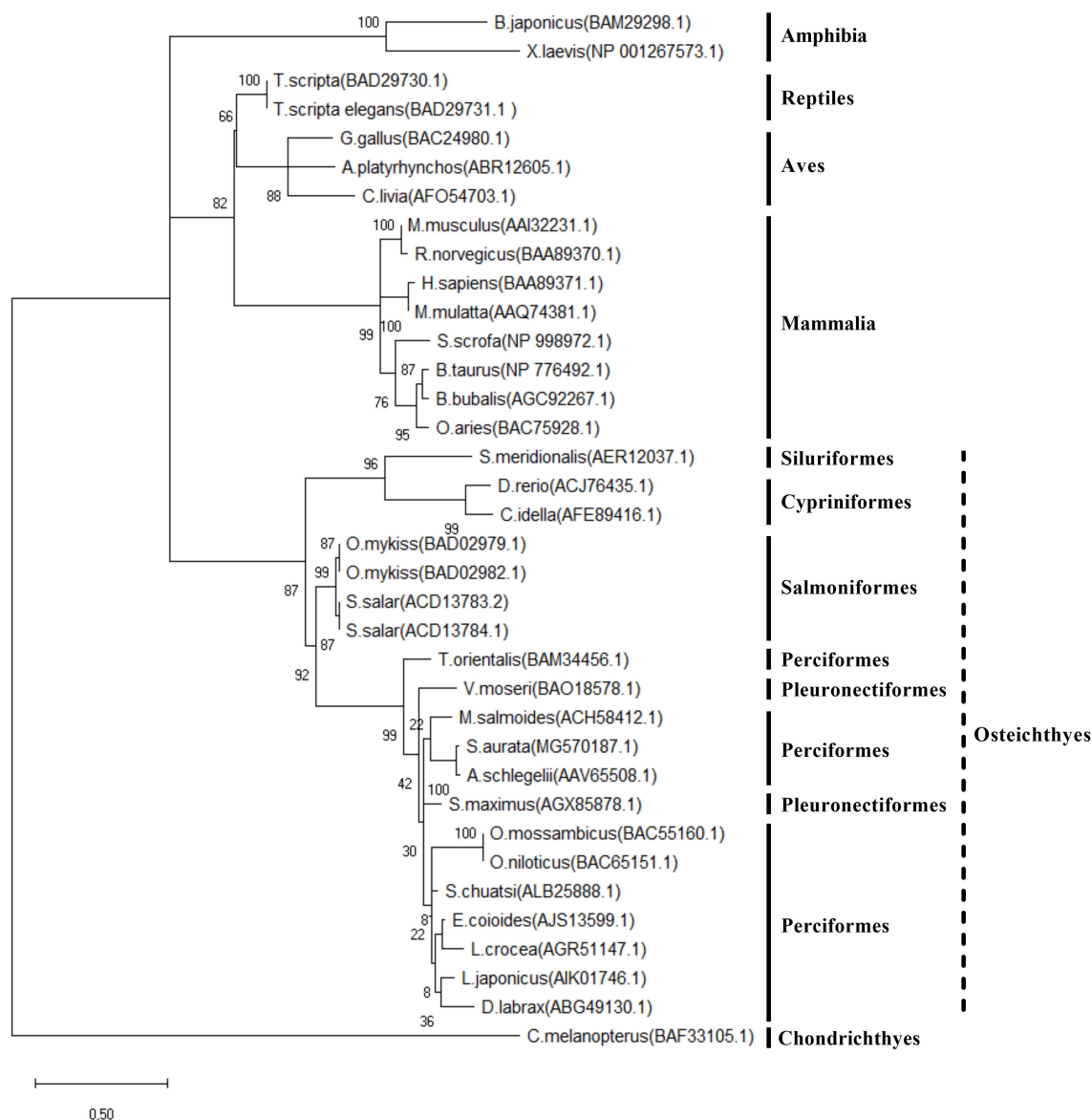
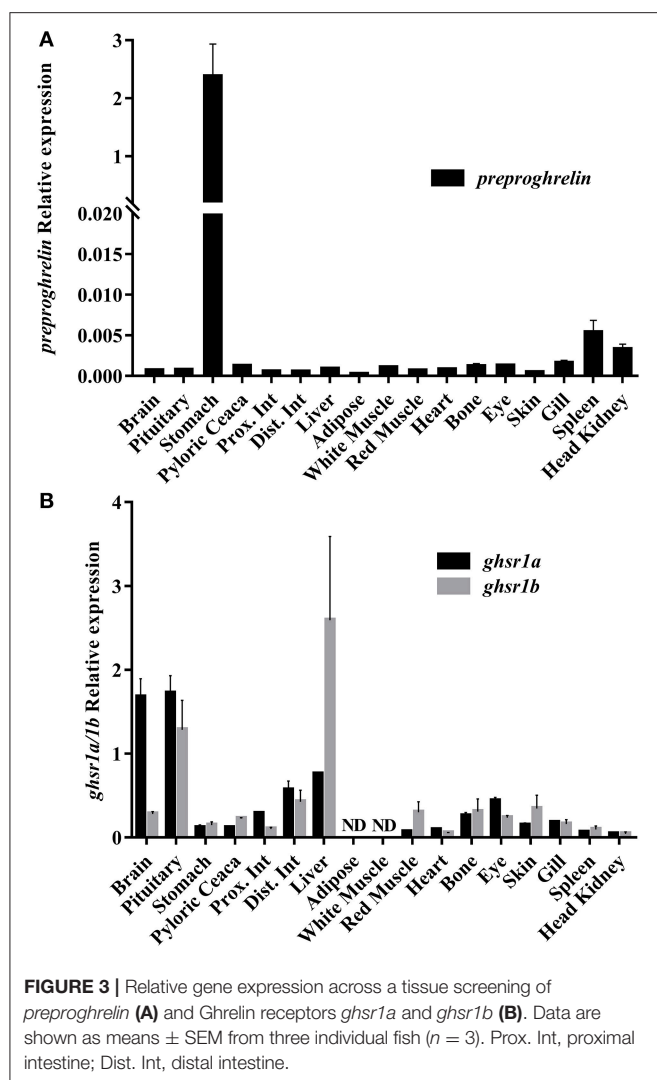


FIGURE 2 | Phylogenetic analysis (unrooted tree) of Preproghrelin among different vertebrates. Multiple alignment was performed using the default settings of the MAFFT tool online (server version (<http://mafft.cbrc.jp/alignment/server/>)) and a phylogenetic tree by Maximum Likelihood phylogeny was built with the MEGA X tool using the JTT + G + I substitution model. *B. japonicus* (*Bufo japonicus*), *X. laevis* (*Xenopus laevis*), *G. Gallus* (*Gallus gallus*), *A. platyrhynchos* (*Anas platyrhynchos*), *C. livia* (*Columba livia*) *T. scripta* (*Trachemys scripta*), *M. musculus* (*Mus musculus*), *R. norvegicus* (*Rattus norvegicus*), *H. sapiens* (*Homo sapiens*), *M. mulatta* (*Macaca mulatta*), *S. scrofa* (*Sus scrofa*), *O. aries* (*Ovis aries*), *B. Taurus* (*Bos Taurus*), *B. bubalis* (*Bubalus bubalis*), *S. meridionalis* (*Silurus meridionalis*), *D. rerio* (*Danio rerio*), *C. idella* (*Ctenopharyngodon idella*), *O. mykiss* (*Oncorhynchus mykiss*), *S. salar* (*Salmo salar*), *T. orientalis* (*Thunnus orientalis*), *V. moseri* (*Verasper moseri*), *M. salmoides* (*Micropterus salmoides*), *S. maximus* (*Scophthalmus maximus*), *S. aurata* (*Sparus aurata*), *A. schlegelii* (*Acanthopagrus schlegelii*), *L. japonicus* (*Lateolabrax japonicus*), *D. labrax* (*Dicentrarchus labrax*), *E. coioides* (*Epinephelus coioides*), *L. crocea* (*Larimichthys crocea*), *S. chuatsi* (*Siniperca chuatsi*), *O. mossambicus* (*Oreochromis mossambicus*), *O. niloticus* (*Oreochromis niloticus*), *C. melanopterus* (*Carcharhinus melanopterus*). Length of the branches corresponds to number of substitutions per site and confidence values (based on a bootstrap number of 1,000) are shown above and below the lines, respectively.



fasting effects were not found either but at 5 h post-prandial the expression levels in the brain were significantly down-regulated (Figure 6D) compared to the initial sampling (–21 days), and similar low expression values were maintained at 1 and 7 days post-feeding.

The mRNA expression profile of both pituitary *ghsrs* isoforms (Figures 6B,C) was similar along the experiment, stable during fasting and down-regulated significantly at 5 h refeeding. After 1 and 7 days, the expression of *ghsl1a* increased significantly reaching the levels as before fasting, while the *ghsl1b* expression remained low until the end. Moreover, the gene expression patterns of both *ghsrs* in the brain (Figures 6E,F) were almost identical, being practically irresponsive to either 21 days of fasting or the onset of feeding.

Gh/Igf-1 axis members

Pituitary *gh* gene expression (Figure 7A), similarly to plasma Gh, progressively increased to reach maximum levels at

1 day post-refeeding, decreasing back to basal levels at day 7.

The liver gene expression of total *igf-1* (Figure 7D) remained stable after fasting and in the early post-prandial period, but after 1 day, the lowest levels were observed, and at day 7 returned to baseline. The *igf-1* splice variants (Figure 7E) showed a similar gene expression profile than that of total *igf-1*, especially *igf-1a* with little effects of fasting and lowest expression levels at 1 day post-refeeding, recovering basal values after 7 days. Moreover, *igf-1b* and *igf-1c* showed a significant post-prandial dip at 2 h, maintaining still lower values at day 1, to return to basal levels at day 7.

Concerning liver Gh receptors, both were significantly down-regulated due to fasting (Figures 7B,C). However, different post-prandial responses were observed: *ghr-1* stopped decreasing at 2 and 5 h, while *ghr-2* expression continued to decline until 2 h, remaining low up to 1 day post-refeeding. The expression of both receptors was then up-regulated at day 7 in comparison to early post-prandial measurements. In the case of Igf-1 receptors, the only isoform detected in liver was *igf-1rb* (Figure 7F). Its expression was not affected by fasting but was significantly down-regulated at 2 h of refeeding, to then recover at 7 days initial expression levels.

The gene expression of four *igfbps* is shown in Figures 7G,H. *igfbp1a* and *igfbp2a* expression had similar stable patterns, except that *igfbp1a* showed a significant abrupt peak in expression 2 h post-refeeding, returning to basal levels at 5 h. The expression of *igfbp4* and *igfbp5b* was detected for the first time in gilthead sea bream liver. Both presented a similar profile, but *igfbp5b* did not show significant changes while the response for *igfbp-4* was more pronounced, with a significant decrease at 5 h and 24 h post-prandial compared to the onset of refeeding. Then, such low expression level was maintained after 1 day post-refeeding and basal levels were recovered after 7 days.

DISCUSSION

Preproghrelin and Ghsrs Characterization

Since its discovery, the *preproghrelin* nucleotide and amino acid sequences have been described in many vertebrate species (36). In the present study, phylogenetic analysis of the gilthead sea bream translated sequence highlighted the conservation of the most characteristic features. In fact, Preproghrelin is considered a well-conserved protein, but with a perceptible evolution among classes and orders. The gilthead sea bream Preproghrelin resulted more closely related to other Sparidae species, flatfishes and European sea bass (*Dicentrarchus labrax*), but more distant to salmonids, cypriniformes, siluriformes and chondrichthyes.

The expression of *preproghrelin* was detected mainly in stomach and pyloric caeca, which agrees with previous studies in mammals and other fish species, establishing that the main source of Ghrelin is the stomach (3, 4, 6, 12, 13). Moreover, weak *preproghrelin* expression was detected in other tissues and organs as in different fish species (5, 6). One of the main targets of Ghrelin is the brain, where it is reported to act in appetite-regulating areas to induce (or decrease in some species) feed

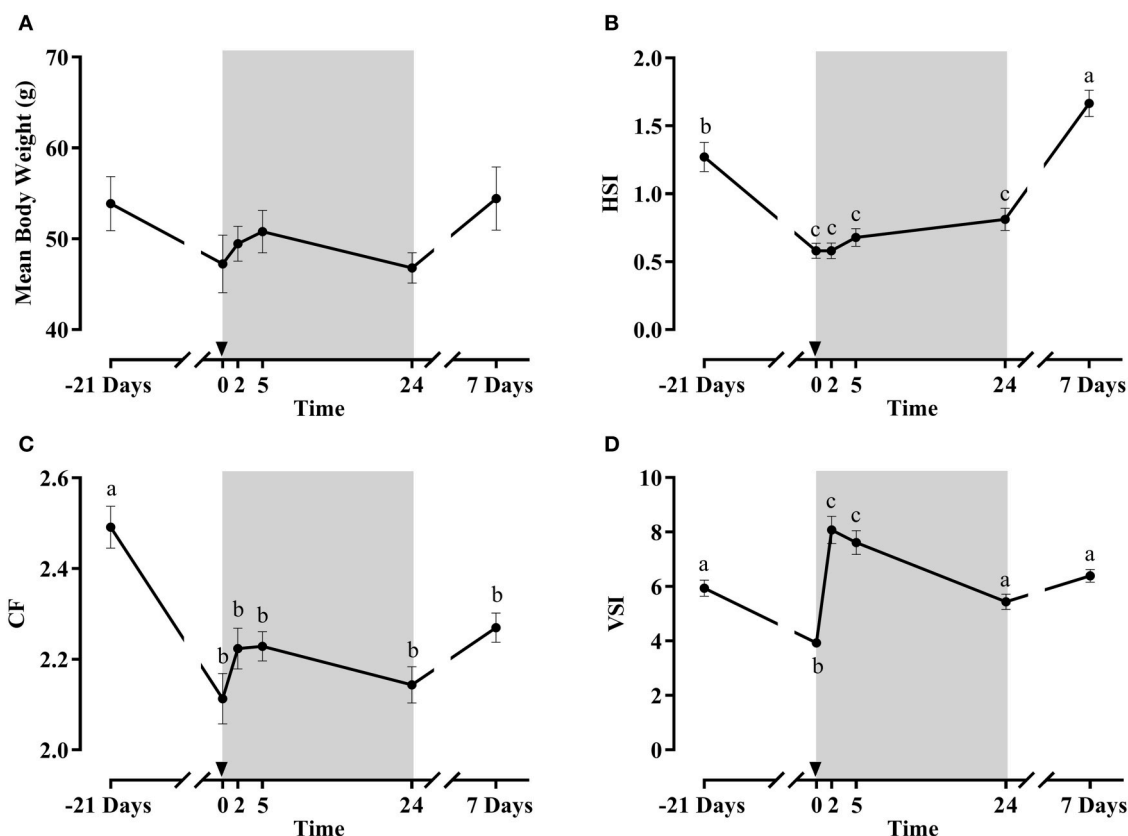


FIGURE 4 | Mean body weight (A), condition factor (CF) (B), hepatosomatic index (HSI) (C) and viscerosomatic index (VSI) (D) of fish during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means $\pm$ SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

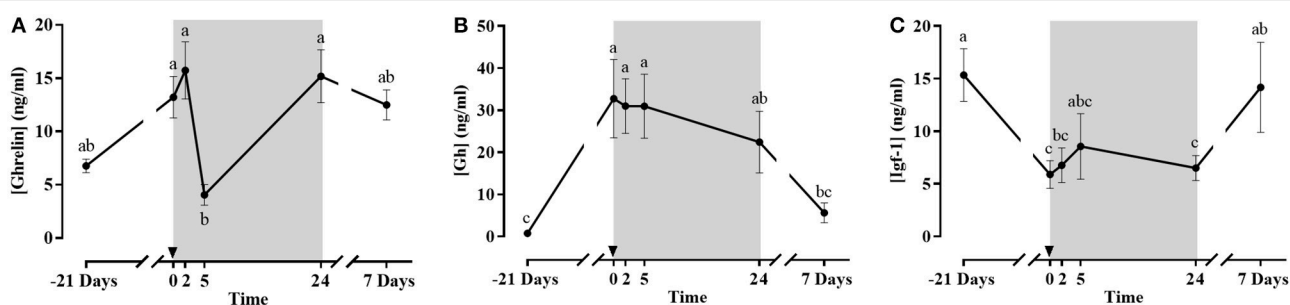


FIGURE 5 | Plasma concentration of Ghrelin (A), Gh (B) and Igf-1 (C) during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means $\pm$ SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

intake (19, 22). Thus, the detection of *preproghrelin* mRNA expression locally in the brain may also contribute to confirm the existing hypothesis that Ghrelin is synthesized both peripherally and centrally (22). In our screening, the low *preproghrelin* mRNA levels detected in the brain may be due to the fact that the whole brain was taken, instead of only the hypothalamus, which is supposed to be the main production site and target in the brain.

The gene expression screening of the two *ghsrs* showed that both are widely distributed among multiple tissues and organs, in line with previous research (16). The tissues with higher expression were pituitary, brain and liver, which support that these are the main targets of Ghrelin action in gilthead sea bream, as in many other vertebrate species (15, 37). Furthermore, as far as we know, this is the first time that it is observed that isoform *a* is more abundant in brain and pituitary, while isoform

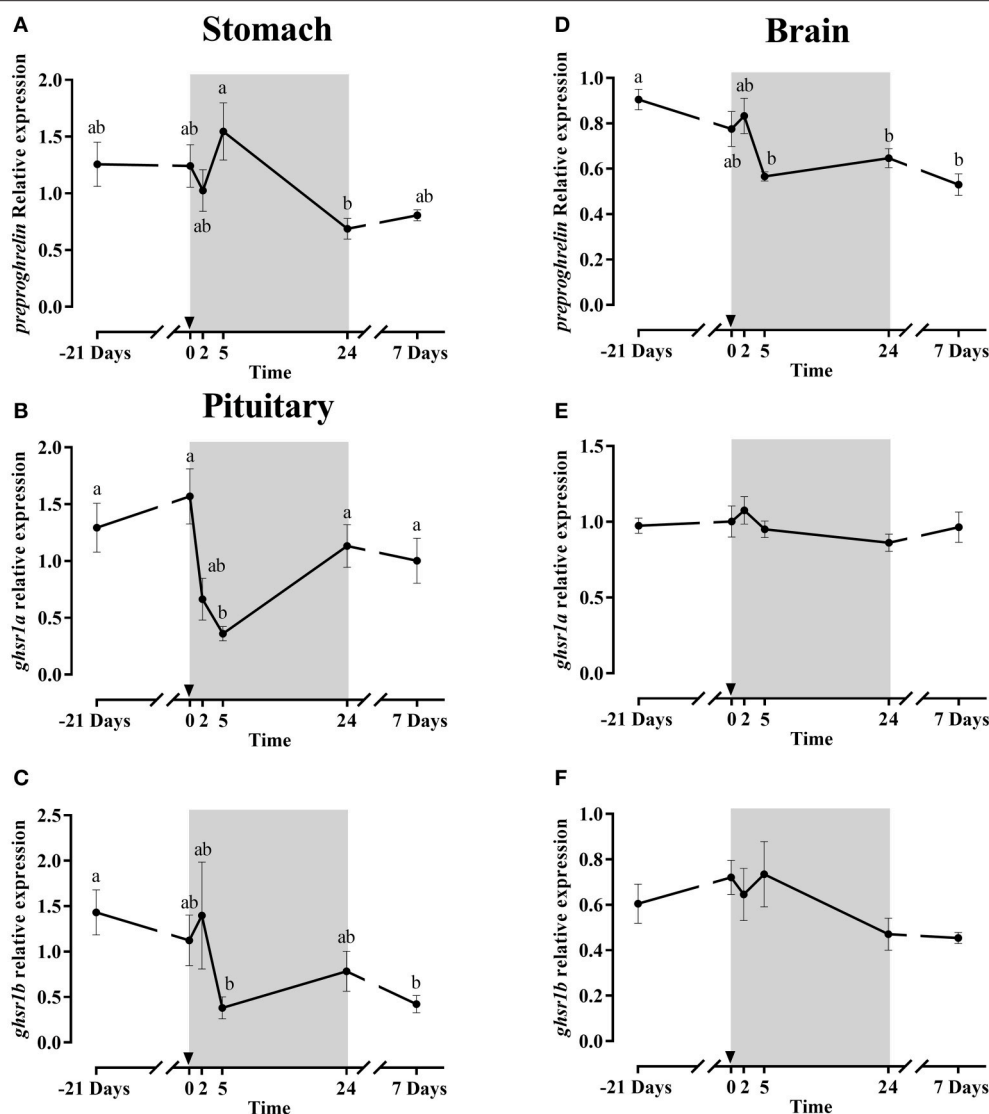


FIGURE 6 | Relative gene expression of stomach *preproghrelin* (A), pituitary *ghslr1a* (B) and *ghslr1b* (C) and brain *preproghrelin* (D), *ghslr1a* (E) and *ghslr1b* (F) during the fasting and refeeding experiment. The postprandial period is shown in gray boxes and the time in hours. Data are shown as means $\pm$ SEM ($n = 6$). Letters indicate significant differences ($p < 0.05$) by one-way ANOVA and Tukey HSD or LSD test.

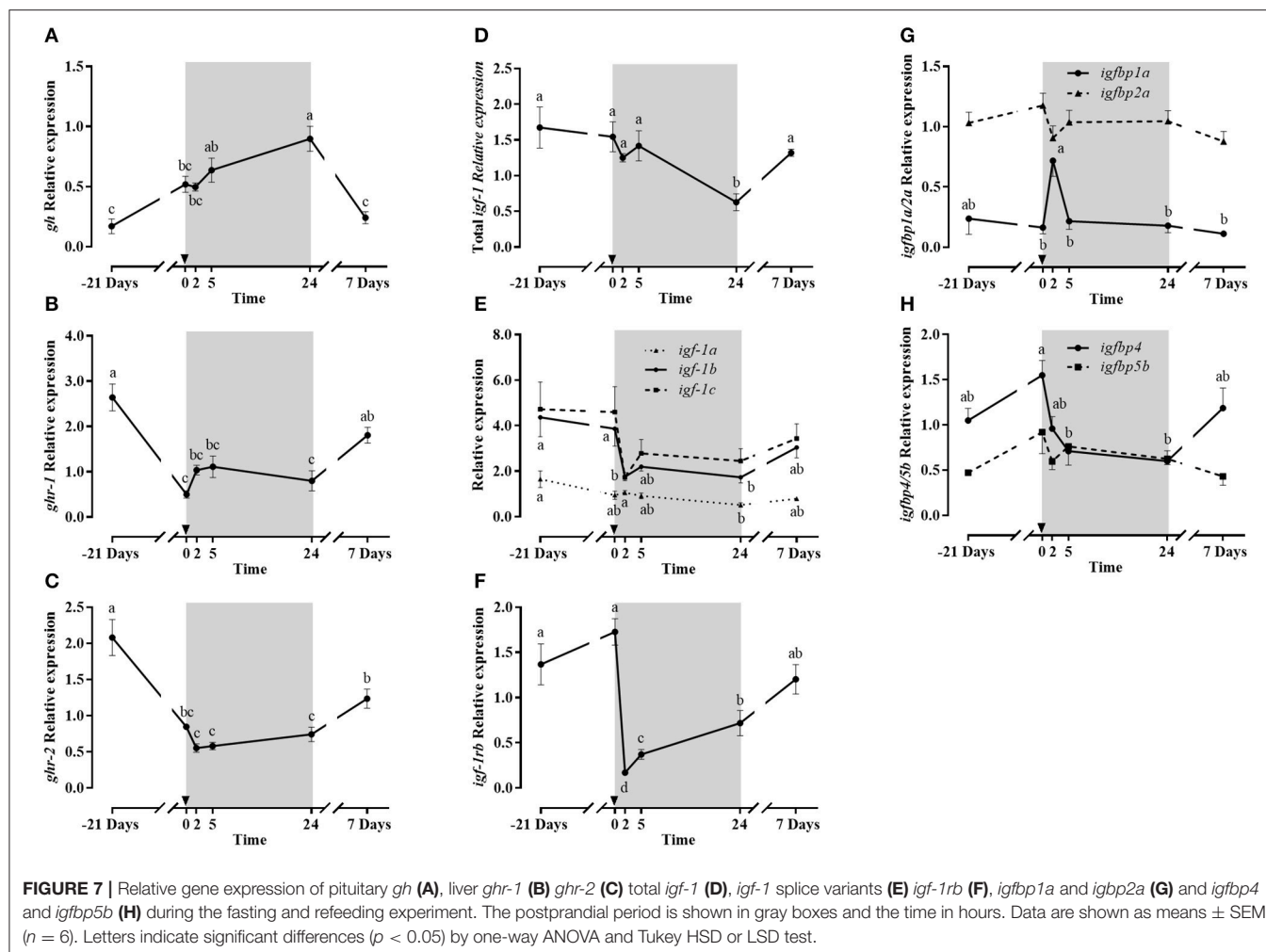
b is more abundant in liver. Such differential expression in these tissues could suggest that Gh secretion requires the presence of the truncated isoform to achieve better regulation, as suggested (15, 16).

Fasting and Refeeding Effects on Growth Performance and Ghrelin

Although gilthead sea bream tolerates long periods of food deprivation well (25, 38–40), the morphometric parameters reduction after 21 days of fasting confirmed that the fish had entered in a catabolic state, which was progressively reverted upon refeeding, as demonstrated by the recovery of the body indexes at the end of the experiment.

The existing literature reveals that the response of Ghrelin to fasting may be, especially in fish, species-specific. Thus, fasting has been reported to up-regulate, down-regulate or unchange the gastrointestinal tract and brain *ghrelin* mRNA levels in diverse fish species (7, 41–45). Such a variety of responses could indicate that other factors, such as sex and age of individuals (44), temperature (46), fasting duration (42) or diet (13) may also affect Ghrelin production. Interestingly, during the development of the present work, Babaei et al. (13) also reported a tissue-specific *preproghrelin* expression response to fasting in gilthead sea bream.

The different response observed to 21 days of fasting with Ghrelin plasma levels and *preproghrelin* mRNA levels in stomach, is consistent with previous fish studies (45, 47) and suggests



that post-transcriptional mechanisms are in place. However, Ghrelin plasma levels were also high at 1 and 7 days post-refeeding probably due to the 24 h fast. In sea bass, a rise in *preproghrelin* expression was observed during the first days of fasting, to then decrease progressively to fed control values after 21 days of fasting (48). In grass carp, a peak of intestinal *ghrelin* expression was described after 7 days of fasting (49). In goldfish, Unniappan et al. (42) found that fasting for 3 and 5 days significantly increased Ghrelin plasma levels, while in gut or hypothalamus *preproghrelin* expression did not increase until after 7 days of fasting. Moreover, in Atlantic salmon, Ghrelin levels were significantly increased after 2, but not 14 days of fasting (50). Together, these observations support the idea that in diverse fish species, the response increasing Ghrelin plasma levels occurs mainly during the early stage of fasting and is not always related to changes in gut gene expression.

Besides, with refeeding Ghrelin plasma levels that were still high at 2 h, were followed by a significant decrease at 5 h, suggesting an inhibitory effect on Ghrelin secretion as food enters the stomach. These decrease in Ghrelin coincided with the

beginning of circulating Gh decline, suggesting the relationship between these two hormones. A similar decrease was also observed at 1 h post-prandial in tilapia (51), and in refed striped bass (52). Moreover, such reduced plasma levels coincided with the peak in stomach *preproghrelin* mRNA levels, whereas the minimum expression 1 day after refeeding corresponded with the recovery of Ghrelin plasma levels, indicating an inverse relationship between the regulation of the gene expression and the circulating hormone. Thus, it appears that during this specific postprandial stage (2, 5, and 24 h) *preproghrelin* gene expression could be regulated by Ghrelin plasma levels.

Unniappan et al. (42) also observed that in goldfish, *preproghrelin* mRNA levels (in gut and hypothalamus) and Ghrelin plasma levels were sensitive to feeding when analyzed periprandially. At 3 h pre-meal, Ghrelin plasma and mRNA levels were high, and 1 and 3 h after feed intake were down-regulated in both tissues. Similar results were observed by Hatf et al. (53) in zebrafish, in which *preproghrelin* mRNA levels in brain and gut were down-regulated 3 h post-meal and increased in fasted fish. These studies are in accordance with the observed decrease in plasma Ghrelin and brain *preproghrelin* mRNA at 5 h

post-feeding in the present experiment, indicating that Ghrelin may be mainly regulated by feed intake also in gilthead sea bream.

Fasting and Refeeding Effects on Ghhrs

The *ghhrs* responded differentially to refeeding in brain and pituitary. The expression in brain remained constant, while in the pituitary decreased progressively up to 5 h to recover at 1 or 7 days of refeeding the expression of *ghsr1a*, and to a lesser extent of *ghsr1b*. In rats, brain and pituitary *ghhrs* were up-regulated in fasting and decreased after refeeding (15, 54, 55). However, the function of Ghhrs in fish and other non-mammalian vertebrates is still not fully understood. Thus, although Ghhrs have crucial roles in the ghrelinergic system and their expression is finely regulated by nutritional condition, hormonal status and environmental factors, their response is highly variable depending on the species especially in fish, in which a higher number of Ghhrs isoforms has been described (19).

Peddu et al. (51) did not find in Mozambique tilapia brain a clear response to fasting in *ghhrs* expression, but at feeding time (just before food administration) both receptors were up-regulated to decrease at 1 and 3 h post-feeding. In the same species, a significant change was not observed in brain *ghsr1a* expression between 1 and 7 days of fasting, while *ghsr1b* increased after 3 but not 5 fasting days (56). In Atlantic salmon, a fasting period of 2 or 14 days did not change *ghsr1a* brain expression (50), neither it did 15 days of fasting in zebrafish *ghhrs* (57). Contrarily, Kaiya et al. (58) found that 7 days of fasting induced a decrease in the expression of *ghsr1a* in the vagal lobe of goldfish. Thus, although species differences exist it seems that there is regulation of *ghhrs* depending on the alimentary condition.

Ghrelin receptors in fish pituitary have been poorly investigated, but low basal expression levels have been found in tilapia (56), goldfish (59, 60) or yellow catfish (61). In the case of grass carp, 14, 21, and 28 days of fasting resulted in increased pituitary gene expression of *ghsr1a* that correlated with increased plasma Gh and *preproghrelin* pituitary gene expression (62). Moreover, these authors found that Ghrelin administration provoked an increase in pituitary *ghsr1a* expression. In the present study, the decrease in *ghhrs* expression during the post-prandial stage was noticeable and related with circulating Ghrelin, pointing to a slowdown of the system during food intake. To summarize, Ghrelin receptors expression in the brain do not show a uniform regulation among fish species and seem to be less influenced by the nutritional condition in comparison to mammals. Furthermore, less is known about pituitary Ghhrs dynamics during fasting in fish, but in gilthead sea bream, both isoforms present a similar response that parallels Ghrelin plasma levels.

Fasting and Refeeding Effects on the Gh/Igf Axis

The rise of circulating Gh during fasting was parallel to *gh* mRNA levels in the pituitary, being significantly high at 5 h post-feeding. The expression of *gh* remained high until 1 day of

refeeding, and similarly to plasma Gh, returned to basal values after 7 days, thus indicating the important and extended effect of fasting in this hormone. This response of Gh to fasting and refeeding has been observed in previous studies in various fish species, such as Chinese perch (*Siniperca chuatsi*), tilapia and black sea bream (*Spondyliosoma cantharus*) (63–65). Plasma Igf-1 also responded to nutritional state, presenting an inverse pattern to that of Gh, decreasing with fasting and slowly increasing with refeeding. Liver total *igf-1* gene expression as well as its splice variants partially recovered after 7 days of refeeding. These results are in line with previous works (63, 66). The inverse correlation between Gh and Igf-1 plasma levels during fasting was pointed out in gilthead sea bream previously (38, 67, 68), and has been described in several other fish species (e.g., coho salmon, chinook salmon, channel catfish, Nile tilapia or gilthead sea bream) in diverse conditions (26, 47, 66, 69–71). Moreover, the results support that the circulating Gh/Igf-1 ratio is a good indicator of metabolic state in gilthead sea bream and that it is clearly affected by feeding condition (67, 72). Picha et al. (52) suggested that during fasting in striped bass, high Ghrelin levels contribute to counteract the negative feedback normally exerted by Igf-1 on Gh release, in order to maintain Gh secretion.

The gene expression of *ghrs* in the liver also reflected the nutritional status. The dramatic down-regulation of both *ghr-1* and *ghr-2* expression, along with increased Gh plasma levels, suggests a Gh liver desensitization during the fasting period (23). After refeeding, a rapid increase in the mRNA levels of *ghr-1*, the isoform mostly related with anabolic processes in this species was observed, and later in the expression of *ghr-2*, indicating that ingested nutrients may have initiated growth promotion (23). Furthermore, liver *igf-1rb* showed a similar tendency to that of *ghrs* after refeeding and its abrupt post-prandial expression drop at 2 h was not recovered until the end of the trial. It is interesting that this response is parallel to *igf-1b* and *c* hepatic gene expression. Down-regulation of liver *igf-1rb* expression was also observed in gilthead sea bream during exercise (26), but as far as we know, this is the first time that this effect is found in refeed fish.

The expression of *igfbps* was stable during fasting while 7 days of refeeding recovered their basal values. Nevertheless, *igfbp-4* presented the highest expression after 21 fasting days in agreement with its Igf-1 conservative function, while the increase of *igfbp-1a* at 2 h post-feeding fitted well with its recognized role in mobilization conditions in this species. Similarly, in a fasting and refeeding experiment in rainbow trout, Gabillard et al. (73) observed different responses for *igfbps*. Hevrøy et al. (50) described the effects of fasting on Ghrelin and Gh/Igf-1 system in Atlantic salmon, in which Igfbp-1 seemed to be a marker of catabolic state. Breves et al. (63) demonstrated different roles of *Igfbps* during fasting, and indicated that Igfbp-1b may operate to reduce Igf-1 signaling during fasting in tilapia. The functional relationship between Gh, Igf-1 and Ghrelin during fasting in fish needs to be further investigated.

To summarize, the full *preproghrelin*, *ghsr1a* and *ghsr1b* nucleotide sequences and their response during fasting/refeeding have been described for the first time in gilthead sea bream.

Both, long term (21 days) and short term (24 h) fasting increased circulating Ghrelin, which showed the lowest values few hours post-prandial. The plasma Ghrelin dip was also reflected by pituitary *ghsrs*, suggesting that Ghrelin's stimulatory action on Gh secretion is modulated by feeding. Plasma Gh levels were elevated in parallel with its pituitary gene expression returning to basal levels after 7 days of refeeding, although at this time circulating Ghrelin was again increased. Taken together, the data suggest that Ghrelin can be a regulator of Gh secretion in gilthead sea bream, but the metabolic state itself and other regulatory molecules may exert important effects. Finally, this study indicates that in gilthead sea bream, Ghrelin secretion is mainly related to the progress of the digestive process, showing a down-regulation in the post-prandial period to rise again just before feeding.

AUTHOR CONTRIBUTIONS

MP-A, EV, and JG conceived and designed the experiments; MP-A, EV, JV-A, AS-M, NR-H, JF-B, JB, IN, EC, and JG performed the experiments; MP-A, EV, JV-A, AS-M, NR-H, IH, JF-B, JB, JC-G, IN, EC, EJ, JP-S, and JG analyzed the data and interpreted the results; JF-B, JB, IN, EC, EJ, JP-S, and JG contributed reagents, materials, analysis tools; MP-A, EV, JV-A, AS-M, NR-H, IH, JF-B, JB, JC-G, IN, EC, EJ, JP-S, and JG wrote and revised the paper.

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DATA AVAILABILITY STATEMENT

The three sequences obtained in the present study are deposited in GenBank (NCBI) under accession numbers: MG570187 for *preproghrelin*; MG570188 for *ghsr1a*, and MG570189 for *ghsr1b*. All relevant data is contained within the manuscript.

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SUPPLEMENTARY MATERIAL

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Nesfatin-1 Regulates Feeding, Glucosensing and Lipid Metabolism in Rainbow Trout

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Nesfatin-1 is an 82 amino acid peptide that has been involved in a wide variety of physiological functions in both mammals and fish. This study aimed to elucidate the role of nesfatin-1 on rainbow trout food intake, and its putative effects on glucose and fatty acid sensing systems. Intracerebroventricular administration of 25 ng/g nesfatin-1 resulted in a significant inhibition of appetite, likely mediated by the activation of central POMC and CART. Nesfatin-1 stimulated the glucosensing machinery (changes in *sglt1*, *g6pase*, *gsase*, and *gnat3* mRNA expression) in the hindbrain and hypothalamus. Central fatty acid sensing mechanisms were unaltered by nesfatin-1, but this peptide altered the expression of mRNAs encoding factors regulating lipid metabolism (*fat/cd36*, *acly*, *mcd*, *fas*, *lpl*, *ppara*, and *ppary*), suggesting that nesfatin-1 promotes lipid accumulation in neurons. In the liver, intracerebroventricular nesfatin-1 treatment resulted in decreased capacity for glucose use and lipogenesis, and increased the potential of fatty acid oxidation. Altogether, the present results demonstrate that nesfatin-1 is involved in the homeostatic regulation of food intake and metabolism in fish.

Keywords: NUCB2, nutrient sensing, hypothalamus, hindbrain, liver, fish

INTRODUCTION

Food intake in vertebrates is subject to a complex regulation involving both peripheral components in charge of transmitting the metabolic status of the organism, and the central nervous system (CNS), responsible for receiving, processing and responding to this information (1). Specific hypothalamic nuclei in the CNS are responsive to changes in energy status, and respond with variations in the expression of key neuropeptides (particularly, agouti-related protein (AgRP)/neuropeptide Y (NPY), and pro-opio melanocortin (POMC)/cocaine and amphetamine-related transcript (CART)), ultimately leading to changes in food intake (2). These hypothalamic neurons, together with neurons from the hindbrain possess nutrient sensing mechanisms able to detect the levels of nutrients, particularly glucose, fatty acids and amino acids, as demonstrated in mammals (2) and in fish (3, 4).

Glucosensing in fish is mainly driven by the canonical mechanism based on glucokinase (GCK), glucose facilitative transporter 2 (GLUT2) and ATP-dependent inward rectified potassium channel (K_{ATP}) (3–6), although GCK-independent glucosensing mechanisms based on the sodium-glucose linked transporter 1 (SGLT-1), liver X receptor (LXR) and sweet taste receptor have been also described in the rainbow trout brain (7, 8). Fatty acid sensing mechanisms described so far in the fish brain are based on carnitine palmitoyltransferase 1 (CPT-1), fatty acid translocase (FAT/CD36), mitochondrial production of reactive oxygen species (ROS), and lipoprotein lipase (LPL) (9–11).

The mechanisms linking the function of nutrient sensing systems with changes in the expression of hypothalamic appetite-regulating neuropeptides are scarcely known in both mammals and fish. Studies available in mammals point to the activation of mechanistic target of rapamycin (mTOR) and the inhibition of AMP-activated protein kinase (AMPK) as mediators between the activation of nutrient sensing systems and modulation of neuropeptides expression. This would occur through the modulation of the transcription factors forkhead box protein O1 (FOXO1), cAMP response element binding protein (CREB), and brain homeobox transcription factor (BSX) (12–14). In fish, available evidence suggests that comparable signaling pathways and transcription factors might be linking nutrient sensing and the expression of neuropeptides (15–17).

Central nutrient sensing mechanisms have been described to be subject of endocrine modulation in mammals (2, 18). In fish, it was reported that the glucosensor mechanism dependent on GCK-GLUT2- K_{ATP} is modulated at the hypothalamic level by insulin (19), leptin (20), ghrelin (21), cholecystokinin (22), and glucagon-like peptide 1 (23). Insulin (24) and ghrelin (25) have been also found to modulate fatty acid sensing mechanisms.

Nesfatin-1 is an 82 amino acid peptide encoded in the N-terminal region of nucleobindin-2 (NUCB2) gene (26). It is mainly expressed in the brain, particularly hypothalamus, although it is also present in a wide variety of peripheral organs (27–30). Specific binding sites for nesfatin-1 were detected both in the CNS and peripheral organs (31). Nesfatin-1 has been mainly studied for its inhibitory action on food intake in both mammals (32–34) and fish (35, 36), but it has also been implicated in the regulation of cardiac functions (37, 38), lipid metabolism (39, 40), glucose homeostasis (33, 41, 42), and reproductive functions (43–45), among others. Available studies in mammals have shown that a subpopulation of NUCB2/nesfatin-1-expressing neurons is activated in the rat brain in response to hypoglycemia (46), and that nesfatin-1 modulates the excitability of glucosensing neurons in the rat brain (34, 47, 48). Furthermore, nesfatin-1 has been reported to be modulated by nutrients in mammals (49) and fish (50, 51). However, as far as we are concerned, the role of nesfatin-1 as a modulator of nutrient sensing systems has not been studied to date in fish.

Therefore, this study first aimed to assess if nesfatin-1 intracerebroventricular (ICV) treatment is anorectic in rainbow trout in a way similar to other mammalian and fish species. Once demonstrated, we aimed to determine the

putative effects of nesfatin-1 on the mechanisms involved in glucose and fatty acid sensing in brain regions implicated in the homeostatic regulation of food intake, i.e., hypothalamus and hindbrain (1, 4). For this, nesfatin-1 was ICV injected and the hypothalamic and hindbrain expression of mRNAs involved in (i) appetite-regulating neuropeptides (*npv*, *agrp*, *pomca1*, *cart*); (ii) glucosensing and glucose metabolism: *glut2*, *sgl1*, *gck*, *pyruvate-kinase (pk)*, *glucose 6-phosphatase (g6pase)*, *phosphoenolpyruvate carboxykinase (pepck)*, *glycogen synthase (gsase)*, *guanine nucleotide-binding protein G(t) subunit alpha transducing 3 (gnat3)*; (iii) fatty acid sensing and metabolism: *fat/cd36*, *ATP-citrate lyase (acly)*, *malonyl-CoA decarboxylase (mcd)*, *fatty acid synthase (fas)*, *cpt-1a/b/c*, *citrate synthase (cs)*, *lpl*, *peroxisome proliferator-activated receptor type alpha and gamma (ppara, pparg)*, *sterol regulatory element-binding protein type 1c (srebp1c)*; and iv) mitochondrial activity: *uncoupling protein 2a (ucp2a)* and *inward-rectifier K channel pore type 6.x-like, kir6.x-like*, was quantified. We also evaluated the phosphorylation status of the proteins mTOR, AMPK α , CREB, and FOXO1. The impact of the ICV treatment with nesfatin-1 was also assessed in the liver since this is the main tissue involved in energy homeostasis. Additionally, hypothalamic sensing of nutrients is known to induce changes in liver metabolism through sympathetic outflow, as described in mammals (2) and fish (1).

MATERIALS AND METHODS

Animals

Female rainbow trout (*Oncorhynchus mykiss*), with a body weight (bw) of 30 ± 5 g, were obtained from a local commercial supplier. Fish were housed in 700 L aquaria with filtered fresh water at $13 \pm 1^\circ\text{C}$ and continuous aeration, and maintained under a 12 h light:12 h darkness (12L:12D) photoperiod (lights on at 07:00 h). Food from a commercial pellet diet specifically designed for salmonids (Ewos Pacific 3 mm, Fish Farm Supply Co, Surrey, Canada) was offered daily at 10:00 h until visual apparent satiety. All studies adhered to the Canadian Council of Animal Care guidelines, and were approved by the Animal Research Ethics Board of the University of Saskatchewan (Protocol Number 2012-0082).

Experimental Designs

Effect of Nesfatin-1 on Rainbow Trout Food Intake

Following a 3-week acclimation period, fish were divided into two experimental groups, control and experimental ($n = 5$ fish/group). Food intake was registered during 1 week before treatment to evaluate basal levels of food intake. For this, a pre-weighed amount of food was offered to fish in each tank. After feeding, the uneaten food was collected, dried and weighed. The amount of food consumed by all fish in each tank was calculated as previously described as the difference from the feed offered (52, 53). On the day of experiment, fish were lightly anesthetized with tricaine methanesulfonate (TMS-222, Syndel Laboratories, Canada), weighed and ICV injected with either saline alone (control group) or containing 25 ng/g of goldfish nesfatin-1

(VPISIDKTKVKLPEETVKESPQNVD TGLHYDRYLREVID-FLEKDQHFREKLHNTDMEDIKQGKLAKELDFVSHHVRTK LDEL; GenScript, Piscataway, USA; experimental group). ICV injections were performed in the third ventricle as previously described (54). Dose of nesfatin-1 used here was chosen based on our previous experiments in goldfish (35, 36). After injections, fish were returned to their corresponding tanks and allowed to recover. Food was recovered at 2, 6, and 24 h post-injection, and food intake was quantified as described above. The experiment was repeated three times, and results shown correspond to the mean of the three experiments.

Effect of Nesfatin-1 on Glucose and Fatty Acid Sensing Systems in Rainbow Trout

Following a 3-week acclimation period, fish were divided into two experimental groups, control and experimental ($n = 9$ fish/group). On the day of experiment, fish were ICV injected with either saline alone (control group) or containing 25 ng/g nesfatin-1 (experimental group), as described in Section Effect of nesfatin-1 on rainbow trout food intake. After 2 h, fish were anesthetized again, sacrificed by decapitation, and samples of hypothalamus, hindbrain and liver were collected, frozen in liquid nitrogen and stored at -80°C until analysis.

Quantification of mRNA Abundance by Real-Time Quantitative PCR (RT-qPCR)

Total RNA from hypothalamus, hindbrain and liver was isolated using Ribozol RNA Extraction Reagent (aMReSCO, Toronto, Canada) and treated with DNase. RNA purity was validated by optical density (OD) absorption ratio (OD 260/280 nm) using a NanoDrop 2000c (Thermo, Vantaa, Finland). Then, an aliquot of 1 μg of total RNA was reverse transcribed into cDNA in a 20 μL reaction volume using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Mississauga, Canada) according to the manufacturer's instructions. Real-time quantitative PCRs were performed using iQ SYBR Green Supermix (Bio-Rad). The specific primer sequences used are shown in **Table 1** and were ordered from IDT (Toronto, Canada). Primers used in this study were previously validated in rainbow trout (8, 25). Genes were amplified in duplicated RT-qPCR runs using a 96-well plate loaded with 1 μL of cDNA and 5×10^{-7} M of each forward and reverse primer in a final volume of 10 μL . RT-qPCR cycling conditions consisted of an initial step of 95°C for 3 min, and 35 cycles of 95°C for 10 s and specific annealing and extension temperature (**Table 1**) for 30 s. A melting curve was systematically monitored (temperature gradient at $0.5^{\circ}\text{C}/5$ s from 65 to 95°C) at the end of each run to confirm specificity of the amplification reaction. Wells containing RNA samples and water instead of cDNA were run for each reaction as negative controls. Only efficiency values between 85 and 100% were accepted (R^2 for all genes assessed were always higher than 0.95). All runs were performed using a CFX Connect Real-Time System (Bio-Rad). The $2^{-\Delta\Delta\text{Ct}}$ method (55) was used to determine the relative mRNA expression in the different samples. Relative quantification of target gene transcripts was done using β -actin gene expression as reference, which was stably expressed in this experiment. The mRNA expression in the control was considered

to be 1, and the expression in experimental fish was calculated as a fold of the expression level in controls.

Analysis of Protein Levels by Western Blot

Tissue samples ($n = 3$ fish) were homogenized in T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Waltham, USA), and proteins were extracted according to the manufacturer's instructions and quantified by Bradford assay. Western blot protocol was performed as previously described (56). The samples (containing 20 μg protein) were prepared in 1x Laemmli buffer containing 0.2% 2-mercaptoethanol (Bio-Rad) and boiled at 95°C for 10 min. Then, the whole sample volume was electrophoresed in 8–16% Mini-PROTEAN[®] TGX[™] precast protein gel (Bio-Rad). Precision plus protein[™] Dual Color Standards (Bio-Rad) was used as molecular weight marker. Following electrophoresis, proteins were transferred to a 0.2 μm pore-size nitrocellulose membrane (Bio-Rad) using the Trans-Blot[®] Turbo[™] transfer system (Bio-Rad), and membrane was blocked in 1x RapidBlock[™] solution (aMReSCO). Then, membranes were incubated overnight with specific primary antibody, all obtained from Cell Signaling Technology (Danvers, USA) except otherwise specified: 1:500 anti-phospho AMPK α (Thr-172), 1:500 anti-AMPK, 1:500 anti-phospho CREB (Ser-133), 1:500 anti-CREB (48h2), 1:250 anti-phospho-FoxO1 (Thr-24), 1:250 anti-FoxO1 (L27), 1:500 anti-phospho-mTOR (Ser-2448), and 1:1000 anti-vinculin (Abcam, Toronto, ON, Canada). All these antibodies cross-react successfully with rainbow trout proteins of interest (57, 58). After washing, membranes were incubated with goat anti-rabbit IgG (H+L) HRP conjugate (Bio-Rad) diluted 1:2,000. For protein visualization the membrane was incubated in Clarity[™] Western ECL substrate (Bio-Rad) and imaged using ChemiDoc[™] MP imaging system (Bio-Rad) with chemiluminescence detection. Protein bands were quantified by densitometry using Image Lab software. Target protein content in each of the samples was calculated as the ratio between the phosphorylated and the total amount of protein, except for the case of mTOR for which phosphorylated levels were normalized to the vinculin content.

Statistics

Statistical differences in mRNA and protein expression were assessed using *t*-test, after data were checked for normality and homogeneity of variance. Data that failed one of these requirements were log-transformed and re-checked. Significance was assigned when $p < 0.05$. All analyses were carried out using SigmaPlot version 12.0 (Systat Software Inc., San Jose, USA) statistics package.

RESULTS

Nesfatin-1 Decreases Food Intake in Rainbow Trout

ICV treatment with nesfatin-1 resulted in a significant decrease in food intake at 6 and 24 h post-injection when compared to the control groups. Reduction of feeding was about 22 and 36%, respectively. No significant differences in food intake were

TABLE 1 | Primers used for quantifying gene expression by RT-qPCR in this study.

Gene	Data base	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)
<i>β-actin</i>	GenBank	NM_001124235.1	GATGGGCCAGAAAGACAGCTA	TCGTCCAGTTGGTGACGAT	59
<i>acly</i>	GenBank	CA349411.1	CTGAAGCCCAGACAAGGAAG	CAGATTGGAGGCCAAGATGT	60
<i>agrp</i>	GenBank	CR376289	ACCAGCAGTCCTGTCTGGGTAA	AGTAGCAGATGGAGCCGAACA	60
<i>cart</i>	GenBank	NM_001124627	ACCATGGAGAGCTCCAG	GCGCACTGCTCTCCAA	60
<i>cpt-1a</i>	GenBank	AF327058	TCGATTTTCAAGGGTCTTCG	CACAACGATCAGCAAACCTGG	55
<i>cpt-1b</i>	GenBank	AF606076	CCCTAAGCAAAAGGGTCTTCA	CATGATGCACTCCCGACAG	55
<i>cpt-1c</i>	GenBank	AJ619768	CGCTTCAAGAATGGGGTGAT	CAACCACCTGCTGTTTCTCA	59
<i>cs</i>	Tigr	TC89195	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	55
<i>fas</i>	Sigenae	tcab0001c.e.065.1.s.om.8	GAGACCTAGTGGAGGCTGTC	TCTTGTTGATGGTGAGCTGT	59
<i>fat/cd36</i>	DFCI	AY606034.1	CAAGTCAGCGACAACCAGA	ACTTCTGAGCCTCCACAGGA	62
<i>g6pase</i>	Sigenae	cay0019b.d.18_3.1.s.om.8.1-1693	CTCAGTGGCGACAGAAAGG	TACACAGCAGCATCCAGAGC	55
<i>gck</i>	GenBank	AF053331	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCTTTGGTCCAG	60
<i>glut2</i>	GenBank	AF321816	GTGGAGAAGGAGGCGCAAGT	GCCACCGACACCATGGTAAA	59
<i>gnat3</i>	Sigenae	CU073912	GCAAGACGTGCTGAGGACCA	ATGGCGGTGACTCCCTCAAA	60
<i>gsase</i>	GenBank	BT073381.1	CGTGGTGAGAGGAAGGAACGTGAGC	CCGTTGAGACCGTGGAGACA	59
<i>kir6.x-like</i>	Sigenae	CA346261.1.s.om.8:1:773:1	TTGGCTCCTCTTCGCCATGT	AAAGCCGATGGTCACTGGGA	60
<i>leptin</i>	GenBank	AB354909.1	GCGGGAGCTTCACCTCTCATT	GAGGTCTGCCAGTCTAGGA	60
<i>lpl</i>	GenBank	AJ224693	TAATTGGCTGCAGAAAACAC	CGTCAGCAAACCTCAAAGGT	59
<i>mcd</i>	Sigenae	BX869708.s.om.10	TCAGCCAGTACGAAGCTGTG	CTCACATCCTCCTCCGAGTC	60
<i>npv</i>	GenBank	NM_001124266	CTCGTCTGGACCTTTATATGC	GTTTCATCATCTGGACTGTG	58
<i>pepck</i>	GenBank	AF246149	GTTGGTGCTAAAGGGCACAC	CCCGTCTTCTGATAAGTCCAA	59
<i>pk</i>	GenBank	AF246146	CCATCGTCGCGTAACAAGA	ACATAGGAAAGGCCAGGGGC	59
<i>pomca1</i>	Tigr	TC86162	CTCGCTGTCAAGACCTCAACTCT	GAGTTGGGTTGGAGATGGACCTC	60
<i>ppara</i>	GenBank	AY494835	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	55
<i>ppary</i>	DFCI	CA345564	GACGCGGGTCACTACTTTA	ATGCTCTTGGCGAACTCTGT	60
<i>sglt1</i>	GenBank	AY210436	GGGCTGAACATCTACCTTGCT	CTCATAACCTCCCACCTCATTG	59
<i>sreb1c</i>	GenBank	CA048941.1	GACAAGGTGGTCCAGTTGCT	CACACGTTAGTCCGCATCAC	60
<i>ucp2a</i>	GenBank	DQ295324	TCCGGCTACAGATCCAGG	CTCTCCACAGACCACGCA	57

ACLY, ATP-citrate lyase; AgRP, agouti-related peptide; CART, cocaine- and amphetamine-related transcript; CPT-1, carnitine palmitoyl transferase type 1; CS, citrate synthase; FAS, fatty acid synthetase; FAT/CD36, fatty acid translocase; G6Pase, glucose 6-phosphatase; GCK, glucokinase; GLUT2, glucose facilitative transporter 2; GNAT3, guanine nucleotide-binding protein G(t) subunit alpha transducing 3; GSase, glycogen synthase; Kir6.x-like, inward-rectifier K channel pore type 6.x-like; LPL, Lipoprotein lipase; MCD, malonyl-CoA decarboxylase; NPY, neuropeptide Y; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate-kinase; POMC-A1, pro-opiomelanocortin A1; PPARα, peroxisome proliferator activated receptor type α; PPARγ, peroxisome proliferator activated receptor type γ; SGLT1, sodium-glucose linked transporter 1; SREBP1c, sterol regulatory element-binding protein type 1c; UCP2a, mitochondrial uncoupling protein 2a.

observed between saline and nesfatin-1-injected fish at 2 h post-injection (**Figure 1**).

Nesfatin-1 Upregulates Anorexigenic Neuropeptides in the Rainbow Trout Brain

ICV administration of nesfatin-1 resulted in a significant increase in the mRNA expression of *pomca1* in both the hypothalamus (1.5-fold) and hindbrain (2.3-fold), and of *cart* in the hypothalamus (3.3-fold), at 2 h post-injection (**Figures 2C,D**). Treatment with the hormone did not significantly alter the mRNA abundance of *npv* or *agrp* in neither the hypothalamus nor the hindbrain (**Figures 2A,B**), and of *leptin* in the liver (**Figure 2E**).

Genes Involved in Glucosensing Are Affected by Nesfatin-1 in Rainbow Trout

Changes in mRNA abundance of parameters related to glucosensing systems after nesfatin-1 treatment in the

hypothalamus, hindbrain and liver of rainbow trout are shown in **Figure 3**. Nesfatin-1 was observed to produce a significant increase in the mRNA expression of *sglt1*, *g6pase*, and *gsase* in the hypothalamus and hindbrain at 2 h post-injection (**Figures 3B,E,G**). Additionally, gene expression of *gnat3* was downregulated in the hindbrain, but not in the hypothalamus, after ICV treatment with the hormone (**Figure 3H**). In the liver, nesfatin-1 was found to significantly decrease the mRNA levels of *glut2*, *sglt1*, *gck*, and *g6pase* (**Figures 3A–C,E**), with no changes in the remaining parameters analyzed.

Nesfatin-1 Modulates the Expression of Genes Involved in Lipid Metabolism in Rainbow Trout

Figure 4 shows the effects of nesfatin-1 on the mRNA expression of parameters related to fatty acid sensing, lipid metabolism and mitochondrial activity in the hypothalamus, hindbrain and liver of rainbow trout. Among the tissues studied, the most affected

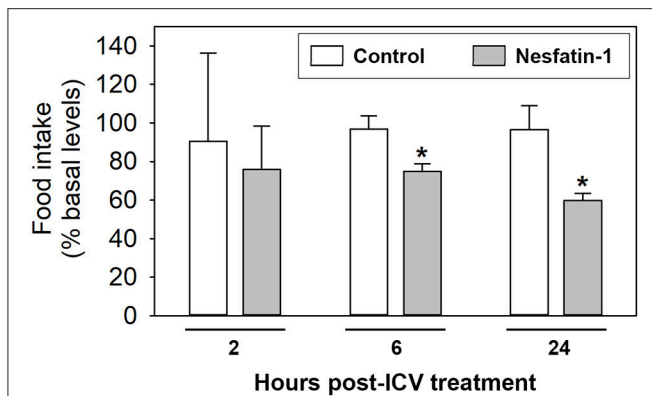


FIGURE 1 | Food intake in rainbow trout 2, 6, and 24 h after intracerebroventricular administration of 1 ml/100 g body mass of saline alone (Control) or containing 25 ng/g of goldfish nesfatin-1 (Nesfatin-1). Levels of food intake are represented as mean + SEM of the percentage of food ingested with respect to baseline levels (calculated as the average of food intake the 3 days previous to experiment). Results correspond to the mean + SEM of the results obtained in three different experiments. Asterisks denote significant differences between control and treated groups assessed by *t*-test ($p < 0.05$).

by ICV administration of nesfatin-1 was the hindbrain, where a significant increase in the gene expression of *fat/cd36*, *acly*, *fas*, *lpl*, *pparα*, and *pparγ* was detected at 2 h (Figures 4A,B,D,G–I). Nesfatin-1 treatment also led to a significant decrease in *fat/cd36* mRNAs and a significant increase in *mcd* and *fas* expression in the hypothalamus (Figures 4A,C). Finally, hepatic expression of *mcd*, *cpt-1b*, *lpl*, and *ucp2a* mRNAs were upregulated by nesfatin-1, while a significant downregulation of hepatic levels of *pparα*, *srebp1c*, and *kir6.x-like* mRNAs was observed (Figures 4C,E,G,H,J–L). Expression of *cs* did not result altered after nesfatin-1 ICV treatment in any of the tissues studied (Figure 4F).

Nesfatin-1 Affects Intracellular Integrative Pathways and Transcription Factors

The effects of ICV administration of nesfatin-1 on the phosphorylation status of key intracellular integrative systems and transcription factors known to be involved in nutrient sensing mechanisms are shown in Figure 5. Nesfatin-1 was found to significantly increase the phosphorylation status of mTOR in the hypothalamus and hindbrain of rainbow trout (Figure 5A). The phosphorylation status of AMPKα and CREB was observed to be downregulated by nesfatin-1 in the hindbrain, while a significant upregulation of the phosphorylation status of AMPKα was detected in hypothalamus (Figures 5B,D). ICV administration of nesfatin-1 did not cause any significant change in the phosphorylation status of FOXO1 in any of the tissues studied (Figure 5C).

DISCUSSION

The present study evaluates the effects of ICV administered nesfatin-1 on food intake, and on the expression of key genes

involved in nutrient sensing mechanisms in the brain and liver of rainbow trout.

Nesfatin-1 Is Anorectic in Rainbow Trout

Our food intake experiment demonstrated that nesfatin-1 ICV treatment leads to a significant reduction in food intake levels in rainbow trout 6 and 24 h post-injection, in agreement with previous observations in other fish species [goldfish, (35, 36)], birds [chicks, (59)] and mammals [mice, (32, 60); rats, (26, 61)]. The upregulation of *pomca1* and *cart* mRNA expression in the trout brain, but the lack of effects on *npv* and *agrp* levels, suggests that the observed anorexigenic effect of nesfatin-1 in rainbow trout is mediated by the enhancement of potent brain appetite-inhibitors rather than by the suppression of central signals stimulating appetite. In mammals, nesfatin-1 inhibits NPY neurons causing their hyperpolarization (62), but has no effect on the mRNA expression of *Pomc* and *Cart* (32), in the hypothalamic arcuate nucleus. This might relate to the relative lower degree of co-localization between nesfatin-1 and NPY compared with that of nesfatin-1 and POMC (63, 64). On the other hand, a stimulatory effect of nesfatin-1 injection on the levels of mRNAs encoding *Pomc* and *Cart* has been observed in the mice hindbrain, specifically in the brainstem nucleus tractus solitarius (NTS) (32). Together, these observations suggest that the mechanisms underlying nesfatin-1 anorexigenic effects are similar in the hindbrain/NTS of fish and mammals, but not in the hypothalamus, where nesfatin-1 seems to modulate different neuronal populations, i.e., NPY neurons in mammals and POMC/CART neurons in fish. In favor of this hypothesis is the observed lack of changes in the brain mRNA expression of *kir6.x-like* in rainbow trout, considering that K_{ATP} channels are thought to mediate nesfatin-1-induced hyperpolarization of NPY neurons in the mammalian hypothalamus (62). Finally, we observed that nesfatin-1 anorexigenic action in rainbow trout seems to be independent of leptin signaling, since mRNA abundance of *leptin* in liver [the main site of synthesis in fish; (4)] did not change after nesfatin-1 treatment, in agreement with what is known in mammals (32, 65).

Nesfatin-1 Stimulates the Glucosensing Capacity in Hindbrain and Hypothalamus

Major findings of this study demonstrated that nesfatin-1 modulates glucosensing in two brain regions (hypothalamus and hindbrain) known to be important in the regulation of glucose metabolism and homeostasis in fish (5, 66), and where nesfatin-1 binding sites have been previously described in mammals (31). Our results show that nesfatin-1 mainly activates glucosensing mechanisms in the rainbow trout brain, as demonstrated by the enhanced expression of *sglt1*, *g6pase*, and *gsase*, and the reduced expression of *gnat3* (only in hindbrain), results in general agreement with those observed in the same regions of rainbow trout subjected to glucose challenges (1, 3). This appears to be the first time in any vertebrates in which nesfatin-1 has been reported to modulate parameters involved in central glucosensing. In mammals, available studies addressed that nesfatin-1 treatment inhibits the firing rate of glucose-inhibited neurons and excites glucose-excitable neurons in the hindbrain

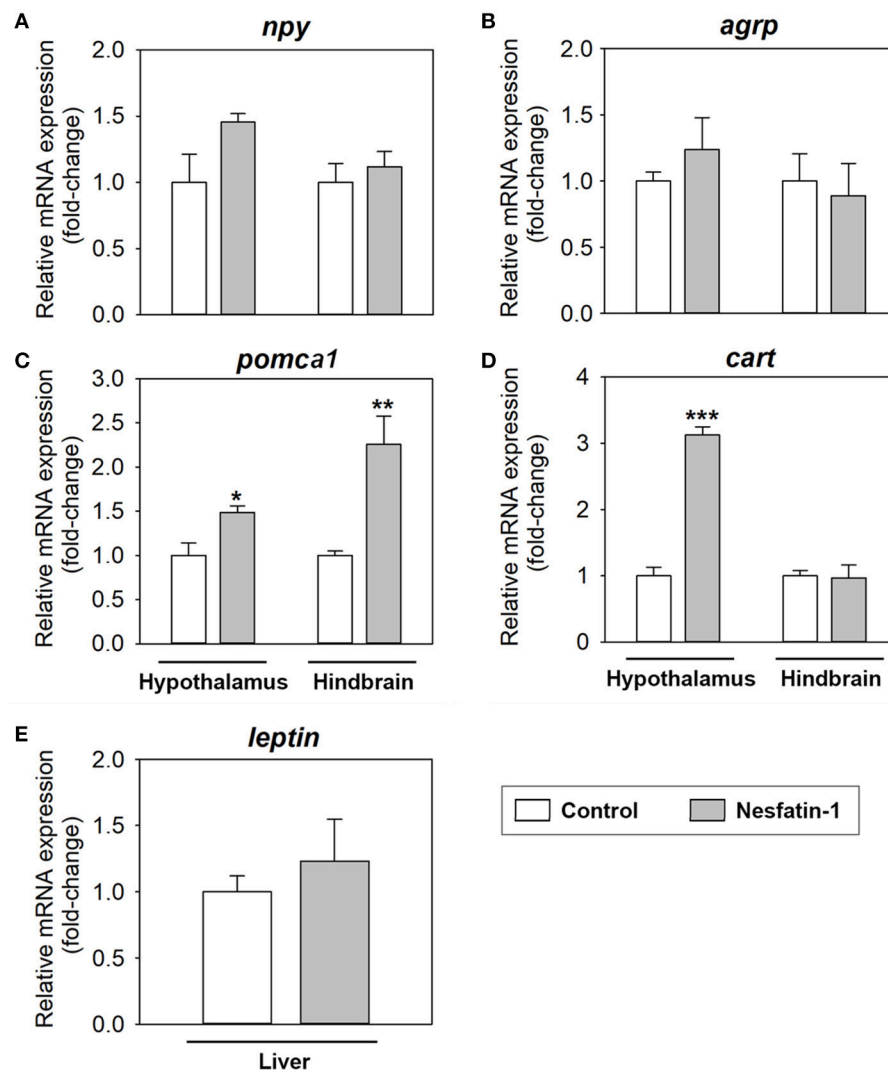


FIGURE 2 | Effects of central administration of nesfatin-1 on the mRNA abundance of key appetite-regulating neuropeptides in the rainbow trout hypothalamus and hindbrain (A–D), and of leptin in the rainbow trout liver (E), at 2 h post-injection. Data obtained by RT-qPCR are shown as mean + SEM ($n = 6$ fish). Gene expression results are referred to control group and are normalized by β -actin expression. Asterisks denote significant differences between control and treated groups assessed by t -test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

(48) and hypothalamus (34, 47), both areas where the presence of nesfatin-1 binding sites have been characterized (67). Our results in rainbow trout demonstrated that the activation of the glucosensing capacity by nesfatin-1 was more marked in the hindbrain compared to the hypothalamus. Glucosensing capacity of the hindbrain is related to its indirect role in the hormonal regulation of food intake (via efferent pathways to the hypothalamus), and especially with its role in the homeostatic regulation of this process (68, 69). Thus, peripheral hormones responsive to food consumption and digestion modulate hunger and satiety mainly through hindbrain circuits (70), therefore supporting the here observed more remarkable response of the hindbrain glucosensing systems after the treatment with nesfatin-1. The less marked nesfatin-1-induced activation of the glucosensing capacity in the hypothalamus could relate

to the reduced number of nesfatin-1 binding sites present in that area (47). Additionally, nesfatin-1 has been reported to increase insulin sensitivity in the rat brain through an increase in insulin receptor/insulin receptor substrate-1/AMPK/Akt/target of rapamycin complex (TORC) 2 phosphorylation (71). Results obtained here are in agreement with this action of nesfatin-1, which ultimately leads to an increase in glucose uptake and metabolism, and therefore a decrease in glucose circulating levels.

Nesfatin-1 Is Not a Modulator of Fatty Acid Sensing

Present results indicate that nesfatin-1 is not a modulator of fatty acid sensing in the brain of the rainbow trout, as the mRNA expression of the parameters involved in fatty acid sensing analyzed did not respond to nesfatin-1 ICV treatment

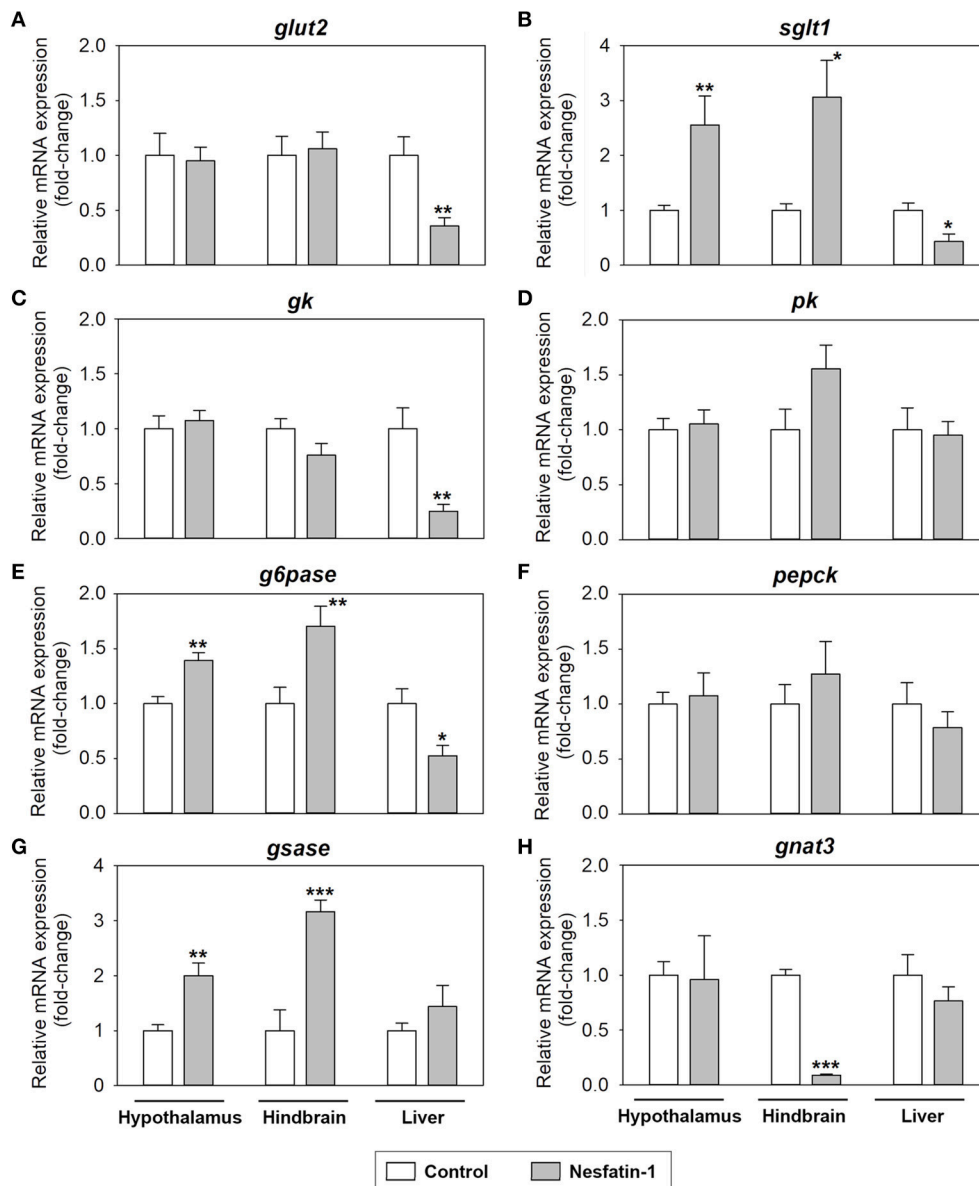


FIGURE 3 | Effects of central administration of nesfatin-1 on the mRNA abundance of parameters related to glucosensing and glucose metabolism in the rainbow trout hypothalamus, hindbrain and liver at 2 h post-injection. (A–H) Data obtained by RT-qPCR are shown as mean + SEM ($n = 6$ fish). Gene expression results are referred to control group and are normalized by β -actin expression. Asterisks denote significant differences between control and treated groups assessed by t -test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

according to the anorexigenic nature of the peptide (1, 3). Thus, given that nesfatin-1 would be physiologically released when enough supply of lipids (and other energy sources) is in the body (67), it would make sense that central fatty acid sensing systems respond with an increase in the rate of entry of fatty acids into the cells and a decrease in the fatty acid synthesis pathways (1, 3). This hypothesis does not go hand in hand with the reduced expression of the fatty acid translocase *fat/cd36* in the hypothalamus and the enhanced expression of the lipogenic enzymes *fas* and *acly*, the enzyme involved in lipid release *lpl*

and the lipogenesis-related transcription factor *ppara* in the hypothalamus and/or the hindbrain after nesfatin-1 treatment. Instead, these results suggest that nesfatin-1 treatment produces an increase in the amount of fatty acids in the brain cells (as suggested by the increased expression of *fat/cd36* and *lpl*) and in the lipogenic activity (increased expression of *fas*, *acly*, and *ppara*), particularly in the hindbrain, which as indicated above is in agreement with the role of this central area in the homeostatic control of food intake and energy balance. This nesfatin-1-induced promotion of lipid anabolism rather

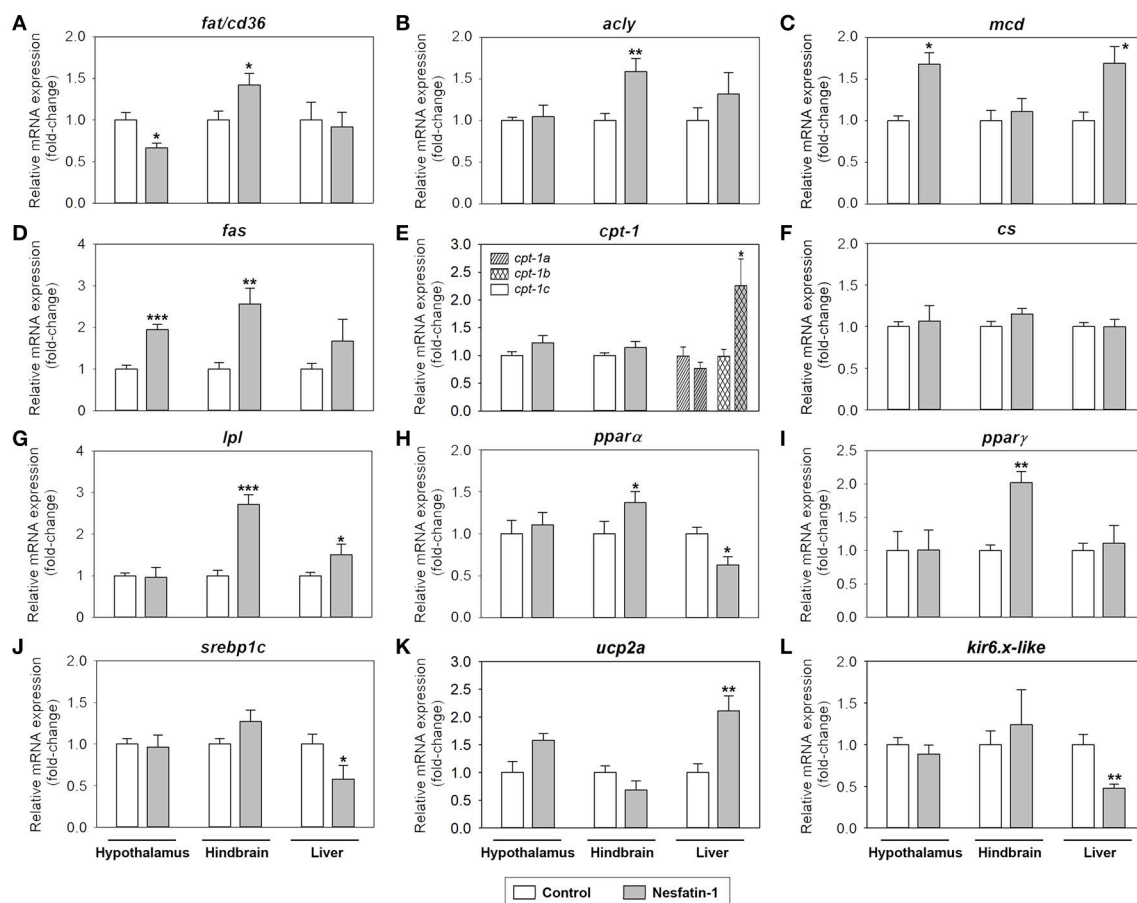


FIGURE 4 | Effects of central administration of nesfatin-1 on the mRNA abundance of parameters related to fatty acid sensing and lipid metabolism (A–J) and mitochondrial activity (K,L) in the rainbow trout hypothalamus, hindbrain and liver at 2 h post-injection. Data obtained by RT-qPCR are shown as mean + SEM ($n = 6$ fish). Gene expression results are referred to control group and are normalized by β -actin expression. Asterisks denote significant differences between control and treated groups assessed by t -test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

than lipid catabolism might relate to an increase in the storage of lipids, a more important energy source for the fish brain compared to glucose (72). However, while a higher amount of fatty acids appears to be in the brain cells in response to nesfatin-1, the lack of significant changes in the mRNA expression of *cpt-1c* (in charge of transporting fatty acids into the mitochondria) and *ucp2a* (related to mitochondrial activity) points to the assumption that such an increase in the levels of fatty acids is not related to their β -oxidation in order to produce energy. This hypothesis seems to be of higher importance in the hindbrain, as it is also supported by the lack of changes in the mRNA levels of *mcd* (promotes the entrance of fatty acids into the mitochondria). This enzyme is however upregulated by nesfatin-1 in the hypothalamus, apparently supporting β -oxidation. It must be taken into consideration that only mRNA abundance was analyzed in the present study, and thus it cannot be discarded that nesfatin-1 is indeed promoting β -oxidation by modulating protein levels and/or enzymatic activity. It can be also hypothesized that nesfatin-1 is instead promoting the accumulation of fatty acids in the neurons to promote neuronal

growth and/or to maintain cell membranes. This hypothesis is supported by the observed increase in the mRNA levels of *ppar γ* , reported to be involved in neuronal development and improvement of neuronal health (73). The present results provide clear evidence to implicate nesfatin-1 in lipid metabolism in the brain, and future studies are needed to further investigate this proposed role.

Administration of Nesfatin-1 into the Brain Elicits Changes in Liver Energy Metabolism

This study demonstrated that nesfatin-1 ICV treatment resulted in a decrease in the capacity of liver to use glucose, as revealed by the reduced expression of *glut2*, *splt1*, *gck*, and *g6pase* compared to the control group. These results suggest that nesfatin-1 may act at central level to elicit changes in the hepatic metabolism (via the autonomic nervous system). In other terms, nesfatin-1 may regulate the function of peripheral organs through the modulation of neural activity in order to maintain homeostasis. In the context of this hypothesis, the signal that the liver

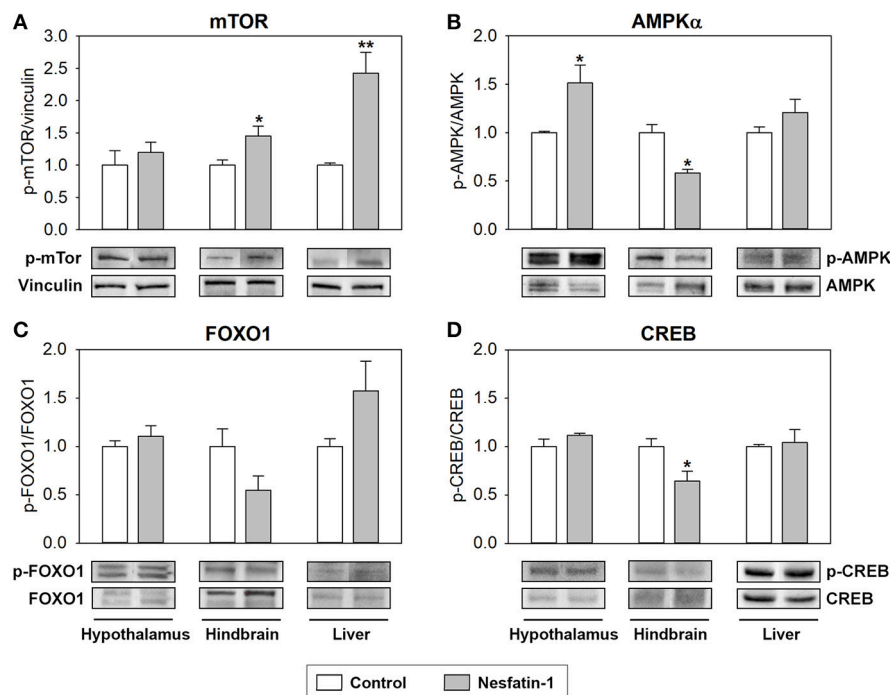


FIGURE 5 | Effects of central administration of nesfatin-1 on the protein phosphorylation status of key intracellular integrative systems and transcription factors in the rainbow trout hypothalamus, hindbrain and liver at 2 h post-injection. **(A–D)** Western blots were performed on three individual samples per treatment, and a representative blot per treatment is shown here. Graphs represent the ratio between the phosphorylated protein and the total amount of the target protein, except for mTOR in which we used vinculin as reference. Asterisks denote significant differences between control and treated groups assessed by *t*-test (**p* < 0.05, ***p* < 0.01).

might be receiving from the central circuitries after nesfatin-1 administration is that the levels of food intake are reduced and therefore it is not necessary to produce and/or metabolize glucose. Previous studies in mammals have shown that ICV administration of nesfatin-1 leads to a decrease in the hepatic mRNA and protein expression and enzymatic activity of PEPCK (gluconeogenic enzyme), while does not modify the expression or activity of GCK and G6Pase (71). Similarly, hypothalamic nesfatin-1/NUCB2 knockdown rats have been shown to have increased hepatic levels of G6Pase and PEPCK (74). These observations demonstrate that central action of nesfatin-1 results in the inhibition of hepatic gluconeogenesis in mammals. Results obtained in the present study suggest that the response model seems to be slightly different in the rainbow trout, where the gluconeogenic potential seems not to be affected by ICV treatment with nesfatin-1 (no changes in *pepck* mRNAs) but instead it appears to be an inhibition in the hepatic use of glucose (decreased expression of *glut2*, *sglt1*, *gck*, and *g6pase*). These differences could relate to the differences between the mammalian and fish systems in terms of glucose metabolism, and the relative intolerance to glucose in carnivorous fish such as the rainbow trout (5, 75).

Regarding fatty acids, the presence of nesfatin-1 in the brain appears to attenuate the lipogenic activity and to stimulate fatty acid oxidation in the rainbow trout liver, as suggested by increased mRNA levels of *cpt-1b*, *lpl*, *mcd* and the mitochondrial activity-related gene *ucp2a*, and a decrease in the expression of

the lipogenesis-related transcriptional factors *pparα* and *srebp1c*, after nesfatin-1 treatment. This is comparable to the direct effects of nesfatin-1 treatment in the mammalian liver (40), but, more interestingly, is again suggesting that afferent signals coming from the brain are informing the liver of the anorectic signal detected, which is translated into an inhibition of fuel production, in this case lipids, for use in peripheral tissues.

Nesfatin-1 in the Brain Elicits Changes in the Phosphorylation Status of Proteins Involved in Intracellular Signaling and Transcription Factors

Changes in these parameters were most abundant in the hindbrain, where glucosensing systems and parameters involved in lipid metabolism were most affected by nesfatin-1. In this brain area, nesfatin-1 treatment resulted in an increase in the phosphorylation ratio of mTOR, while a decrease occurred in the phosphorylation ratio of AMPKα and CREB. These changes are not consistent with the few studies available in mammals showing that nesfatin-1 treatment upregulates CREB in a neural cell line (76) and reduces the phosphorylation of mTOR in the dorsal motor nucleus of the vagus (77). However, they are similar to those observed when levels of glucose and/or fatty acids increase in fish (16, 78), a scenario that would match the expected effects for an anorectic signal such as nesfatin-1. In the hypothalamus,

only phosphorylation status of AMPK α was affected by nesfatin-1 and in the opposite direction than in the hindbrain. It seems that the more attenuated response of the glucosensing system to nesfatin-1 in the hypothalamus compared to the hindbrain is not enough to trigger significant changes in intracellular signaling pathways and transcription factors in the hypothalamus. Finally, in the liver, ICV treatment with nesfatin-1 resulted in a significant increase in the phosphorylation status of mTOR, in accordance with previous observation in mammals also obtained after ICV treatment with nesfatin-1 (71). This activation of hepatic mTOR after nesfatin-1 ICV treatment can be related with triggering the less use of fuels in this tissue, as discussed above.

In summary, this study demonstrates an anorexigenic action for nesfatin-1 in rainbow trout, likely mediated by the activation of central anorexigens (i.e., POMC and CART), and shows for the first time a role for this peptide in the modulation of glucosensing mechanisms and lipid metabolism in central locations of this fish species. Overall, results obtained in the brain (hypothalamus and hindbrain) point out that nesfatin-1 might stimulate glucosensing systems. Nesfatin-1 also appears to increase the levels of fatty acids in the brain cells, and we hypothesized that this could relate to promoting neural development and/or helping in maintaining cell membranes. The more marked effects of nesfatin-1 in the hindbrain might relate to a higher presence of nesfatin-1-expressing neurons and nesfatin-1 binding sites in this area in mammals (48). The presence of nesfatin-1 in the brain also seems to produce a decrease in the use of glucose, and to attenuate the lipogenic activity and stimulate fatty acid oxidation in the liver, thus apparently modifying energy

expenditure in agreement with the anorectic nature of nesfatin-1. Together, this study shows for the first time that nesfatin-1 has an important role in the homeostatic regulation of food intake and is likely involved in energy expenditure in fish. Further studies are required to delve into the knowledge of the proposed novel role for nesfatin-1 in lipid metabolism in the fish brain.

AUTHOR CONTRIBUTIONS

AB conducted experiments, analyzed data, prepared manuscript. CV helped in conducting experiments. JB helped in conducting experiments. JS contributed to experimental design, assisted with data analysis and edited manuscript for submission. SU contributed to experimental design, provided funding, assisted with data analysis and edited manuscript for submission.

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A *Rhodnius prolixus* Insulin Receptor and Its Conserved Intracellular Signaling Pathway and Regulation of Metabolism

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The insulin signaling pathway is a modulator of metabolism in insects and can regulate functions associated with growth and development, as well as lipid and carbohydrate balance. We have previously reported the presence of an insulin-like peptide and an insulin-like growth factor in *Rhodnius prolixus*, which are involved in the homeostasis of lipids and carbohydrates in post-feeding and non-feeding periods. In the present study, we have characterized the first insulin receptor (IR) to be discovered in *R. prolixus*, Rhopr-IR, and investigated its intracellular signaling cascade and its role in nutrient control. We identified a candidate protein sequence within *R. prolixus* putative peptidome and predicted its conserved features using bioinformatics. Tissue-specific expression analyses indicated that the Rhopr-IR transcript is differentially-expressed in all tissues tested, with the highest values observed in the central nervous system (CNS). Treatment of insects with the IR kinase activator BpV(phen), glucose, or porcine insulin resulted in the activation of protein phosphorylation in the fat body, and stimulated the phosphorylation of protein kinase Akt, an evolutionarily conserved key regulator of the intracellular insulin signaling cascade. We also observed activation of Akt and phosphorylation of its downstream targets glycogen synthase kinase 3 β (GSK3 β) and the transcription factor FOXO for several days following a blood meal. We used dsRNA to knockdown transcript expression and examined the resulting effects on metabolism and intracellular signaling. Furthermore, knockdown of the Rhopr-IR transcript increased lipid levels in the hemolymph, while reducing lipid content in the fat body. Interestingly, the levels of carbohydrates in the hemolymph and in the fat body did not show any alterations. The activation of Akt and phosphorylation of FOXO were also reduced in knockdown insects, while the phosphorylation pattern of GSK3 β did not change. Our results support the identification of the first IR in *R. prolixus* and suggest that Rhopr-IR signaling is involved in hemolymph nutrient homeostasis and fat body storage both in post-feeding and in non-feeding stages. These metabolic effects are likely regulated by the activation of Akt and downstream cascades similar to mammalian insulin signaling pathways.

Keywords: Rhopr-IR, Akt, Gsk3b, FOXO, lipid, insect, RNAi, Western blot

INTRODUCTION

The insulin signaling pathway is an evolutionarily conserved regulator of physiological functions related to metabolism and is well-known for balancing glucose uptake and storage in mammals. However, the pathway is found in all metazoans and has a broad pleiotropic nature, playing additional roles in reproduction, development, growth, and life span (1, 2). The signaling cascade is initiated by the activation of an insulin receptor (IR) upon binding of insulin, insulin-like peptides (ILPs), or insulin-like growth factors (IGFs). IRs are part of the receptor tyrosine kinase (RTK) family, which contain tyrosine kinase domains within their cytoplasmic portion. In humans, the insulin receptor is expressed in two isoforms, denoted IR isoform A (IR-A) and IR isoform B (IR-B), due to the alternative splicing of exon 11 found within the IR gene (3, 4). Whereas, the shorter IR-A is mainly expressed in cells of the central nervous system, hematopoietic cells, and several fetal tissues (4, 5), the longer IR-B isoform is primarily expressed in muscle, liver and adipose tissue (4). Although previous work suggests that human IR-A displays a higher binding affinity to insulin than IR-B (6), it has also been found that IR-A can bind with high affinity to insulin growth factor II (IGF II) as well (5). These isoforms also display differential signaling in response to insulin. Whereas, IR-A stimulation in mammalian pancreatic β cells leads to the increased transcription of the insulin gene through the involvement of the PI3K class Ia, p70 s6 and Ca^{2+} /calmodulin kinases, insulin signaling at IR-B results in the activation of PI3K class II, the downstream kinase Akt, and resulting transcription of the glucokinase (β GK) gene, resulting in the regulation of metabolic events (7).

Upon receptor activation, the intracellular kinase domains of both IR isoforms initiate the auto-phosphorylation of the IR, which recruits adaptor proteins termed insulin response substrates (IRS) that facilitate a cascade of tyrosine phosphorylation (8–10). Once activated, the IRS can trigger different downstream signaling pathways by interacting with either Grb2 (growth factor receptor bound protein-2) or PI3K (phosphatidylinositol-3 kinase). The activated Grb2 protein initiates the Ras-MAPK (mitogen-activated protein kinase) pathway by associating with the son-of-sevenless (SOS) protein leading to the recruitment of Ras and the activation of its GTPase function. The activated Ras protein then initiates the serine/threonine kinase activity of the MAPK pathway, leading to mitogenic responses within target cells (11). Stimulation of MAPK and its downstream response elements results in cell growth and division by activating the MEK/ERK complex via the Ras and Raf proteins (12). In contrast, PI3K activates the PI3K/PKB (protein kinase B) pathway (12, 13), leading to the production of PIP_3 (phosphatidyl-inositol triphosphate), which in turn promotes the activation of downstream kinases such as Akt 2 (PKB) and PKC (protein kinase C) (13). Activated Akt phosphorylates different downstream targets such as GSK3 β (glycogen synthase kinase 3 β), as well as select isoforms of the FOXO (Forkhead box) transcription factor family that control cell cycle arrest, apoptosis, and nutrient metabolism, among other functions (14). Phosphorylation of GSK3 β stimulates the

storage of glucose by facilitating the formation of glycogen (15), while the phosphorylation of FOXO relieves cell cycle arrest, inhibits apoptosis, and promotes cell growth (16).

In vertebrates, there are three known receptors in the IR subfamily: the insulin receptor, which binds to insulin; the type 1 IGF receptor, which binds to IGF I and II; and the orphan IR-related receptor (17). In contrast, only one or two IRs have been identified in most invertebrate species investigated, including insects. Single IRs were described in *Drosophila melanogaster* (18), in the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti* (19, 20), and in the dung beetle *Onthophagus nigriventris* (21). The presence of two IRs was reported in the honey bee *Apis mellifera* (22), in the fire ant *Solenopsis invicta* (23), in the brown plant hopper *Nilaparvata lugens* (24), in the beetle *Tribolium castaneum* (25), and in the citrus aphid *Aphis citricidus* (26). Unlike IRs, the number and sequences of ILPs and IGFs can vary among insect species, ranging from one single insulin-related peptide in *Locusta migratoria* (27), to eight ILPs identified in *D. melanogaster* (DILPs) (18), to more than 30 ILP genes discovered in *Bombyx mori* (28). Much like in other animals, insulin signaling controls a variety of processes in insects, including lipid and carbohydrate homeostasis (29–31), wing size (26), caste differentiation (22, 23), fecundity (20), and reproduction (25).

We have previously reported the presence of one ILP and one IGF in the blood-gorging insect *R. prolixus*. We found that the ILP, namely Rhopr-ILP, is only produced in neurosecretory cells in the brain and is involved in lipid and carbohydrate homeostasis during post-feeding and non-feeding periods (32). In contrast, Rhopr-IGF is expressed in a variety of tissues, with the highest transcript levels found in the fat body. We found that Rhopr-IGF also contributes to hemolymph nutrient balance in addition to regulating wing and body size (33). Here, we identify and characterize an IR in *R. prolixus*. The Rhopr-IR transcript is expressed in all tissues investigated, with highest expression levels identified in the central nervous system (CNS). We discovered that receptor activation is responsive to insulin stimulation and initiates the activity of Akt, resulting in the coordinated phosphorylation of downstream targets GSK3 β and FOXO. Finally, knockdown of Rhopr-IR transcript renders an imbalance in hemolymph and fat body lipid homeostasis.

MATERIALS AND METHODS

Identification of a Candidate Insulin Receptor Protein Sequence

The predicted peptidome of *R. prolixus* (available at rprolixus.vectorbase.org) was scanned using different insulin receptor protein sequences (*Homo sapiens* XP_011526290.2, *D. melanogaster* AAC47458.1, *Onthophagus nigriventris* AFQ20827.1) using the program Geneious 8.1.7 (34). The correspondent candidate mRNA sequence was identified by BLASTing the putative protein sequence against *R. prolixus* transcriptome (available at rprolixus.vectorbase.org) on Geneious 8.1.7. The conserved features and domains of the Rhopr-IR were identified using the online service InterPro: protein sequence analysis and classification (www.ebi.ac.uk/

interpro). The tyrosine kinase active site and signature were predicted using the online database Prosite (<https://prosite.expasy.org/>). The transmembrane region was confirmed using the TMHMM Server v. 2.0 (www.cbs.dtu.dk/services/TMHMM/) and Phobius predictor (phobius.sbc.su.se), which was also used for predicting the signal peptide. Glycosylation and phosphorylation sites were predicted using the NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc/) and the NetPhos 3.1 Server (www.cbs.dtu.dk/services/NetPhos/), respectively.

Phylogenetic Analysis of Insulin Receptor Protein Sequences

The predicted insulin receptor protein sequence from *R. prolixus* was aligned with 57 other sequences from 54 different vertebrate and invertebrate species using the program MUSCLE 3.8 (Multiple Sequence Comparison by Log-Expectation—www.ebi.ac.uk/Tools/msa/muscle).

The evolutionary history of insulin receptors was inferred by analyzing the tyrosine kinase domain within the alignment described above using the maximum likelihood method (35) on the program Molecular Evolutionary Genetics Analysis version 7.0 [MEGA7, (36)]. The bootstrap consensus tree was inferred from 500 replicates and branches corresponding to partitions reproduced in <50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The distances were estimated using a JTT model with a discrete Gamma distribution, 4 categories (+G, parameter = 0.4310 for the tyrosine kinase tree, parameter = 1.0151 for the extracellular domains tree).

Insects

Fifth instar *R. prolixus* were used throughout the study. The colony was kept at 25°C, under 50% humidity, and insects were fed on defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada) once per instar, or as indicated.

Analysis of Rhopr-IR Relative Transcript Expression in 5th Instar *R. prolixus*

The relative expression of Rhopr-IR was quantified using real-time quantitative PCR (qPCR) in 10 different tissues from unfed 5th instars. RNA was extracted using a Total RNA mini kit (BioBasic, Markham, ON, Canada), followed by cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied-Biosystems, Fisher Scientific, Toronto, ON, Canada). Primers for the amplification of Rhopr-IR (Supplementary Table 1) were designed to amplify fragments of similar size across all experimental and reference genes (β -actin, α -tubulin, rp49) (37, 38). All the qPCR reactions were performed using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada). Relative expression was calculated using the $\Delta\Delta C_t$ method (39).

Alternatively, semi-quantitative reverse transcriptase-PCR (RT-PCR) was performed to confirm the presence of the transcript in tissues with low relative expression. The same Rhopr-IR primers used for real-time PCR (Supplementary Table 1) were used and actin was amplified as a

transcript reference. RNA from unfed 5th instars was extracted, as mentioned above. The result of the RT-PCR was visualized on 1.2% agarose gels.

Fat Body Collection

Fat bodies were collected from both unfed and recently fed 5th instars to investigate the signaling of the Rhopr-IR pathway under endogenous or exogenous stimulation. For unfed insects, the ventral and dorsal fat bodies were removed 4–5 weeks after feeding as 4th instars (5 pairs combined per trial) under *R. prolixus* glucose-free physiological saline (NaCl 150 mM, KCl 8.6 mM, CaCl₂ 2.0 mM, MgCl₂ 8.5 mM, NaHCO₃ 4.0 mM, HEPES 5.0 mM, pH 7.0). For post-feeding signaling analysis, ventral and dorsal fat bodies were removed 1 day before feeding, and 2–4 h and every day for 5 days after feeding (5 pairs combined per time point per trial). Once removed from the insects, tissues were immediately stored in cold phosphate-buffered saline (PBS) and frozen at –20°C until sample preparation for Western blotting (see below).

For unfed insects treated with BpV(phen) (Millipore-Sigma, Milwaukee, WI, USA) or porcine insulin (Millipore-Sigma, Oakville, ON, Canada), the ventral and dorsal fat bodies (5 pairs combined per trial) were removed under *R. prolixus* glucose-free saline 30 min post-injection with either 1 μ L of *R. prolixus* saline (with or without 34.0 mM glucose), BpV(phen) (10^{-3} M), or porcine insulin (0.1 or 1 μ g). Tissues were stored immediately in cold PBS and frozen at –20°C until lysed and used for Western blotting.

Tissue Lysis and Protein Quantification

Fat body tissues collected from unfed and recently fed insects were thawed from storage at –20°C (in PBS) and were immediately submerged in cold, freshly-made lysis buffer (aprotinin, 0.31 nM; leupeptin, 21 nM; pepstatin A, 1.5 nM; phenylmethylsulfonyl fluoride, 1 mM, EDTA, 5 mM; EGTA, 1 mM; sodium fluoride, 10 mM; sodium orthovanadate, 1 mM; in RIPA buffer [150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 in double-distilled or MilliQ water]). For post-feeding analysis of fat bodies, tissues were collected 1 day before feeding, and 2–4 h and every day for 5 days post-feeding and were stored –20°C (in PBS) until use. After thawing, tissues were first weighed collectively (per trial per day), followed by incubation in cold, freshly-made lysis buffer. Once in lysis buffer, samples were sonicated 5 $\times$ for 3 s each, followed by a 2-h incubation at 4°C with constant agitation. Samples were then centrifuged for 25 min at 4°C and 17,000 g. The resulting infranant was collected and used for Western blotting. Protein quantification was done on all lysed tissue samples prior to gel electrophoresis using the BCA protein quantification assay (Pierce™ BCA Protein Assay Kit, ThermoFischer Mississauga, ON, Canada).

Gel Electrophoresis and Western Blotting

After protein quantification, lysed fat body samples from unfed and recently fed insects were subjected to gel electrophoresis.

Protein bands were separated under reducing conditions on hand-cast (TGX Stain-Free™ FastCast™ acrylamide solution, Bio-Rad, Mississauga, ON, Canada; prepared according to protocol specifications) or pre-made (Mini-PROTEAN® Stain-Free™ gels, Bio-Rad) 12% stain-free SDS-polyacrylamide gels and loaded in equal amounts across all wells (amounts loaded specified per experiment). All gels were run for 60–90 min at a constant voltage (120 V) in Tris/Glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3; Bio-Rad). Proteins were then transferred to a low-fluorescence PVDF (LF-PVDF) membrane in Transfer buffer over 3 min, using a Trans-Blot® Turbo™ Transfer System (all reagents/materials: Bio-Rad). Membranes were then blocked overnight in PBS-T (1xPBS containing 0.1% Tween-20) and 5% bovine serum albumin (BSA). Blots were incubated overnight in primary antibody (1:1000 dilution in PBS-T with 3% BSA) at 4°C, against the following antigens: anti-Akt [Akt (pan) (C67E7) rabbit monoclonal antibody, Cell Signaling Technology, Beverly, MA, USA]; anti-pAkt (phospho-Akt (Ser473) (D9E) XP® rabbit monoclonal antibody, Cell Signaling Technology); anti-GSK3β (phospho-GSK-3Beta (Ser9) (D85E12) XP® rabbit monoclonal antibody, Cell Signaling Technology); anti-FOXO (phospho-FOXO (Ser256) rabbit polyclonal antibody, Cell Signaling Technology); anti-actin (rabbit polyclonal antibody, Millipore-Sigma, Oakville, ON, Canada); or anti-tubulin (mouse monoclonal antibody, Life Technologies, Burlington, ON, Canada). After incubation, primary antibodies were washed-off with PBS-T followed by incubation in secondary antibody (1:5000, horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies, Cell Signaling Technology) for 1–2 h at room temperature with constant agitation. Blots were then washed with PBS-T and visualized using enhanced chemiluminescence (Clarity™ Western ECL Substrate, Bio-Rad). Blots were imaged on a ChemiDoc XRS system and analyzed using Image Lab 5.0 (Bio-Rad software and systems) using the “intense band” automatic exposure setting in the “high resolution” and/or “high sensitivity” default blot protocol options, in order to optimize the exposure time for intense bands and prevent overexposure of the blot/minimize detection of background noise. For the complete blots corresponding to those used in subsequent figures please see **Supplementary Data**.

Double-Stranded RNA Design and Synthesis

A 474-base pair template, designed in the 5′ region of the open reading frame of Rhopr-IR transcript, was used to synthesize a double stranded RNA molecule (dsIR) using the T7 Ribomax Express RNAi System (Promega, Madison, WI, USA), according to the manufacturer protocol. Gene specific primers (GSP) were combined with GSP containing the T7 RNA polymerase promoter sequence (**Supplementary Table 1**). As an experimental control, a dsRNA molecule based on the Ampicillin Resistance Gene (dsARG) from the pGEM-T Easy Vector system (Promega, Madison, WI, USA) was used throughout the study (38, 40).

Knockdown of Rhopr-IR Transcript Expression Using Double Stranded RNA

To knockdown the expression of Rhopr-IR in unfed 5th instar *R. prolixus*, 1 µg of dsARG or dsIR in 1 µL of ultrapure water was injected into the insect hemocoel using a Hamilton micro syringe (Hamilton Company, Reno, NV, USA). Insects were dissected at 3 and 10 days post-injection and Rhopr-IR transcript expression was measured using quantitative PCR. The knockdown percentage in dsIR-injected insects was calculated relative to the expression of the transcript in dsARG-injected insects. In another experiment, at 3 days after injection, insects were fed on defibrinated rabbit blood, separated in groups of 40 individuals of dsARG or dsIR- injected insects. Each group was allowed to feed for 25 min.

Hemolymph Collection From Rhopr-IR Knockdown Insects

For unfed insects, hemolymph was collected at 7 days post-dsRNA injection, and for the recently fed insects, hemolymph was collected at 4 days post-feeding (7 days post-injection). Hemolymph samples were obtained upon immobilizing insects, after which 5 µL were collected from the cut end of a leg of each insect using a micro-pipette. Samples were immediately placed in 50 µL of 10% trichloroacetic acid (TCA) and centrifuged for 5 min at 20°C, 8000 g. Supernatants were then transferred to new microcentrifuge tubes and subsequently used for carbohydrate level measurements. Pellets containing lipids associated with lipoproteins were resuspended in 200 µL isopropanol and used for lipid level measurements.

Fat Body Collection of DSRNA-Injected Insects

For unfed insects, fat bodies were collected at 7 days post-dsRNA injection, and for recently fed insects, fat bodies were collected at 4 days post-feeding (7 days post-injection). The ventral fat body sheet covering the abdominal segments was removed under *R. prolixus* physiological saline (containing 34.0 mM glucose) and placed in 200 µL isopropanol or 200 µL 10% TCA. Samples were sonicated for 5 s each and centrifuged for 10 min at 4°C, 8000 g. Following centrifugation, 20 µL of each isopropanol supernatant were transferred to new tubes containing 180 µL isopropanol for total fat body lipid content measurements, while 50 µL of each TCA supernatant were transferred to new tubes for measuring total fat body carbohydrate content.

To evaluate the effects of Rhopr-IR knockdown on fat body growth and IR signaling, both unfed and recently fed insects were injected with 1 µg of dsARG or dsIR in 1 µL of ultrapure water. For unfed insects, the ventral and dorsal fat bodies were removed (5 pairs per trial) at 4 days post-injection. In another experiment, insects injected with dsARG or dsIR were fed 4 days post-injection and ventral and dorsal fat bodies were collected between 2 and 4 h and every day for 5 days after feeding (5 pairs per time point per trial). Once removed, all tissues were immediately stored in PBS and frozen at −20°C until undergoing the same preparation for Western blotting, as described above.

Lipid and Carbohydrate Measurements From Hemolymph and Fat Body Samples

Lipid (41) and carbohydrate (32) measurements were carried out using Lipid and Anthrone-based assays, as previously described.

Statistical Analyses

Results are shown as means $\pm$ standard errors. The statistical significance of the data was calculated using either a Student's *t*-test or a one-way ANOVA followed by Dunnett's multiple comparisons test, where specified. Results were considered statistically different when $p < 0.05$. All analyses were carried out using the programs SigmaPlot (Systat Software, San Jose, California, USA) or GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

RESULTS

Analysis of Rhopr-IR Amino Acid Sequence and Prediction of Conserved Features and Domains

An insulin receptor candidate sequence was identified within the *R. prolixus* peptidome (RPRC006251-PA) along with its coding mRNA sequence (RPRC006251-RA), which was used for designing the primers for Rhopr-IR quantification using qPCR and for dsRNA production. The protein sequence was previously annotated on the UniprotKB database under the general classification of "Receptor Protein-Tyrosine Kinase" (T1HQC7_RHOPR). The Rhopr-IR monomer is composed of two subunits (A and B) that are connected and stabilized by disulfide bonds, and its candidate sequence was found to be 1,246 amino acids long (Figure 1; Supplementary Figure 1). According to the multiple sequence alignment performed (Supplementary Figure 2), the predicted sequence of Rhopr-IR is missing the intracellular C-terminal tail that follows the tyrosine kinase catalytic domain and further investigation is needed in order to characterize this portion of the receptor that is likely to be present in the protein. The Rhopr-IR sequence also contains a signal peptide with a cleavage site between residues 25 and 26, followed by a leucine-rich domain, a cysteine-rich domain, and a second leucine-rich domain, which together incorporate the 12 predicted disulfide bonds likely involved in establishing the structure of the ligand binding region (10). Three fibronectin type III (FnIII) domains follow the second leucine-rich domain and contain the cysteine residues (Cys) that are likely involved in the dimerization of the active receptor and in the connection of subunits A and B in the monomer. FnIII-1 is located within subunit A and contains one Cys (528) that likely forms a disulfide bond with the equivalent residue located in the other subunit A of the Rhopr-IR dimer. FnIII-2 starts in subunit A, ends in subunit B, and contains three Cys (668, 682, 699) that likely form disulfide bonds with their equivalent residues in the other subunit A of the active receptor dimer. FnIII-3 is located in subunit B and is predicted to contain one disulfide bond between Cys784 and Cys798, which is probably involved in stabilizing the structure of the monomer. The disulfide bond that connects

subunits A and B is predicted to be formed by Cys647 in FnIII-2 and Cys865 in FnIII-3.

The transmembrane domain follows FnIII-3 in subunit B and is 24 amino acids long. The tyrosine kinase catalytic domain is located in the intracellular portion of subunit B and contains a tyrosine kinase-specific active site, defined by the sequence F-V-H-R-D-L-A-A-R-N-C (1097–1107), a proton donor/acceptor site (Asp1101), and two ATP-binding sites (Arg1105–Asn1106 and Asp1119). A receptor tyrosine kinase class II signature is also present in subunit B and is defined by the sequence D-I-Y-E-T-D-Y-Y-R (1125–1133). Furthermore, there are five tyrosine residues that are potentially phosphorylated in the intracellular portion of the receptor, and ten asparagine residues in the extracellular portion that are potentially glycosylated in the mature receptor.

Phylogenetic Analysis of Rhopr-IR

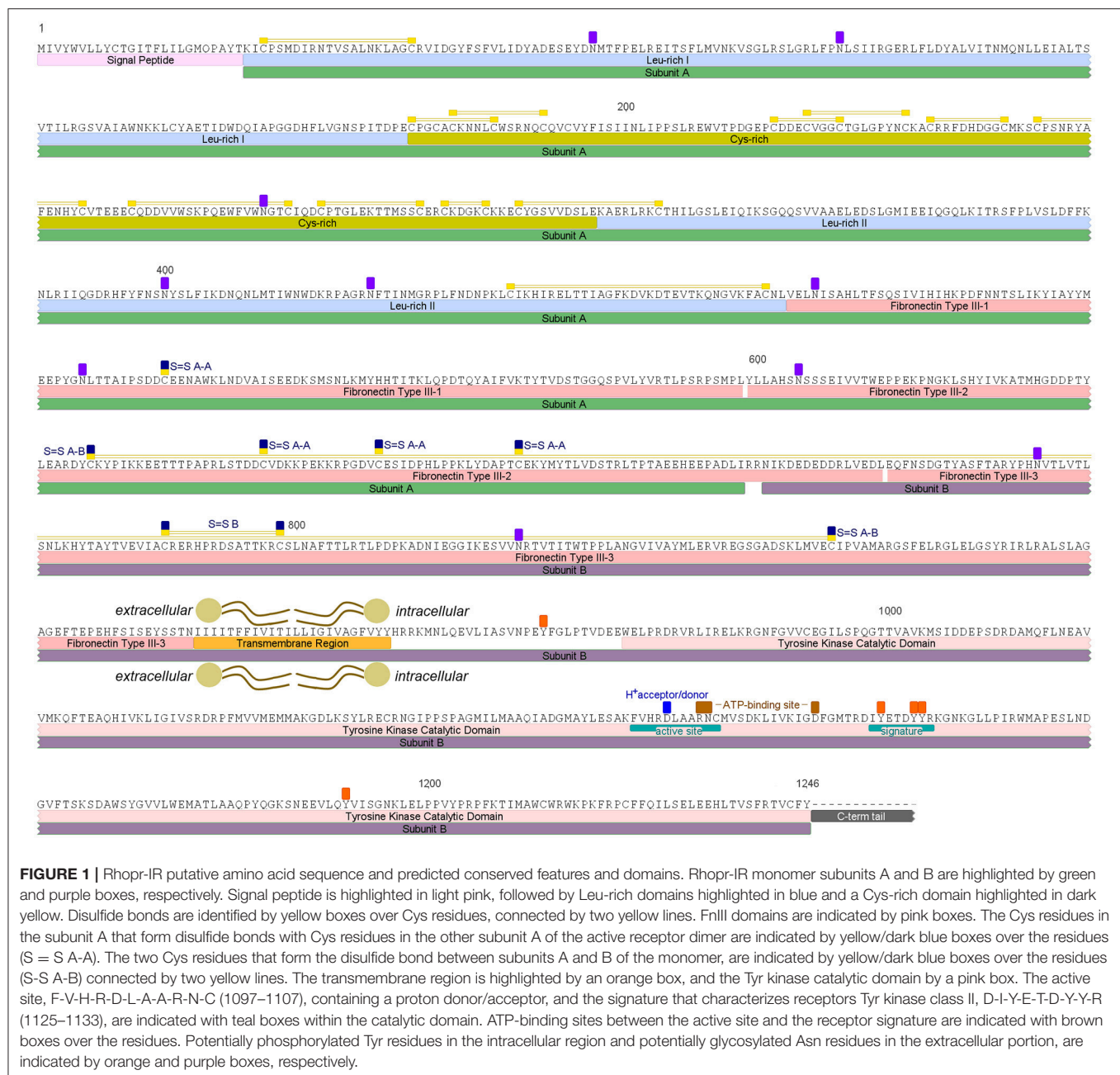
The alignment of the insulin receptor protein sequences was constructed using the online tool MUSCLE and a highly conservative region was selected to generate a maximum likelihood phylogenetic tree (Supplementary Figure 2). The tree was generated using the intracellular portion of the sequence containing the tyrosine kinase catalytic domain (Figure 2). *R. prolixus* Rhopr-IR grouped with other hemipterans, suggesting the existence of close relationships to both the bed bug (*C. lectularius*) and to the brown marmorated stink bug (*H. halys*).

Characterization of Tissue-Specific Expression of Rhopr-IR Transcript in 5th Instar *R. prolixus*

The tissue-specific relative transcript expression of Rhopr-IR was investigated using qPCR (Figure 3) and the presence of the transcript in tissues with the lowest relative expression was confirmed using semi-quantitative RT-PCR (Supplementary Figure 3). Rhopr-IR transcript was present in all tissues analyzed, with the highest relative expression identified in the CNS, followed by the salivary glands, the anterior midgut, and the posterior midgut. The remaining tissues (foregut, hindgut, fat body, leg muscles, dorsal vessel, and Malpighian tubules) displayed very similar and lower levels of Rhopr-IR transcript expression.

The fat Body of *R. prolixus* Demonstrates IR Signaling Through a Phosphorylation Cascade

To investigate the signaling activity of Rhopr-IR, insects were first injected with either *R. prolixus* glucose-free saline or 10^{-3} M BpV(phen) (in glucose-free saline). This activator is well characterized as a specific activator of mammalian IRs, which is achieved through the BpV(phen)-mediated inhibition of the protein-tyrosine phosphatase 1B (PTP1B) responsible for negatively regulating basal IR activity (42). Once inhibited, PTP1B is incapable of catalyzing the dephosphorylation of mammalian IR proteins, leading to an increase in the levels of basal auto-phosphorylated receptor. In mammals, this leads to an increase in the intracellular levels of phosphorylated tyrosine species (43). In the present study, fat bodies were harvested from



injected insects after 30 min, and the general phosphorylation of cellular tyrosine (Tyr) residues was assessed. As seen in **Figure 4A**, treatment with BpV(phen) induced a global increase in intracellular Tyr phosphorylation, as indicated by an increase in the intensity of the anti-pTyr bands relative to those in saline controls. The activation of Rhopr-IR by BpV(phen) was further supported by the observed increase in the phosphorylation of the IR downstream target Akt (pAkt; **Figure 4B**), compared with low apparent pAkt detection in saline controls. Thus, it appears that the mammalian IR activator BpV(phen) can also activate an IR-like receptor in *R. prolixus*, and that the activation of Rhopr-IR subsequently induces a tyrosine phosphorylation cascade typical

of other IR signaling cascades in mammals and insects alike (19, 44, 45). Furthermore, it appears that BpV(phen) treatment also induces the phosphorylation of the potential Rhopr-IR downstream protein target Akt, and thus likely stimulates the coordinated phosphorylation of other proteins in the Rhopr-IR pathway.

Endogenous Rhopr-IR Activation Exhibits a Distinct Pattern Following Feeding

Previous reports indicated that an ILP released from neurosecretory cells in the CNS peaked within 4 days post-feeding, implying the stimulation of whole-body insulin-like

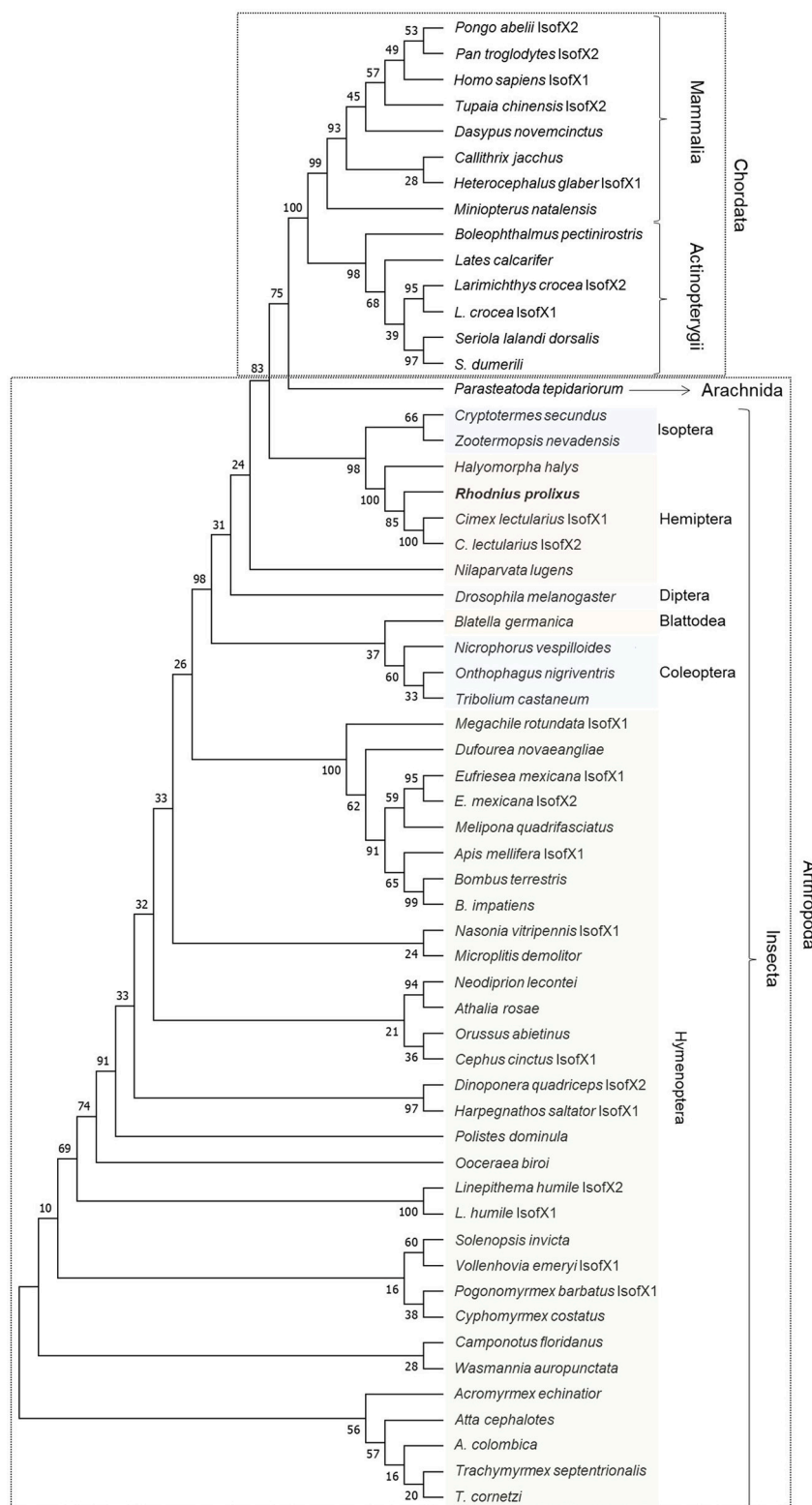
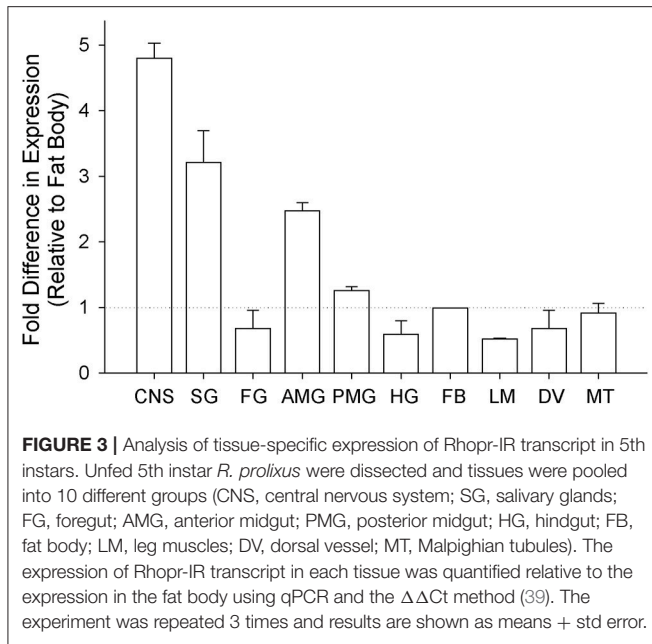


FIGURE 2 | Maximum likelihood inference for phylogenetic analysis of insulin receptor sequences from vertebrates and invertebrates using the tyrosine kinase domain. The phylogenetic tree shows the relationships among 58 tyrosine kinase domain sequences of insulin receptors from 54 different species of vertebrates (Chordata) and invertebrates (Arthropoda), including Rhopr-IR sequence. The tree was generated by maximum likelihood inference using the JTT + G model and the bootstrap consensus tree was inferred from 500 replicates. The analyses were conducted in MEGA7 (36).

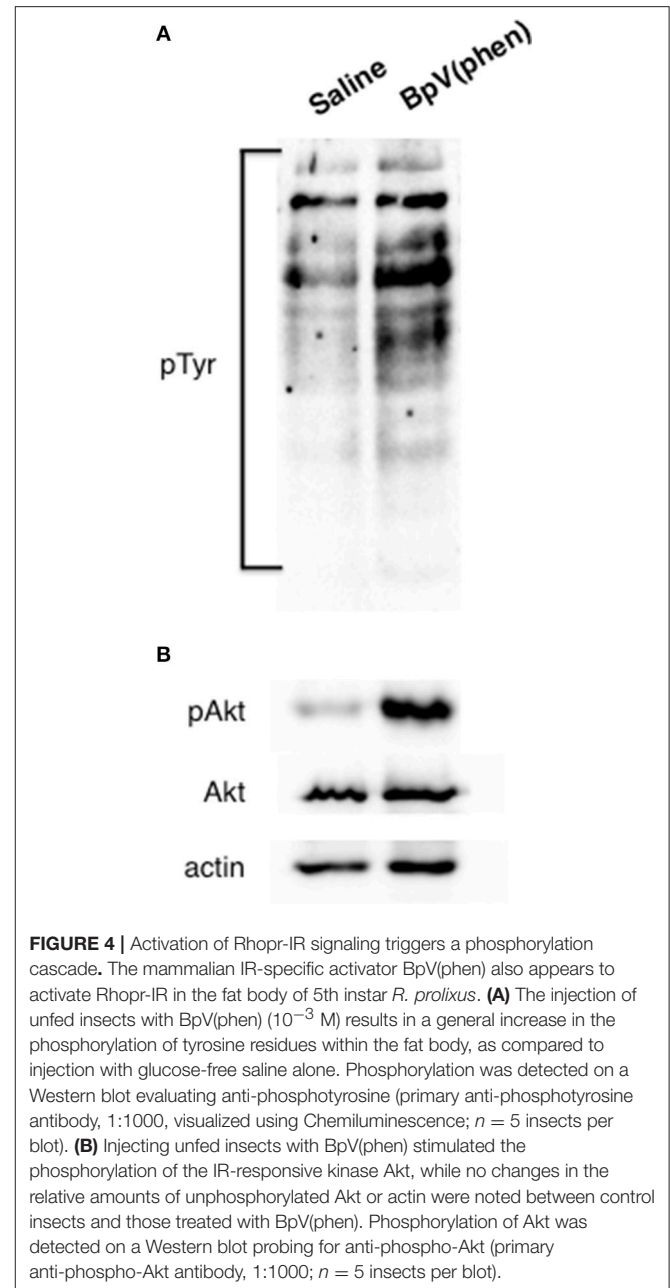


signaling in this time frame (46). Therefore, we investigated the activation of the Rhopr-IR pathway within the fat body of 5th instar insects after a blood meal, compared with unfed controls. As seen in **Figure 5A**, there is a distinct pattern of pathway stimulation as soon as 4 h after feeding, as indicated by the increased intensity of phospho-Akt, -GSK3 β , and -FOXO bands, compared to relatively no detection of phosphorylation in unfed insects. The most significant phosphorylation of Akt and GSK3 β occurred between 1 and 2 days post-feeding (**Figures 5B,C**), while the phosphorylation of FOXO, located downstream in the IR pathway, peaked at 2 days after feeding (**Figure 5D**). The phosphorylation of all pathway components decreased markedly at 3–4 days post-feeding and remained at low levels for up to 7 days after ingestion of the blood meal (data not shown). The coordinated phosphorylation of these pathway components suggests the stimulation of Rhopr-IR signaling in the fat body by Rhopr-ILP after a blood meal.

The weight (mg) and protein concentration ($\mu\text{g}/\mu\text{l}$) of 5th instar fat bodies were also determined for 5 days post-feeding compared to unfed (**Figure 6**). It was observed that fat bodies significantly increased in weight and protein concentration as early as 4 days post-feeding, and there was a phenotypic observation of increased lipid accumulation. However, the involvement of the Rhopr-IR pathway activation in regulating nutrient uptake post-feeding is yet to be investigated.

Exogenous Activation of Rhopr-IR by Mammalian Insulin

To further characterize the activity of Rhopr-IR, we investigated the stimulatory effects of mammalian insulin on the IR pathway within *R. prolixus* fat bodies. As seen in **Figures 7A,B**, injecting 5th instar *R. prolixus* with 0.1 μg porcine insulin led to a significant increase in Akt phosphorylation within 30 min (n



= 3 trials, with 5 insects per trial), relative to control insects injected with *R. prolixus* glucose-free saline. This increase in pAkt levels would suggest an increase in the activity of other downstream components of the Rhopr-IR pathway, although the phosphorylation of these proteins was not investigated. Higher concentrations of porcine insulin did not appear to stimulate phosphorylation as compared to controls. As expected, injecting insects with glucose-containing saline led to a slight but non-significant increase in fat body Akt phosphorylation (**Figures 7A,B**), indicating that the IR pathway is also likely responsive to hemolymph glucose fluctuations. However, the full extent of the effects of carbohydrate levels on Rhopr-IR expression and signaling is yet to be investigated.

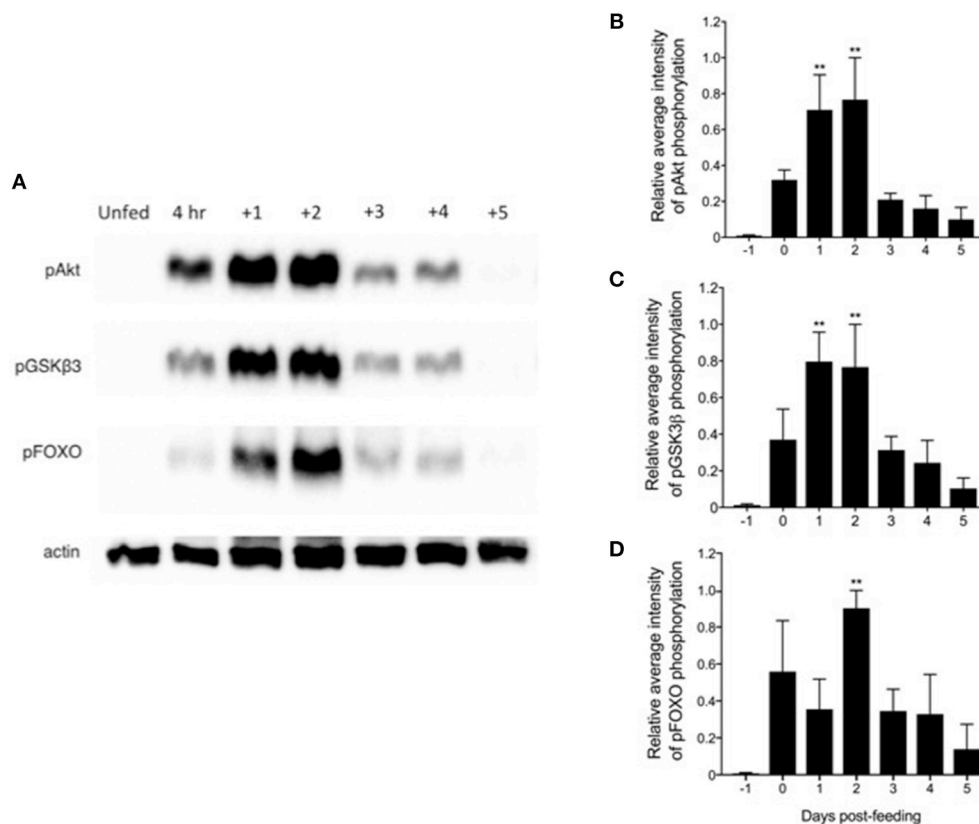


FIGURE 5 | Feeding triggers a coordinated phosphorylation cascade within a Rhopr-IR associated pathway. **(A)** Fat bodies were dissected from unfed 5th instar *R. prolixus* 1 day prior to feeding ("Unfed"), between 2 and 4 h immediately after feeding ("4 h"), and up to 5 days after feeding on a rabbit blood meal ($n = 5$ insects per day per trial; 3 trials completed in total). Western blots were conducted to probe for anti-phospho-Akt (pAkt), anti-pGSK β 3, and anti-pFOXO (primary antibodies, 1:1000; visualized using Chemiluminescence), which form part of a downstream pathway stimulated by Rhopr-IR signaling. The phosphorylation of all three proteins increased significantly post-feeding, with pAkt **(B)** and pGSK β 3 **(C)** reaching peak phosphorylation as soon as 1 day post-feeding, and with pFOXO **(D)** reaching peak relative phosphorylation levels 2 days post-feeding. The experiment was repeated 3 times ($n = 5$ insects per trial) and results are shown as means + std error. **($p < 0.01$) indicates statistically significant difference, inferred using one-way ANOVA with Dunnett's multiple comparisons test.

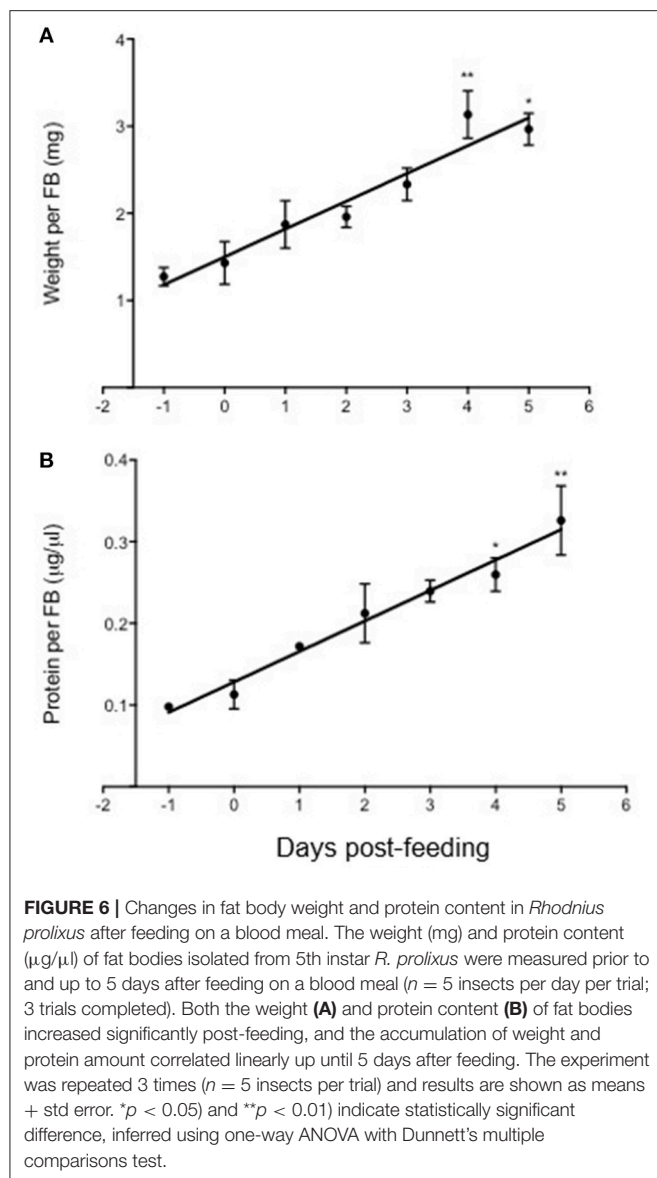
Knockdown of Rhopr-IR Expression

RNA interference was used to knockdown the expression of Rhopr-IR (**Supplementary Figure 4**). Insects were injected with dsIR or control dsARG, and transcript expression was quantified in four different tissues at 3 and 10 days after injections. The CNS and midgut showed a reduction in expression of around 70% by day 3, which was also observed at day 10 post-injection. In the fat body, the expression was knocked down by 40% at day 3 and by 80% at day 10. In contrast to other tissues, the knockdown of expression in the leg muscles at day 3 post-injection was higher than that observed at day 10, which were 50 and 40%, respectively.

Insects injected with dsRNAs were fed on rabbit blood at 3 days post injections in two separate groups, dsARG and dsIR, and were weighed before and after feeding to investigate the effects of Rhopr-IR knockdown on the amount of ingested blood. Although the post-feeding average body weight of Rhopr-IR knockdown insects was slightly lower than that of the control group, no significant difference was observed between the groups (**Supplementary Figure 5**).

Metabolic Regulation of Lipids and Carbohydrates in Rhopr-IR Knockdown Insects

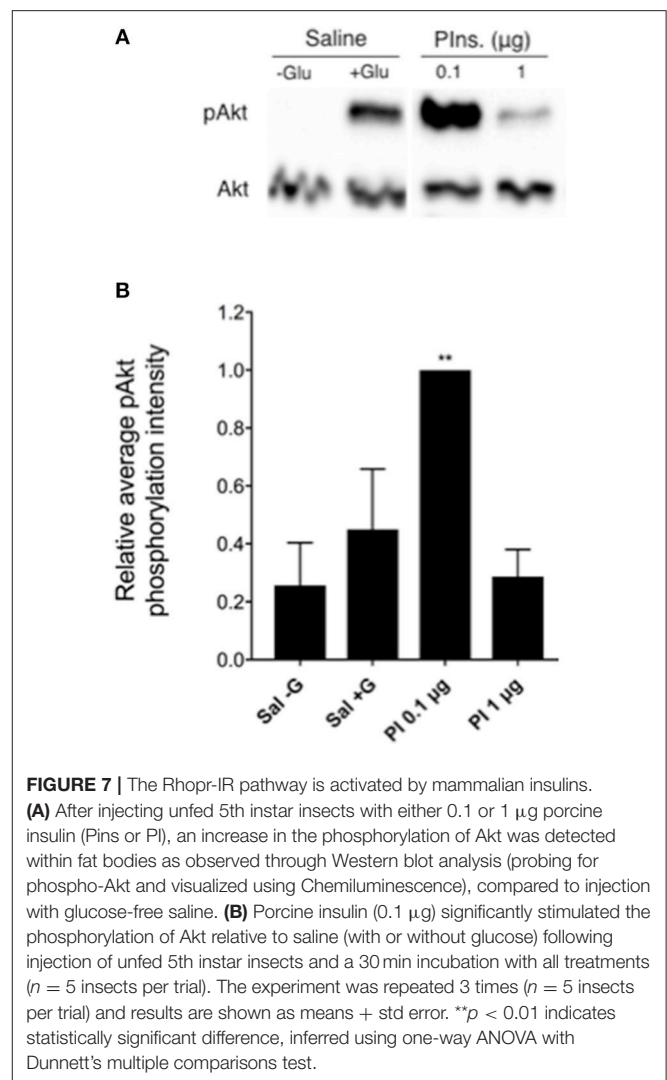
The levels of lipids and carbohydrates were measured at 7 days post dsRNA injection in unfed insects and in dsRNA injected insects at 4 days post-feeding. In unfed insects, the hemolymph lipid level increased following the knockdown of Rhopr-IR transcript (**Figure 8A**), while there was a reduction in the fat body lipid content in knockdown insects compared to the dsARG-injected controls (**Figure 8B**). In contrast, no differences in carbohydrate levels were observed in either the hemolymph or fat bodies of unfed insects following dsIR or dsARG injections (**Figures 8C,D**). A similar scenario was observed for recently fed insects, where hemolymph lipid levels increased (**Figure 9A**) and fat body lipid content decreased in Rhopr-IR knockdowns relative to dsARG controls (**Figure 9B**). Similar to experiments on dsRNA injected unfed insects, no difference was seen in the hemolymph and fat body carbohydrate content between Rhopr-IR knockdowns and dsARG controls (**Figures 9C,D**). Our results



suggest that Rhopr-IR is involved in fat body lipid storage and hemolymph lipid homeostasis in post-feeding and non-feeding periods. Interestingly, we did not see differences in either stored or circulating carbohydrates between knockdown and control insects.

Silencing of Rhopr-IR Using DSRNA Suppresses IR Pathway Activation

Considering the transient knockdown of Rhopr-IR expression observed after treating insects with dsRNA, the effects of dsIR injection on the Rhopr-IR signaling cascade was also investigated. Unfed 5th instars were injected with 1 μg of either dsARG or dsIR and after 4 days, fat bodies were removed and analyzed for the phosphorylation of IR pathway components. As seen in **Figure 10**, the fat bodies of animals injected with dsIR displayed lower levels of Akt and FOXO phosphorylation compared with



dsARG controls, indicating that the transient knockdown of Rhopr-IR likely resulted in reduced IR-related signaling for at least 4 days post-treatment. Interestingly, the phosphorylation state of GSK3 β remained unchanged. Overall, these results support the ability of dsRNA to reduce IR expression and related signaling in *R. prolixus* and support the coordinated activity of different upstream and downstream pathway components in mediating IR signaling. Further work should be conducted to determine the prolonged effects of dsIR treatment on fat body IR protein content and associated physiological effects in unfed and fed insects.

DISCUSSION

The insulin pathway is an evolutionarily conserved cellular signaling system found in all metazoans. The activation of all identified IRs is achieved through the binding of insulin or an ILP, whose sequences can differ markedly between animal classes. However, despite variances in sequence and structure, both

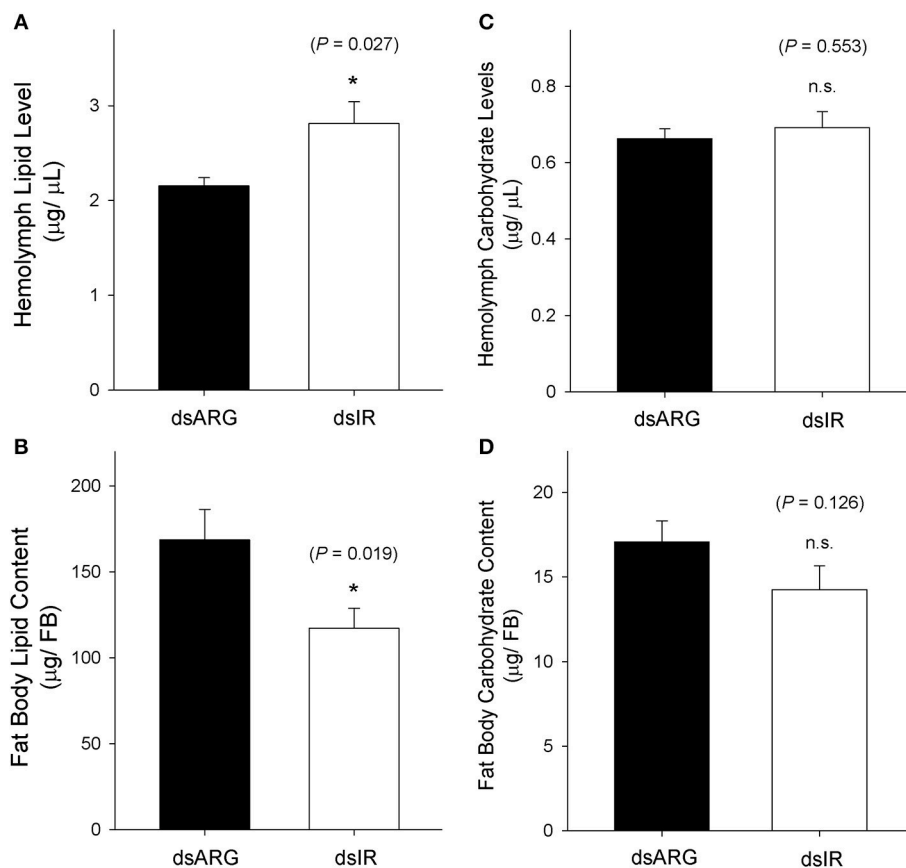


FIGURE 8 | Lipid and carbohydrate measurements of unfed Rhopr-IR knockdown insects. 5th instar *R. prolixus* were injected with 1 µg of dsARG or dsIR in 1 µL of ultrapure water and hemolymph and fat body samples were collected 7 days later. Significance of results is inferred comparing dsIR-injected insects to dsARG-injected insects. **(A)** Increase in hemolymph lipid level in dsIR-injected insects ($n = 21$ dsARG, $n = 20$ dsIR). **(B)** Decrease in fat body lipid content in dsIR-injected insects ($n = 12$ dsARG, $n = 14$ dsIR). **(C)** Hemolymph carbohydrate levels ($n = 18$ dsARG, $n = 17$ dsIR). **(D)** Fat body carbohydrate content ($n = 16$ dsARG, $n = 17$ dsIR). Results are shown as means + std errors. An asterisk (*) indicates statistically significant differences between dsARG- and dsIR-injected insects.

vertebrate and insect IRs have been shown to regulate similar phosphorylation cascades that control physiological functions related to macronutrient homeostasis, storage, and mobilization. In insects, the various pathways controlled by IR also influence other processes such as ecdysteroid production (47, 48), ovarian maturation and egg production (19), senescence, and age-related changes in locomotor behavior (49). Although ILPs and IRs have been identified in a variety of invertebrate species, a comprehensive characterization of each pathway component and their role in physiological functions is far from complete.

We have identified an IR in *R. prolixus*, namely Rhopr-IR, which activates conserved intracellular kinases and modulates lipid homeostasis in both post-feeding and non-feeding states. Through the analysis of its sequence, it appears that Rhopr-IR is a glycoprotein composed of two subunits (A and B), and contains the conserved ligand-binding, leucine-rich, cysteine-rich, and kinase domains found in vertebrate and *D. melanogaster* IRs (10, 50). When compared to human IR, one of the most studied IRs (51), Rhopr-IR displays highly conserved structural features, including the extracellular portion that contains cysteine

residues that form disulfide bonds in positions homologous to those in human IR (**Supplementary Figure 1**). Within subunit A, there are 12 predicted disulfide bonds that stabilize each monomer and contribute to the structure of the classical binding surface of insulin. Other predicted disulfide bonds located in the Fibronectin Type III domains connect subunits A and B of each monomer and subunits A of the monomers to form the active dimeric receptor (52). The 1,248-amino acid predicted sequence of Rhopr-IR was compared to IR sequences from 54 other species, including vertebrates and invertebrates. We found that the region with the highest sequence similarity was located in the intracellular tyrosine kinase domain, therefore we analyzed the evolutionary history of Rhopr-IR using this domain, which suggested a close relationship to other hemipteran IRs. It has been previously shown that the intracellular components of the insulin signaling pathway are very conserved throughout evolution in regards to function and sequence (10, 53).

In our investigation, we found that the Rhopr-IR transcript is present in all tissues tested, supporting the idea that the insulin signaling pathway is involved in a variety of physiological

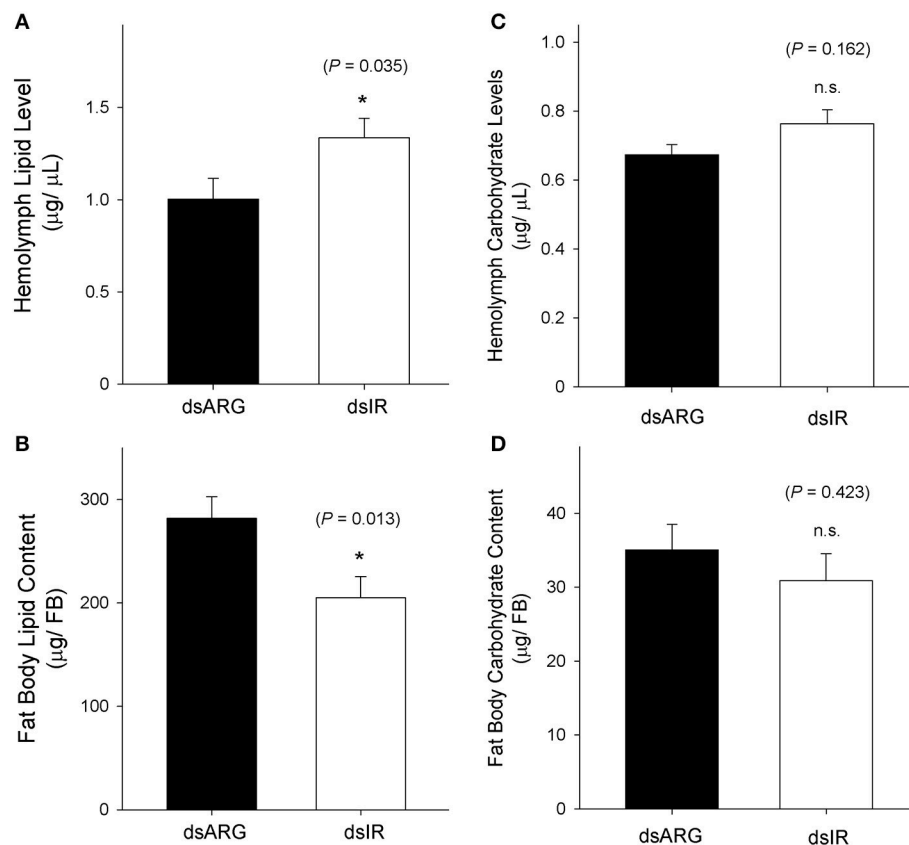
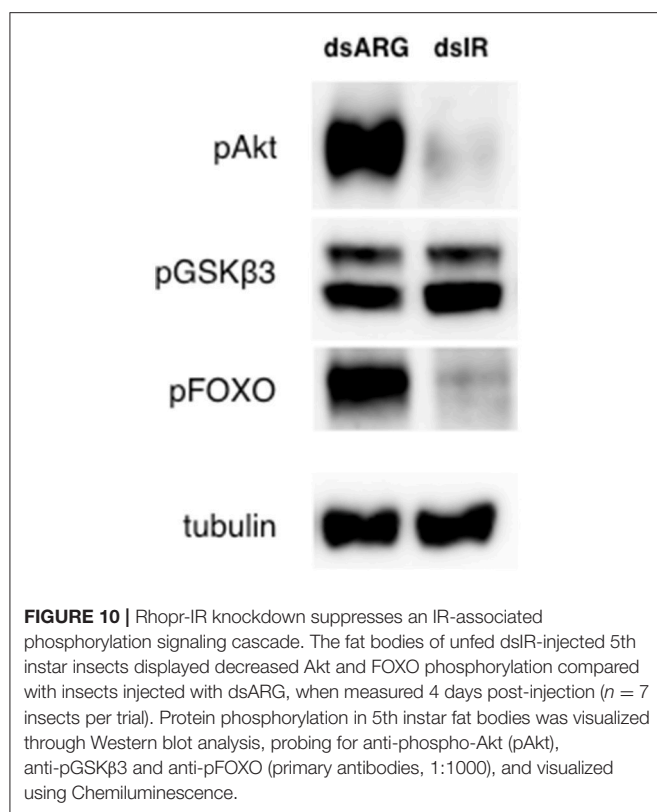


FIGURE 9 | Lipid and carbohydrate measurements in recently fed Rhopr-IR knockdown insects. 5th instar *R. prolixus* were injected with 1 µg of dsARG or dsIR in 1 µL of ultrapure water and fed on defibrinated rabbit blood 3 days after injections. Hemolymph and fat body samples were collected 4 days post-feeding. Significance of results is inferred comparing dsIR-injected insects to dsARG-injected insects. **(A)** Increase in hemolymph lipid level in dsIR-injected insects ($n = 26$ dsARG, $n = 27$ dsIR). **(B)** Decrease in fat body lipid content in dsIR-injected insects ($n = 25$ dsARG, $n = 20$ dsIR). **(C)** Hemolymph carbohydrate levels ($n = 22$ dsARG, $n = 22$ dsIR). **(D)** Fat body carbohydrate content ($n = 10$ dsARG, $n = 9$ dsIR). Results are shown as means + std errors. An asterisk (*) indicates statistically significant differences between dsIR and dsARG injected insects.

processes, as seen in other insects. Previous studies used Northern blot analysis and *in situ* hybridization to identify the expression of an IR within the CNS and ovaries of larval and adult *D. melanogaster*, as well as to localize the receptor within imaginal disks in growing embryos (54). Transcript expression of IR was seen in new and maturing ovary follicles and nurse cells of mosquitoes (19), as well as in ovaries of the honey bee *A. mellifera* (55). Interestingly, there has been very little investigation into the localization of the IR within fat body and skeletal muscles in other insects, although the signaling of ILPs on these and other tissues has been thoroughly characterized, suggesting the presence of both IR transcript and protein. Surprisingly, we have identified a relatively low level of Rhopr-IR transcript expression in the fat body relative to other tissues in unfed 5th instars. It is possible that receptor expression is modulated by nutrient status and intake, and as a result there may be a differential expression pattern observed within the fat body after feeding. It should also be noted that the tissue localization of Rhopr-IR transcript proposed in this study relates to the last nymphal stage of *R. prolixus* and not the adult insect. It is possible that

IR transcript expression may differ markedly between these life stages, considering the reproductive activity and multiple feeding cycles associated with the adult stage.

The pharmacological activation of Rhopr-IR was subsequently investigated to classify its function compared with previously identified insect IRs. It is important to reiterate that IR stimulation typically triggers the activation of two main intracellular pathways: the PI3K/Akt/FOXO cascade, and the Ras-MAPK pathway. However, while the PI3K/Akt pathway regulates processes involving glucose uptake and metabolism (56), the IR-dependent MAPK pathway is involved in mediating mitogenic and cell cycle responses in mammals (11), among other functions. For the purposes of the present study, we chose to investigate the PI3K/Akt branch of the IR signaling network overseeing nutrient storage and metabolism, as there is great interest in studying the post-feeding physiological responses of the blood-feeding *R. prolixus* disease vector. However, it would be of interest to further explore the identification of a MAPK-like pathway downstream of the Rhopr-IR and its role in regulating cell growth and proliferation in future research.



In the present work, we chose to first investigate basal IR activation by stimulating receptor activity using the mammalian IR-specific activator BpV(phen), as well as a mammalian insulin. BpV(phen) relieves the negative regulation of basal autophosphorylation of IR by inhibiting PTP1B, a phosphatase that catalyzes the dephosphorylation of the autophosphorylated receptor (42, 57). Consequently, by inhibiting PTP1B, an expected increase in IR-mediated tyrosine phosphorylation is typically observed, as well as the increased phosphorylation of proteins containing pTyr sites downstream of the IR receptor (43). We observed both an increase in general tyrosine phosphorylation as well as phosphorylation of the kinase Akt, which is a conserved component of IR signaling in mammals, *C. elegans*, and *D. melanogaster* (58). The coordinated increase in tyrosine and Akt phosphorylation reinforce our characterization of Rhopr-IR as a receptor tyrosine kinase, similar to the IRs identified for vertebrate insulins and other insect ILPs. Stimulation of insect IR-like pathways by other pervanadate compounds [similar to BpV(phen)] have also been reported in the mosquito *A. aegypti* (47), where an increase in ecdysteroid production, normally controlled by the insulin pathway, was observed. Thus, as a result of the stimulation of IR-like signaling induced by the injection of insects with BpV(phen), we believe that the mechanisms controlling basal IR activity in insects is similar to what is seen in mammals, and that bisperoxovanadium compounds are capable of stimulating IR-like receptors in *R. prolixus* as well as other insect species.

It should be noted that BpV(phen) activates IR through an indirect mechanism and does not act on the receptor itself,

as previous studies identified molecular interactions of BpV compounds as potent inhibitors of the tumor suppressor phosphatase phosphatidylinositol 3,4,5-triphosphate 3-phosphatase (PTEN) (59). Hence, in order to support the observed increase in the phosphorylation of downstream IR proteins (such as Akt) when treated with BpV(phen), we furthered our investigation of Rhopr-IR activity by injecting insects with porcine insulin and evaluated the responsiveness of its potential downstream pathway components. We previously reported the effects of porcine insulin on the modulation of nutrient balance in the locust *L. migratoria* (60). Furthermore, porcine insulin was also found to dose-dependently stimulate the phosphorylation of embryo-specific proteins in *D. melanogaster* (50) and to directly compete with an ILP from *Manduca sexta* in binding to an insulin antibody (61). Other studies have investigated the ability of bovine insulin to stimulate ecdysteroid synthesis in *B. mori* (48), and to stimulate a decrease in circulating carbohydrates in the cockroach *Periplaneta americana* (62). Here, we observed that treatment with porcine insulin led to an increase in the phosphorylation of Akt in the fat body of *R. prolixus* after a half-hour incubation. Interestingly, injecting insects with larger amounts of insulin resulted in a decrease in the detected phosphorylation of Rhopr-IR pathway components. It is possible that Rhopr-IR experienced desensitization when stimulated by higher concentrations of porcine insulin, leading to an observed decrease in pathway activation. This hypothesis is supported by a previous study conducted in isolated rat adipocytes, where an apparent time-dependent and dose-dependent decrease in IR proteins was observed after treatment with large insulin concentrations for 4 h, coupled with a measurable decrease in the insulin-dependent glucose transport (63).

Alternatively, it is possible that larger concentrations of insulin stimulated the maximal activation of pathway phosphorylation in a shorter timeframe, which may not have been captured by the 30-min time point used in this study. Through kinetic analysis, it has been found that insulin induced 50% maximum stimulation of IR autophosphorylation within 30 s of incubation with partially purified IR β -subunit, with near-maximal activation achieved after a 5-min incubation (64). This study further observed a dose-dependent inhibition of the autophosphorylation of the IR β -subunit by concentrations of insulin <100 nM. Therefore, it is likely that porcine insulin was capable of activating Rhopr-IR and stimulating the ILP-responsive pathway as seen in other insects but induced either receptor sensitization or rapid kinetic effects not detectable by the methodology used in the present study. The receptor-ligand specificity of insect IRs, and specifically Rhopr-IR, for vertebrate insulins and insulin-like ligands should be further investigated.

We further sought to map a potential signaling cascade triggered by Rhopr-IR by investigating the timing and coordination in phosphorylation of components downstream of Akt. The phosphorylation of Akt and its substrates GSK β 3 and FOXO was barely detectable using Western blot analysis in unfed 5th instars, whereas feeding on a blood meal triggered a coordinated increase in the phosphorylation of all three proteins. As FOXO is the most downstream target of the pathway, it was

unsurprising that peak levels of phosphorylation appeared to be reached 2 days post-feeding, while maximum phosphorylation of Akt and GSK3 β was observed 1 day after feeding. Previously, it was observed that the maximum release of ILPs into circulating hemolymph may be reached at 3 to 4 days post-feeding in *R. prolixus*, as monitored through immunofluorescence (46). Thus, the coordinated phosphorylation of Akt, GSK3 β , and FOXO in the present study implies an increase in the activity of the IR within the same time frame as putative endogenous ILP release following feeding. These three protein targets are part of the PI3K/Akt/PKB pathway, which is stimulated by vertebrate IRs and other insect IRs such as within the plant hopper *Nilaparvata lugens* (24), the mosquito *A. aegypti* (65), the moth *B. mori* (66), and *D. melanogaster* (67–69). Along these lines, we believe that Rhopr-IR shares the conserved PI3K/Akt signaling cascade observed in other insulin-mediated pathways.

However, it should be noted that Akt activation and signaling is not exclusive to the PI3K/Akt axis of the IR pathway. In mammals, the activity of Akt has been implicated in several diverse cellular processes including cell survival (due to the inhibition of the apoptosis-inducing FOXO transcription factor), cell growth, proliferation, metabolism, cell migration, and cancer proliferation (70, 71). Aside from IR-mediated stimulation, the phosphorylation and activation of Akt is also regulated by G-protein coupled receptors (GPCRs), as observed in mammals through a muscarinic acetylcholine GPCR whose Akt-related signaling mediates mammalian cell survival (72). It has also been found that ecdysteroids and ecdysteroid receptor signaling also regulate Akt activation and overall IR pathway signaling in *Drosophila* (73), in relation to coordinating physiological responses to insect growth and development. Stimulation of ecdysteroidogenesis in *B. mori* is also partially accomplished by Akt activation mediated by the prothoracicotropic hormone (PTTH) signaling at its receptor, PTTH-R (74). Furthermore, IGF signaling in both mammals and invertebrates also triggers the activation of the PI3K/Akt pathway (18, 75). Similar to IR stimulation, the activity of IGF at its cognate tyrosine kinase receptor (IGF-1R) also regulates metabolism and cell survival, as well as development-related growth, aging, and skeletal muscle atrophy and growth (76–79). Thus, it is possible that the observed activation of pAkt levels in the present study could have been influenced by one or several of these competing pathways. Moving forwards, we believe that the signaling induced by Rhopr-ILP and -IGF at the Rhopr-IR within the fat body, and particularly the effects of these signaling molecules on Akt activation and pathway response to nutrient sensing and storage in *R. prolixus*, should be investigated.

Physiologically, the coordinated phosphorylation of Akt, GSK3 β , and FOXO mediated by the activation of IR signaling results in cell growth, glucose uptake and increased nutrient storage, as observed in mammalian cells and in *Drosophila* (69, 80–84). The serine/threonine kinase Akt is activated upon its phosphorylation, resulting in the pAkt-mediated phosphorylation and inactivation of GSK3 β (15, 85) and FOXO (86). The inhibition of GSK3 β stimulates glycogen synthesis, lipid and glucose storage, while the inhibition of FOXO prevents the transcriptional promotion of apoptosis and allows for cell

growth, as observed in *D. melanogaster* (69). We observed that the post-feeding increase in Rhopr-IR pathway activity also coincides with an increase in fat body weight and apparent lipid accumulation. By silencing the expression of Rhopr-IR using dsRNA, we detected a decrease in Akt and FOXO phosphorylation in unfed insects 4 days post-injection, which also correlated with a decrease in fat body lipid content and increase in hemolymph triglyceride levels. This effect of Rhopr-IR knockdown on circulating and stored lipids was repeated in recently fed insects, and it was observed that dsIR-injected animals had significantly less lipid stored in the fat body and more lipids in their hemolymph when compared to dsARG-injected controls.

As there was no apparent effect of dsRNA injection on the amount of blood ingested by insects, it is likely that dsIR knockdown partially silenced the Rhopr-IR signaling leading to the depressed activation of Akt and resulting increased activity of FOXO. Without the insulin signal, FOXO remains active in the nucleus and stimulates the expression of a variety of genes such as PEPCK (phosphoenolpyruvate carboxykinase), a key enzyme involved in propagating the gluconeogenesis signal in the absence of stored nutrients (87). In *D. melanogaster*, FOXO stimulates the expression of the triacylglycerol (TAG) lipase gene *dLip4* (88) and the mitochondrial acyl-CoA synthetase gene *pudgy* (89), which are directly involved in lipid metabolism. In *D. melanogaster*, it was also observed that the expression of a constitutively active FOXO homolog lacking its regulatory Akt phosphorylation sites resulted in the suppression of IR-induced triglyceride storage (69), rendering similar results to our observations of reduced lipid storage in Rhopr-IR knockdown insects. Furthermore, it was observed that treatment of diapausing *A. aegypti* females with dsFOXO resulted in significantly lower lipid accumulation compared with untreated controls, and there was a noticeable reduction in the number of fat body cells in dsFOXO animals (90). The cellular apoptotic and anti-proliferative activities mediated by FOXO most likely counteract fat body cell growth and ultimately work to reduce lipid storage. Therefore, arresting FOXO function by activating Rhopr-IR and Akt results in fat body cell growth and lipid accumulation. Future work should focus on simultaneously monitoring FOXO phosphorylation and transcriptional activity along with cell growth and lipid storage, in order to corroborate this hypothesis.

Contrasting the lipid imbalance observed between fat body and hemolymph, we did not see any changes in carbohydrate distribution post Rhopr-IR knockdown, either in unfed or in recently fed insects. Additionally, GSK3 β phosphorylation levels in the fat body of unfed insects were not drastically altered by dsRNA injections, suggesting that the reduction in transcript expression did not interfere in the signaling that regulates carbohydrate homeostasis. Although GSK3 β is a substrate for Akt and therefore has its activity modulated by this kinase, it has been shown that PKA (protein kinase A) and other kinases in the Wnt pathway can also phosphorylate and inactivate this enzyme (91, 92). GSK3 β was proven to be essential for glycogen metabolism but not lipogenesis during oogenesis and embryogenesis in *R. prolixus*, in response to both insulin and Wnt receptors (93). Also, triatomine insects, such as *R. prolixus*,

mainly rely on lipids as their motor energy supply, having a remarkably low concentration of sugars in the hemolymph when compared to other insects (94). Thus, it is interesting yet unsurprising that silencing Rhopr-IR yields a more sensitive response in regulating the homeostasis of lipid levels and related signaling cascades, with a smaller effect observed on pathway components that regulate carbohydrate nutrient balance. We have recently reported the presence of two ILPs in *R. prolixus*, Rhopr-ILP and Rhopr-IGF, and their involvement in energy homeostasis. We observed that both hormones are involved in the control of lipid and carbohydrate levels in the hemolymph, but only Rhopr-ILP seems to be involved in the regulation of lipid and carbohydrate content in the fat body (32, 33). It has been shown that insulin signaling in *D. melanogaster* adult adipocytes can activate GSK β 3 independently from FOXO depending on developmental stage (95). We could speculate that knocking down the expression of Rhopr-IR, but not its ligands, is reducing lipid uptake while increasing lipolysis through FOXO activation. At the same time, the lower Rhopr-IR expression levels could be promoting carbohydrate homeostasis by preventing GSK β 3 activation.

In conclusion, we have identified a candidate IR sequence in *R. prolixus* genome, namely Rhopr-IR, that is expressed in all tissues tested, suggesting that this receptor is likely involved in a variety of physiological processes. This work also demonstrates that a mammalian insulin and an IR activator have the ability to trigger an intracellular phosphorylation cascade, indicating that the conserved domains of Rhopr-IR can

interact with mammalian activators. These observations further support the likely conserved nature of IR signaling between mammals and insects. Furthermore, Rhopr-IR regulates lipid homeostasis in hemolymph and fat body likely through the modulation of pathway protein activity including the kinase Akt and the transcription factor FOXO, as seen in vertebrate and invertebrate models. Our results contribute to the understanding of energy homeostasis control in *R. prolixus* as well as to the characterization of the evolutionarily conserved insulin signaling system in insects.

AUTHOR CONTRIBUTIONS

MD and SD designed the study, conducted the experiments and wrote the manuscript. IO and AL aided in the design of the study, revised the manuscript, and supervised the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2018.00745/full#supplementary-material>

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Gut Microbiota and Energy Homeostasis in Fish

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The microorganisms within the intestinal tract (termed gut microbiota) have been shown to interact with the gut-brain axis, a bidirectional communication system between the gut and the brain mediated by hormonal, immune, and neural signals. Through these interactions, the microbiota might affect behaviors, including feeding behavior, digestive/absorptive processes (e.g., by modulating intestinal motility and the intestinal barrier), metabolism, as well as the immune response, with repercussions on the energy homeostasis and health of the host. To date, research in this field has mostly focused on mammals. Studies on non-mammalian models such as fish may provide novel insights into the specific mechanisms involved in the microbiota-brain-gut axis. This review describes our current knowledge on the possible effects of microbiota on feeding, digestive processes, growth, and energy homeostasis in fish, with emphasis on the influence of brain and gut hormones, environmental factors, and inter-specific differences.

Keywords: fish, microbiota, feeding, energy, regulation

INTRODUCTION

Microbiota/Microbiome

The microbiota can be defined as the collection of microorganisms that occupy a particular environment whereas the term “microbiome” refers to the collection of genomes of the microorganisms within the microbiota (1). These microbial communities include commensal, symbiotic, and pathogenic microorganisms (2). Multicellular organisms, including plants and animals live in close association with microorganisms, and harbor such complex microbial communities in and on themselves, from the skin surface to the gastrointestinal tract (GIT) (2). The microbiota may be contracted and developed through exposure to environmental factors. Given the potentially large impact of the microbiota on the host health, an increasing number of studies have been carried out to characterize and determine the mechanisms of action of these microbes.

The diverse microbial community that colonizes the GIT (gut microbiota) plays a critical role in modulating the host's physiology (3–5). The gut microbiota has lived in symbiotic association with the vertebrate host for millions of years, the host providing a nutrient-rich environment for the microbiota, and the microbiota providing metabolic, protective, and structural functions for the host (4–6). The gut microbiota is often considered as an “extra organ,” as it plays a key role in the intestinal development and physiology, as well as overall development, growth and health (3). Recent studies suggest that the gut microbiota is involved in energy homeostasis by regulating feeding, digestive and metabolic processes, as well as the immune response (1, 6–8). In particular, the gut microbiota influences the brain-gut axis, the bidirectional communication between the GIT and the brain (9–11), by affecting both gut and brain (12) and thus helps to maintain host homeostasis.

The function of the gut microbiota and the subsequent physiological responses of the host depend on the composition of the microbes that are present in the intestinal track (11). There is a wide variation in the composition of fish gut microbiota between species and individuals, but several phyla have been shown to be dominant, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (13). To date, most of the studies on gut microbiota have focused on mammals, in particular rodents, and in comparison, little is known about the host-microbe interactions in fish (5). There are several limitations to using mammalian models, including husbandry constraints, and the use of isogenic strains. Owing to their short life cycles and high offspring numbers, and their diversity in genetics, physiology and immunological features, which can be easily manipulated, fish may represent valuable models to study microbiota in vertebrates (14, 15). In addition, studies on fish gut microbiota may help improve welfare of fish and aquaculture practices. However, notable differences exist between mammals and fish with regards to metabolism and energy expenditure (2) and variations in host-microbe interactions and in contributions to maintaining host homeostasis could be expected between fish and mammals.

This review describes our current knowledge on the role of the fish gut microbiota in the regulation of host physiology, with emphasis on feeding, digestion and metabolism, as well as its influence on stress responses, reproduction and development, and immune responses. Environmental and host-specific factors affecting the fish gut microbiota composition and actions are also discussed, as well as future implications of fish gut microbiota manipulation and potential research directions for this growing field.

PHYSIOLOGICAL ROLES OF GUT MICROBIOTA

Feeding/Digestion/Metabolism

Studies in mammals show that microorganisms within the GIT are involved in the regulation of appetite/ingestion, digestion, and metabolism (16–19). For example, germ-free mice lacking a gut microbiota are leaner than normal control mice even when consuming more calories (17). Furthermore, these mice have lower levels of appetite-regulating hormones such as leptin and ghrelin (17), indicating that the gut microbiota is involved in the regulation of appetite and metabolism. Microbial secretions, including specific metabolites such as short chain fatty acids (SCFAs), indoles, propionate, butyrate, and acetate (20) affect digestive processes and metabolism. The microbiota also interacts with GIT neurotransmitters [e.g., serotonin (21), and the catecholamines dopamine, norepinephrine (22)] and thus influence their effects on gastrointestinal (GI) motility, function and hormone release, as well as feeding behavior (23, 24). Conversely, serotonin and catecholamines released from enteric neurons can influence the microbiota present in the gut and alter release of cytokines and bacterial molecules (25).

Some of these metabolites can act on enterocytes and regulate their intestinal barrier function (26), absorptive capacity [e.g., monosaccharide absorption (27)], and nutrient uptake and storage [e.g., altered enzymatic activity in the gut and fat storage (28)], thus influencing metabolism [(e.g., cholesterol metabolism and adipogenesis) (29)]. Furthermore, metabolites from the gut microbiota can modify the secretory activity of enterocytes, thus affecting the production gut peptides that modulate gut motility and enzyme secretion (30, 31). For example, SCFAs receptors have been shown to interact with the enteroendocrine L cells containing the gut hormone Peptide YY (PYY) to influence the colonic PPY expression in rats (32), and further influence metabolism. These microbial compounds can also influence feeding behavior [e.g., (17, 33, 34)] directly, by entering the circulation and reaching the brain, or indirectly, by either by activating vagal terminals or by modulating the release of appetite-regulating gut peptides (e.g., CCK, ghrelin, gastrin), which in turn, affect the release of central appetite-regulating neuropeptides (e.g., neuropeptide Y, NPY; proopiomelanocortin, POMC) (33, 35, 36) (**Figure 1**). The exact mechanisms ruling the communication between the gut microbiota and the brain (termed the “microbiota-gut-brain axis”) and how changes within the gut microbiota may impact neuropeptide systems in the brain are still unclear (31).

To date, very few studies have been conducted in fish with regards to the influence of microbiota on feeding and metabolism, but they provide clues to some similarities with mammals in this regard.

The influence of microbiota on food intake has been examined in a few studies correlating feeding rates and changes in microbiota. However, results are inconsistent and difficult to compare, as several studies and several additives are used. For example, zebrafish fed with *Lactobacillus rhamnosus* have reduced appetite compared to control fish (37, 38). However, carp fed a diet supplemented with fructo-oligosaccharide (FOS) display changes in microbiota composition (increased levels of heterotrophic aerobic bacteria and lactic acid bacteria) but no changes in feeding rates compared to fish fed a control diet (39).

The potential effect of the gut microbiome on metabolism has been examined in a few fish species. In grass carp (*Ctenopharyngodon idella*), many biosynthesis, and metabolism pathways of carbohydrates, amino acids and lipids change as the composition of microbiota changes (40). In zebrafish, the colonization of the gut by microorganisms promotes epithelial absorption of fatty acids (41) and fish with intact microbiota have increased lipid accumulation in the intestinal epithelium, and increased expression of genes related to lipid metabolism compared to germ-free fish who lack microbiota (42). In addition, Japanese flounder (*Paralichthys olivaceus*) fed a diet supplemented with *Bacillus clausii* display higher weight gain, feed efficiency and growth performance compared to fish fed control diets (43). The authors suggest this could be attributed to increased food intake and improved nutrient digestibility (43). All this data suggests a strong influence of the microbiota in fish metabolism.

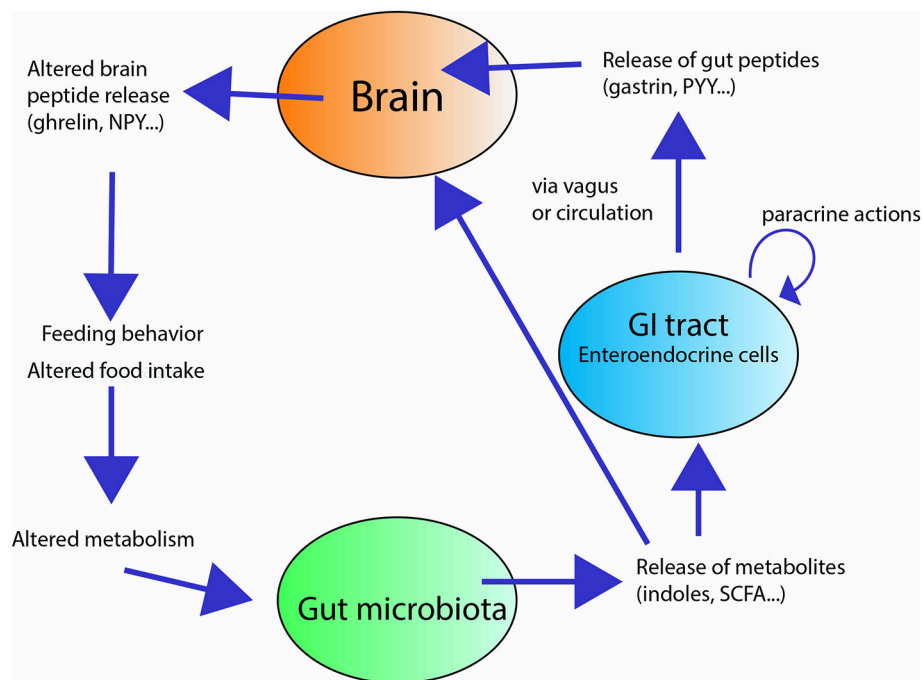


FIGURE 1 | Overview of the gut-microbiota-brain axis in feeding and digestion. The gut microbiota (green circle) releases metabolites in response to substrates present in the gut lumen. These metabolites locally stimulate the enteroendocrine cells (blue circle) of the gastrointestinal tract (GIT) and/or reach the brain (orange circle). The stimulated enteroendocrine cells release gut peptides, which act locally in the GIT and affect brain feeding centers, altering neuropeptide release, and modifying feeding behavior and energy homeostasis.

Other Functions Related to Energy Homeostasis

Stress Response

The stress response is mediated by several hormones and is a result of the bi-directional communication between the brain and peripheral organs (2). Stress in fish can be caused by a number of environmental factors (including poor water quality, high levels of particulates, suboptimal photoperiod, oxygen levels, temperature), high population density, poor diet/ malnutrition, as well as transportation and handling (44).

When stress occurs, the hypothalamic-pituitary-adrenal (HPA) axis releases corticotrophin-releasing hormone (CRH), which stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which stimulates the secretion of adrenal glucocorticoids to prepare the body to cope with stress (45).

In fish, as in mammals (45, 46), the microbiome affects the HPA axis, the stress response and behavior, in particular, anxiety-like and locomotor behaviors, which might in turn affect feeding behavior and energy homeostasis. For example, in zebrafish, enhancing the microbiota (by means of pro and prebiotics—see below) reduces anxiety-like behavior (47) and decreases the stress response, by lowering CRH expression and cortisol levels (48). Disruption of the gut microbiota might thus decrease the ability of the fish to forage for food and decrease feeding by increasing levels of stress hormones, which have been shown to inhibit feeding [e.g., rainbow trout *Oncorhynchus mykiss* (49); goldfish *Carassius auratus* (50)].

Conversely, stress can change the structure of the intestinal mucosa and induce alterations in the intestinal mucus, thus affecting absorption of nutrients as well as the gut immune system (or gut-associated lymphoid tissue, GALT), leading to infections by opportunistic pathogens (51). In fish, acute stress such as netting, induces an increased sloughing off of mucus and the removal of autochthonous bacteria which play a protective role against potential pathogens (52). Overall, stress results in modifications of gut microbiota and may alter immune response and increase the risk of colonization/invasion by pathogens and infection (2), which might decrease feeding rates, as seen in other fish [e.g., goldfish (53); chinook salmon (*Oncorhynchus tshawytscha*) (54, 55)]. However, different fish species may cope with stress in alternative ways, so that the effects of stress on gut microbiota may differ among species (52).

Reproduction/Development

Reproduction

Reproduction is closely related to energy homeostasis, as it is energetically costly, and can only be successfully accomplished when sufficient energy stores are available (56).

Studies have shown the gut microbiota may contribute to the development of gonads and subsequent reproductive success of the host. For example, when administered continuously from birth to sexual maturation, *Lactobacillus rhamnosus* alters the gut microbiota and accelerates larval development of zebrafish by improving growth and sex differentiation (57, 58). Adult female zebrafish treated with *L. rhamnosus* display an

increase in the number of vitellogenic follicles and higher gonadosomatic indexes (GSI), higher numbers of ovulated eggs and higher expression levels of reproductive hormones (kisspeptin, GnRH3, leptin) compared to control fish, therefore increasing the likelihood of reproductive success (58). Similarly, in ornamental livebearer fish species (59) and goldfish (60), supplementation of feed with probiotics increases GSI, fecundity and fry production of spawning females and length and weight of fry. Although the mechanisms mediating the actions of gut microbiota in host reproduction are still under investigation, it is likely that these mechanisms involve the regulation of feeding, food absorption and energy homeostasis.

Development

The development of tissues and organs which make up an animal is largely influenced by the presence and composition of the microbiota, in particular the development of the digestive and nervous systems (61), both crucial for appropriate ingestion and absorption of food.

The relationship between the fish gut microbiota and development of the host can be seen through clear patterns in the composition of gut microbiota during fish development (62). In grass carp (*Ctenopharyngodon idella*), bacterial communities vary between the eggs and the larvae, *Proteobacteria* and *Bacteroidetes* being dominant in the eggs and in the larvae, respectively, and bacterial diversity increases as the fish develops from egg to larvae (63). An increase in diversity has also been reported from the larval to the adult stage, as seen in grass carp, Chinese perch (*Siniperca chuatsi*) and southern catfish (*Silurus meridionalis*), also suggesting that the gut microbiota variation levels increase with fish development (62). In zebrafish, epithelial cell proliferation in the developing gut is stimulated by the presence of microbiota, providing direct evidence of the role of the gut microbiota in GIT development (64). Furthermore, the GIT of germfree zebrafish displays incomplete development and impaired function, which can be reversed by the inoculation of bacteria (14).

Evidence suggests that the microbiota is also involved in the neurological development, and is required for normal neurobehavioral development in the early life of the zebrafish. Fish with microbiota disruptions following antibiotic administration show abnormal locomotive activity (65), which might affect feeding behavior and foraging. The mechanisms ruling this interaction are still unknown.

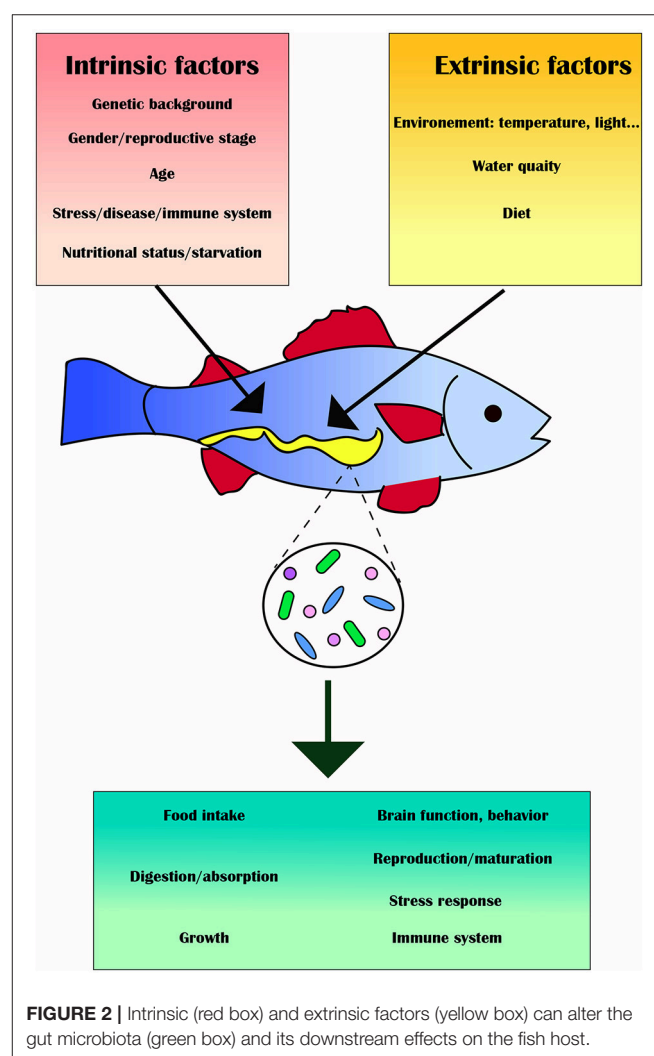
Immune Responses

Pathogens might disrupt brain and intestinal functions and hamper feeding and growth (66). It has been proposed that the microbiota protects the host from colonization and proliferation of environmental pathogens, a process known as “colonization resistance” (67, 68). Although mechanisms behind this resistance are not clear, it has been suggested that commensal bacterial species compete with pathogens for niche space and produce and secrete antimicrobial peptides (67). Any disruption of the intestinal balance mucosa may thus lead to infections and activation of the GALT (69). The associated commensal

microbiota of the mucosal immune system makes an important contribution to the immunity and metabolism of host fish, as the gut microbiota plays a major role in the development and maturation of the GALT (70, 71). For example, in both rainbow trout (70) and gilthead seabream (*Sparus aurata*) (72), administration of beneficial microorganisms (probiotics) enhances both the intestinal microbiota and the immune response.

FACTORS AFFECTING FISH GUT MICROBIOTA

Biotic (e.g., genotype, physiological status, pathobiology, life style) and abiotic (e.g., environmental) factors may affect the fish gut microbiota and influence its composition and diversity, as well as its function and metabolic activity, thus affecting feeding, growth, energy storage and health of the fish (73) (**Figure 2**). This section will review these intrinsic and extrinsic factors and provide specific examples in which the gut microbiota of various fish has been altered as a result.



Environmental Factors

Initially, fish embryos develop in a relative constant bacteria-free environment (within the egg or the mother). Fish are thus theoretically microbe-free at hatching, and gut microbes acquired post-hatch originate from surrounding environments (74). After hatching, fish are submitted to changing environmental factors (e.g., water composition and quality, and temperature), which can greatly influence the gut microbiota throughout their lifespan (52).

For example, the composition of gut bacteria differs between fish inhabiting freshwater and marine ecosystems (52). Generally *Aeromonas* and *Pseudomonas* predominate freshwater fish, whereas in marine fish, *Vibrio* is the most common genus (52). In black molly (*Poecilia sphenops*), an increase in salinity induces changes in dominant bacterial taxa in the microbiomes (75) and in rainbow trout, an increase in temperature results in an increase in microbial growth (76).

The gut microbiota of a species can also fluctuate over short time scales such as within 1 day (77) and days, or longer time periods (months or years) and this may be the result of seasonal variations (78).

Seasonal changes, accompanied by changes in temperature might induce alterations in food consumption due to variations in nutrient loads in the water column (79). As a consequence, the composition of the microbiota may be associated with particular seasons. For example, the gut bacterial load of tilapia decreases in winter compared to other seasons (79), and in the gut of hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*), *Pseudomonas*, *Micrococcus*, and *Flavobacterium* are only present in the winter (79).

Rearing conditions have also been shown to influence the composition of the gut microbiota. For example, in Atlantic salmon, fish held in two different holding conditions (indoor recirculating aquarium facility and cage culture in an open freshwater “loch” environment) have different microorganism compositions within their gut microbiota (80).

Pollutants and toxins present in the environment may also influence the fish microbiota. For example, common carp (*Cyprinus carpio*), exposed to waterborne copper (81) and zebrafish exposed to polystyrene microparticles (82) display disturbances of the intestinal microbiota related to immunity, which increase their susceptibility to pathogens and inflammation (microbiota dysbiosis). Other kinds of environmental chemicals such as pesticides [e.g., (83)], heavy metals [e.g., (84)] and antibiotics [e.g., (85)], can induce gut microbiota dysbiosis associated with changes in the intestinal mucus layer and inflammation in fish, thereby reducing the ability of absorb nutrients.

Host-Specific Intrinsic Factors

The variations in microbiota composition among fish might be due to several factors, including phylogeny, genetics and sex, age/life stages, and diet/feeding habits (86).

Genetics/Sex

Genetic background influences the gut microbiota and intra- and inter-specific variations in microbiota have been demonstrated.

Interspecies differences in the composition of gut microbiota among individuals of the same species may be present. For example, in rainbow trout, some bacterial groups are associated with specific families, perhaps due to different habitats or different diets (87). Inter-specific differences bacterial community structure are also seen, even if species exposed to the same environment [e.g., four freshwater larvae of silver carp, grass carp, bighead carp, and blunt snout bream (88)].

The sex of the fish may influence the gut microbiota through sex-specific host-microbe interactions, diet preferences or immune responses (89). For example, differences in the gut microbiota between sexes of the threespine stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*) have been reported (89), but the gut microbiota of the zebrafish is similar between sexes (61, 90). However, given the small number of published reports and the wide variation in studies, the underlying mechanisms are not yet understood.

There are still questions on whether genetics or the environment has greater influence on the gut microbiota. To date, host genetics has been considered the most influential in shaping the fish gut microbiota. Channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) raised under constant environmental and husbandry conditions have similar gut microbiota compositions, suggesting that a shared environment can overcome differences in host genetics (91).

Age/Sexual Maturity

Differences in the microbiota composition have been identified between juvenile and sexually mature individuals (44, 52). Zebrafish juveniles have higher bacterial richness in their gut microbiotas than elderly adult fish (44, 62), suggesting an increase response of gut microbiota to higher levels of circulating sex hormone levels in adult compared to juvenile fish (44). Furthermore, the GALT may interact differently with the gut microbiota in juvenile and mature zebrafish as this system is not fully developed in the juveniles (44). Similarly, in southern catfish, gut microbial diversity increases as the host ages (92).

Changes in the gut microbiota have also been shown during the early life development stages of fish. In Atlantic salmon (*Salmo salar*), intestinal microbiota compositions vary between embryonic stages, with embryonic communities having lower richness and diversity compared to those of hatchlings (93). In Malaysian Mahseer (*Tor tambroides*), larval, juvenile, and adult stages have higher gut microbiota diversity than fingerling and yearling stages (94).

It is possible that modifications in diet contribute to the differences between juvenile and mature fish gut microbiota. Fish of different ages and sexual stages might have different nutrient requirements and might adjust their diets and feeding rates to obtain adequate energy intake [e.g., in Gibel carp, juvenile fish require more proteins than pre-adults (95)]. As the diet and gut microbiota composition change with age, is likely that the nature of the contribution of the gut microbiota to host homeostasis also changes. However, further research is needed to explain this

potential relationship between fish age, fish gut microbiota and host energy homeostasis.

Feeding Habits/Diet

Feeding habits can greatly influence the structure and composition of the gut microbiota (52). Gut bacterial diversity is generally lower in carnivores, and progressively increases in omnivores and herbivores (71). For example, the most abundant bacteria in herbivorous fish include *Clostridium*, *Citrobacter*, and *Leptotrichia*, *Cetobacterium* and *Halomonas*, in omnivorous fish, and *Clostridium*, *Cetobacterium*, and *Halomonas* in carnivorous species (96–98). This trend has been found in both marine and freshwater fish, suggesting that the trophic level is likely one of the most influential factor affecting the gut microbiota composition (86).

The gut microbiota can also vary within species of the same trophic level. For example, the gut microbiota of four herbivorous Asian carp species (silver carp, *Hypophthalmichthys molitrix*; bighead carp, *Hypophthalmichthys nobilis*; grass carp, *Ctenopharyngodon idella*; and common carp, *Cyprinus carpio*) reared in the same environmental conditions exhibits inter-specific differences, in particular with regards to the relative abundance of the cellulose degrading phyla *Firmicutes*, most probably due to species-specific diets (99).

A limited number of studies show that modifying the diet of the fish can result in alterations of the gut microbiota, but this is not always the case. Diets containing guar gum, (non-starch polysaccharide) fed to the omnivorous mullet, *Mugil liza* (100) or soy proteins to the carnivorous rainbow trout (101) induce alterations in the bacterial quantity and composition in the GIT. In zebrafish, administration of dietary nucleotides results in modifications of the microbiota and reduction in fatty acid oxidation in muscle and liver as well as lower inflammatory tone (102). However, in channel catfish, different diets with different protein sources including animal and plant meals only have minimal effects on gut microbiota (103).

Extreme dietary changes, such as fasting also shape the fish gut microbiota. During times of fasting, morphological changes in the GIT occur due to the reduced nutrient uptake, which may account for changes observed in the gut microbiota (101). Furthermore, a depletion of nutrients induces changes in gut microbiota composition to favor bacterial species/communities that use more diverse energy sources and are capable of survival under limited nutrient conditions (52). Microbiota gut diversity and richness are usually higher under feeding conditions than under fasting conditions as seen in zebrafish (58) and in leopard coral grouper (*Plectropomus leopardus*) (104). In grouper, the dominant phyla are *Proteobacteria* in fasting and *Firmicutes* in fed conditions (104) and in the Asian seabass, *Lates calcarifer*, fasting induces a significant enrichment of *Bacteroidetes* and a depletion of *Betaproteobacteria* (105).

The act of feeding itself can also influence the microbiota. In rainbow trout, a short time after feeding (3 h) is not sufficient to cause any significant changes in the bacterial composition, but can cause changes in species richness and relative abundance (106). Similarly, in Southern catfish, although the diversity remains the same, the relative abundance of bacterial phyla differs

at 3, 12, and 24 h after feeding, suggesting that short-term food digestion may alter community structure, but has less effect on the microbial composition (92).

GUT MICROBIOTA MANIPULATION AND APPLICATIONS

The role of the fish gut microbiota in host physiology has become increasingly evident with growing research in this field. Several experimental methods have been used to assess the role of the fish gut microbiota via the manipulation of these communities, including gnotobiotic, antibiotic, probiotic and prebiotic, and symbiotic studies (Table 1).

Gnotobiotic Fish

Gnotobiotic animals (or gnotobiotics) are animals with a known microbiota composition. These include germ-free (or axenic) animals and axenic animals that have been inoculated with known microorganisms. Studies involving gnotobiotic fish allow the control over many variables that affect the development of the microbiota and analysis of host responses to specific gut microorganisms (14, 52). The disadvantages of this type of study is the complex procedures involved in the production and maintenance of gnotobiotics (52).

Antibiotics

Antibiotics (or anti-bacterials) can be considered environmental factors affecting the gut microbiota. In the aquatic environment, they may be found naturally or as pollutants discharged as metabolites [such as sulfamethoxazole (SMX) and oxytetracycline (OTC)] through feces or urine of treated humans or animals (107). Mosquitofish (*Gambusia affinis*) exposed to antibiotics display lower community diversity and taxonomic composition from both skin and gut microbiomes, compared to untreated fish (108) and in zebrafish, exposure to OTC results in a disruption of the intestinal microbiota (107). Antibiotics can be used to manipulate the microbiota, as they kill or inhibit the growth of specific bacteria. The administration of antibiotics does not completely eliminate gut microbiota communities but can cause significant changes in the microbial composition.

The use of antibiotics in aquaculture for disease prevention and treatment is common. However, antibiotics may disrupt the microbial communities and increase disease susceptibility (56, 118). Furthermore, antibiotics can bioaccumulate in animal tissues (56) and lead the development of drug-resistant bacteria, which can be passed along the food chain (119, 120). Due to the growing awareness of the disadvantages of antibiotics, strict regulations have been established in the aquaculture industry and alternative methods are being developed and tested (121). Treatment of fish with beneficial microorganisms (probiotics) is a promising solution to antibiotics, as these probiotics inhibit the colonization of potential pathogens by producing antibacterial peptides and competing for nutrients with detrimental bacteria (122). Probiotics may thus reverse the negative effects of antibiotics and improve fish health. For example, in black molly *Poecilia sphenops*, successful colonization of two probiotic species

TABLE 1 | Fish gut microbiota via the manipulation techniques used in research (see references in text).

Microbiota manipulation	Objective	Potential use	Advantages	Disadvantages	Examples of host species examined	Representative references
Gnotobiotic	Establish a germfree (gut microbiota absent) host or a known microbiota composition (predefined microbiota)	Examine the effects of an absence of microbiota (germ free) on the host physiology or the effects of specific gut microorganisms and/or a predefined microbiota on the host	Control over multiple variables and analysis of host responses to specific changes in microbiota	Complex procedures required to produce and maintain gnotobiotics	Zebrafish	(52) (14)
Antibiotic	Inhibit or eliminate targeted gut microbiota bacteria and/or bacterial pathogens	Bacterial disease prevention/treatment	Bacterial disease prevention/treatment	Potential to disrupt microbial communities and increase disease susceptibility, and bioaccumulation	Zebrafish, Mosquito fish Black molly	(107) (108) (109)
Probiotic	Establish beneficial gut microbiota bacteria	Use in aquaculture, improve fish health (digestion/growth), health management, disease prevention	Enhancement of immune function of the host, resistance to pathogens, and overall health	No known disadvantages. Probiotics are considered safe overall.	Zebrafish Rainbow trout Malaysia masheer	(110) (111) (112)
Prebiotic	Stimulate growth of probiotic bacteria	Use in aquaculture, improve fish health (digestion/growth), health management disease prevention	Enhancement of immune function of the host, resistance to pathogens, and overall health	No known disadvantages. Prebiotics are considered safe overall.	Rainbow trout Nile tilapia Common carp fry	(113) (114) (115) (116)
Symbiotic	Establish prebiotic and probiotic bacteria	Use in aquaculture, improve fish health (digestion/growth/immune system), health management, disease prevention	Improvement of immune response (better than probiotics alone) and increase in growth and feed utilization in host.	No known disadvantages. Symbiotics are considered safe overall.	Nile tilapia	(117)

(*Phaeobacter inhibens* and *Bacillus pumilus*) reverses the negative impacts of antibiotics, and decreases mortality rates (109).

Pro-, Pre- and Symbiotics

Probiotics are live or dead component of a microbial cells which confer a health benefit to the host through promoting beneficial intestinal bacterial species, whereas prebiotics are non-digestible food ingredients that selectively stimulate the growth of probiotics (73). The supplementation of probiotics and/or prebiotics into the diet of fish is believed to result in beneficial alterations of the gut microbiota and subsequent changes in metabolism and energy expenditure that are beneficial for the host (119, 123, 124). The administration of these supplements also enhance immune function of the host and increase its resistance to pathogens, enhancing general health and indirectly favoring feeding and growth (125). These effects highlight the potential for probiotics/prebiotics to enhance fish health by manipulating the fish gut microbiota.

Commonly used probiotics in aquaculture include members of the *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium*, *Shewanella*, *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacter*, *Pseudomonas*, *Clostridium*, and *Saccharomyces* genera (56). Multispecies probiotics may be more effective than single-strain probiotics as different strains present in multispecies probiotics increase the chance of survival in the

gut [as seen in rainbow trout (111)]. Probiotics increase the number of beneficial gut bacteria. For example, feeding fish with probiotics results in a higher abundance of core gut bacteria in zebrafish (110), and an increase in bacterial diversity in rainbow trout (111) and Malaysian mahseer (112).

Although exceptions exist, probiotics generally promote feed efficiency and growth in fish [e.g., tambaqui *Colossoma macropomus* (126), Japanese flounder (43), tilapia (123), carp (127), red seabream *Pagrus major* (128), trout (111)], likely by increasing nutrient absorption and perhaps feeding. An increase in absorption results from changes in intestinal morphology, with higher absorptive surface areas and higher microvilli densities in the intestine [e.g., tilapia (129), zebrafish (38), Malaysian mahseer (130)]. The effects on growth might be mediated by changes in the expression of growth-related genes. For example, following probiotics treatment, growth hormone (GH) expression levels increase in pituitary of Malaysian mahseer (112) and in liver of yellow perch (*Perca flavescens*) (131), and insulin-growth factor (IGF-1) expression (112) is upregulated in the liver of Malaysian mahseer (112) and yellow perch (131) and in body of European seabass (132).

Overall, the effects of probiotics on feeding have been little examined to date and remain unclear. In tambaqui (133), fish fed probiotics (*Bacillus subtilis*) and control diets have similar feeding ratios, and probiotics induce a

reduction in appetite in zebrafish [*Lactobacillus rhamnosus* (38)] and an increase in food consumption in pacu [*B. subtilis* (134)]. The discrepancies in results are likely due to variations in the nature and doses of probiotics used. These alterations in feeding might be partially due to the modulation of the expression of genes related to appetite. In larval zebrafish, administration of the probiotic *Lactobacillus rhamnosus* induces a decrease in brain NPY expression and an increase in adipose tissue leptin expression (38), and in goldfish, ghrelin intestinal expression is down-regulated in *Lactobacillus acidophilus* fed fish compared those of fed control diets (135).

In comparison to probiotics, prebiotics have received less attention with regards to their potential for aquaculture. However, overall, studies show that prebiotics have beneficial effects on growth performance, digestive enzyme activity, as well as disease and stress resistance of the host (136). Common prebiotics used in fish include inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), and mannanoligosaccharides (MOS) (137). Prebiotics have been shown to increase growth performance and feed utilization in some fish [e.g., rainbow trout, (113, 114), pacu *Piaractus mesopotamicus* (138), Nile tilapia (115), Caspian roach (*Rutilus rutilus*) (139)], but this is not always the case. For example, growth performance is not affected in common carp fry (*Cyprinus carpio*) fed inulin (116) or in pacu fed β -glucan (134). The increase in growth seen in some studies might be in part due to modifications in the intestinal structure (i.e., increases in intestinal villi height and digestive enzyme activities), as seen following probiotics administration. An improved immune system function [e.g., rainbow trout (114), Caspian roach (139)] and improved stress response [zebrafish (48)] most probably also largely contribute to a better growth performance of the fish.

Prebiotics and probiotics may also be administered in combinations, known as synbiotics (140, 141). Studies show that synbiotics improve the survival and implantation and metabolism of probiotic health-promoting bacteria in the GIT (142). Synbiotics have been shown to increase growth performance and feed utilization in the host, which may be a result of providing the host with energy and nutrients, and/or enhanced digestion processes (143). For example, following the supplementation of a probiotic (*Bacillus licheniformis*) and a prebiotic (yeast extract), growth performance is increased in Nile tilapia (*Oreochromis niloticus*), and this is accompanied by an increase in feed intake and feed utilization (118). The combination of probiotics and prebiotics in the diet also results in better immune responses than probiotics alone [e.g., rainbow trout (117)].

CONCLUSIONS

Several studies strongly suggest that the fish gut microbiota influences the overall health of the host fish with regards to overall physiology, digestion, stress response, reproduction, and the immune system.

Relatively few studies on the effects of the microbiota on energy homeostasis have been conducted to date and large variations exist between results, making them difficult to compare.

First, given the possible influence of genetics and the environment, and the low number of species examined, many more species need to be examined before conclusions can be made. Second, a variety of methods has been used for studying the fish gut microbiota, and the results obtained may vary depending on the experimental methods used, highlighting the need to develop appropriate standardized methods to describe fish microbiota (52). Studying the influence the gut microbiota may have on fish energy balance is challenging, as several different mechanisms of action are responsible, involving both local and endocrine pathways, different physiological systems (e.g., stress, immunity...) and molecules (hormones, metabolites...), and that all these systems interact (e.g., gut-microbiota-brain axis communication). Furthermore, each microorganism within the microbiota might have different actions. In addition, compared to terrestrial animals, fish are more exposed to constant environmental changes that could affect the microbiota.

Manipulating the gut microbiota of fish has great potential for aquaculture use to improve growth. However promising, the future of probiotics/prebiotics faces several challenges, including appropriate modes of treatment (oral, or in the water) and doses, the characterization of mechanisms of action of individual probiotic organisms, and quality control and regulation (144, 145). The fish model can also be useful to understand the gut microbiota in other vertebrate species such as humans. Zebrafish (58) and threespine stickleback (14) have been widely used as they are small fish that can be easily maintained in laboratory conditions, and have rapid development and generation times. In addition, their genomes are readily available and display structural and functional genetic similarities to humans.

Therefore, although progress has been done, much remains to be resolved using fish models for gut microbiota. Nonetheless, the research conducted to date has offered great insights into the mechanisms by which these communities are able to regulate the fish host, and provided insights into improving aquaculture practices, and better understanding the host microbe relationships among other vertebrates including humans, and the development of potential pathological treatments.

AUTHOR CONTRIBUTIONS

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

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Regulation of Feeding and Metabolism by Neuropeptide F and Short Neuropeptide F in Invertebrates

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Numerous neuropeptide systems have been implicated to coordinately control energy homeostasis, both centrally and peripherally. However, the vertebrate neuropeptide Y (NPY) system has emerged as the best described one regarding this biological process. The protostomian ortholog of NPY is neuropeptide F, characterized by an RXRF(Y)amide carboxyterminal motif. A second neuropeptide system is short NPF, characterized by an M/T/L/FRF(W)amide carboxyterminal motif. Although both short and long NPF neuropeptide systems display carboxyterminal sequence similarities, they are evolutionary distant and likely already arose as separate signaling systems in the common ancestor of deuterostomes and protostomes, indicating the functional importance of both. Both NPF and short-NPF systems seem to have roles in the coordination of feeding across bilaterian species, but during chordate evolution, the short NPF system appears to have been lost or evolved into the prolactin releasing peptide signaling system, which regulates feeding and has been suggested to be orthologous to sNPF. Here we review the roles of both NPF and sNPF systems in the regulation of feeding and metabolism in invertebrates.

Keywords: neuropeptide F, neuropeptide Y, short neuropeptide F, feeding behavior, neuropeptide evolution, G protein coupled receptor, protostomes, neuromodulation

INTRODUCTION

The nomenclature of the two distinct neuropeptide families, short neuropeptide F (sNPF) on the one hand, and neuropeptide F (NPF) on the other hand, has led to confusing data and conclusions in literature. Whereas, NPF shares a common bilaterian ancestor with vertebrate neuropeptide Y (NPY), the sNPF system appears to be protostomian-specific according to (1). In this review, we summarize work on the regulation of feeding and metabolism by NPF and sNPF in invertebrates and attempt to clarify annotation ambiguities that have become apparent upon large-scale phylogenomic analyses of NPF and sNPF systems across bilaterians (1, 2).

The NPY Family in Vertebrates

NPY is part of a large neuropeptide family that besides NPY, includes peptide YY (PYY) and the pancreatic polypeptide (PP) (3). The 36 amino acid NPY was isolated in 1982 from pig brain extracts (4) and was found to be widely distributed in the CNS of vertebrates (5, 6). Whereas, NPY is found at all levels of the brain-gut axis, PYY, and PP seem to be predominantly expressed by endocrine cells of the digestive system (7). The three peptides share a characteristic

secondary structure called the pancreatic polypeptide-fold (PP-fold) (8) that is fundamental for the full activation of the NPY G protein coupled receptors (NPYRs). These GPCRs comprise the Y1, Y2, Y4, Y5, and Y6 subtypes (9, 10). The Y1, Y2, and Y5 receptor isoforms preferentially bind NPY and PYY (11), while Y4 is specific for PP (12). The Y6 receptor exists as a truncated inactive protein in most mammals, and is only a functionally active receptor in mice and rabbits (13). While NPY centrally promotes feeding and reduces energy expenditure, PYY and PP mediate satiety. In addition, NPY family neuropeptides have various other functions that go beyond the regulation of feeding and appetite (14).

Discovery of NPF and sNPF Neuropeptides in Invertebrates

The first invertebrate NPY-like peptide was discovered in 1991 in the tapeworm *Moniezia expansa* using a C-terminally directed pancreatic polypeptide (PP) antiserum (15). HPLC purification of the PP-immunoreactive (IR) extract followed by automated Edman degradation sequencing identified a 39 amino acid tapeworm peptide that displays sequence similarities with the 36 amino acid vertebrate NPY (15). Based on its C-terminal sequence that ends with a phenylalanine (F) instead of a tyrosine (Y), this peptide was named neuropeptide F instead of neuropeptide Y. After this discovery, NPF-like peptides have been identified in other flatworms and in molluscs (16–20), all of which typically display an RPRF-amide C-terminal sequence and a length ranging from 36 to 40 amino acids.

The first sNPF peptides were discovered in insects, including the Colorado potato beetle *Leptinotarsa decemlineata* and the desert locust *Schistocerca gregaria* (21, 22) using antisera raised against *M. expansa* “long” NPF (23). These insect peptides consist of only 8 to 10 amino acids instead of 36 to 40 amino acids as typical for vertebrate NPY and flatworm or mollusc NPF. Based on their carboxyterminal RLRfamamide sequence, which is similar to the RPRfamamide motif of the “long” NPFs from flatworms and molluscs (15), they were designated “short” NPFs or sNPFs (24).

Discovery of NPF and sNPF Receptors in Invertebrates

NPF receptors (NPFs) were initially cloned from the brain of the pond snail *Lymnaea stagnalis* (25) and subsequently from *Drosophila* larvae (26, 27). Both receptors retained the typical features of vertebrate NPYRs and they showed the highest homology to the mammalian NPYR 2 isoform (28). Upon expression of these NPFs in Chinese Hamster Ovary (CHO) cells, it was found that the respective NPF peptides inhibit forskolin-stimulated adenylyl cyclase activity, in accordance with vertebrate NPYRs signaling through $G_{i/o}$ small proteins (25, 26, 29). In addition, the *Drosophila* NPF receptor can be activated by mammalian NPY-type neuropeptides when expressed in *Xenopus* oocytes (27).

The first sNPF receptor (sNPF) was cloned from *D. melanogaster* (30) and later on from the fire ant *Solenopsis invicta* (31) and the mosquito *Anopheles gambiae* (32). Different *Drosophila* sNPF variants elicit a calcium response in CHO

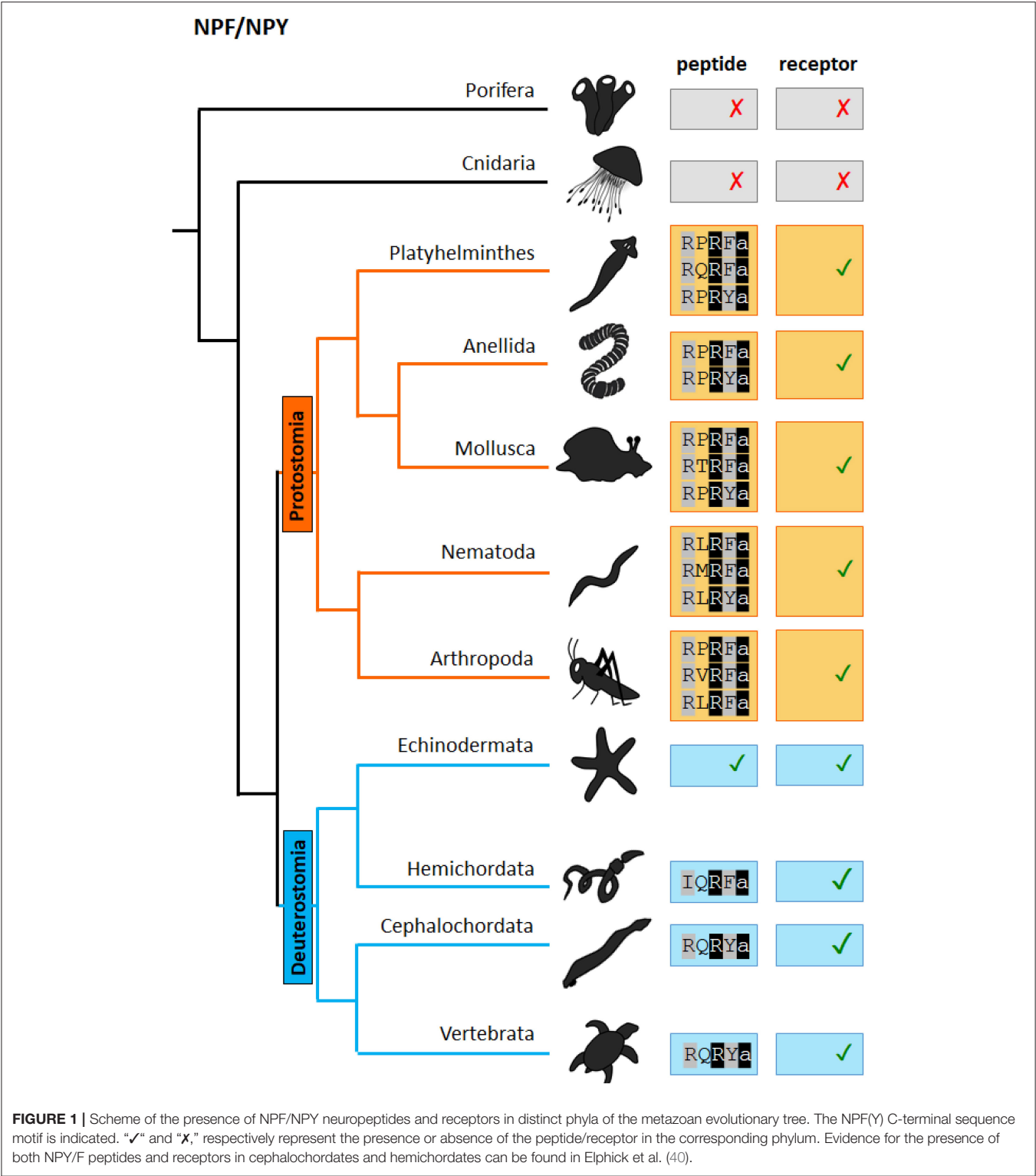
cells (30, 33) or in *Xenopus* oocytes (26, 34) when these are transformed to express the *Drosophila* sNPF.

Issues With Nomenclature

After the cloning of a long 36 amino acid NPF neuropeptide precursor in *Drosophila melanogaster* (35) and the sequencing of the *Drosophila* genome (36), it became evident that not all NPF/NPY-immunoreactive peptides that were designated as NPF were actually long NPFs such as those isolated from *M. expansa* and *D. melanogaster*. This led to the introduction of the terms long NPF (or simply NPF) and short NPF (sNPF) (24, 37). Alignment studies of neuropeptide precursors from both vertebrates and invertebrates pointed out the diversity in the consensus sequences for sNPF and NPF (38, 39), and suggested that short NPFs are restricted to protostomian phyla, while long NPFs are conserved across bilaterians.

In the past, several studies in insects have demonstrated overlapping functions of NPF and sNPF signaling with respect to feeding and metabolism, suggesting a common evolutionary origin of sNPF and NPF neuropeptides and receptors. This hypothesis was also supported by structural similarities between both invertebrate sNPF and NPF receptors and vertebrate NPY receptors (NPYR) (in particular with vertebrate NPY2R) (28, 39). Recent phylogenomic analyses of neuropeptide receptor families in bilaterians, however, show that sNPF and NPF receptors share only a distant common ancestor, explaining the structural similarities between both.

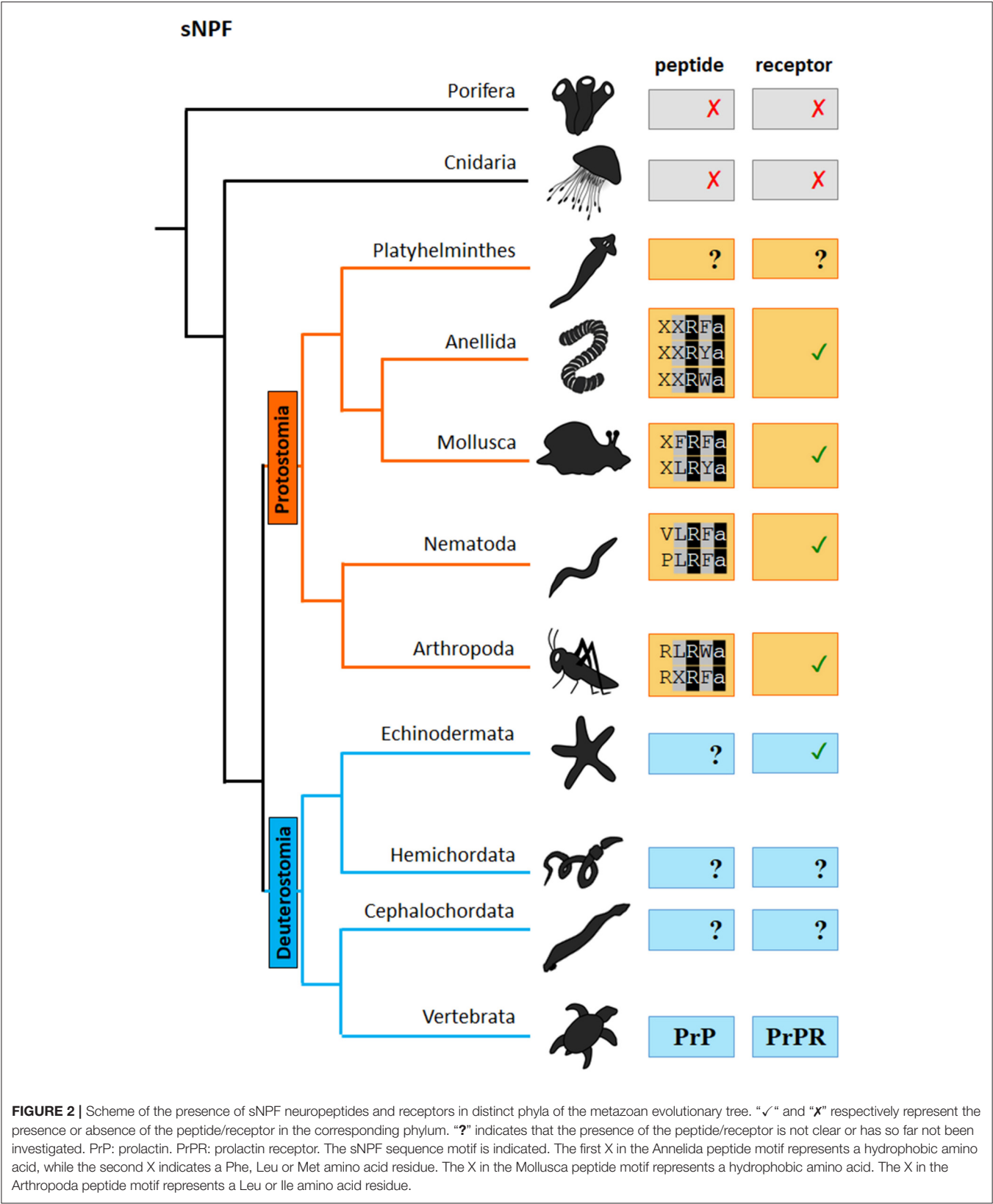
Both NPF and sNPF signaling systems in invertebrates have been implicated in the regulation of a diverse array of biological processes including reproduction, growth, nociception, circadian clock, learning, feeding and metabolism and they function mainly as neuromodulators or neurohormones [For an extensive review see (39)]. Here we will review past research on NPF and sNPF in the regulation of feeding-related behaviors and metabolism in protostomes. For each discussed phylum, we will briefly introduce the initial characterization of the sNPF and NPF signaling systems, followed by the current knowledge on their function(s) in feeding and metabolism. We will make a clear distinction between NPF and sNPF and will point to annotation ambiguities where appropriate. Clarifying annotation errors is not a goal here but will be crucial to further understand the evolution of the functions of sNPF and NPF neuropeptides signaling systems. Although the current picture is still not entirely clear due to insufficient genome information from all phyla, we now have increased insight in the evolutionary history of sNPF and NPF systems (**Figures 1, 2**). It is clear that NPF and sNPF are distinct neuropeptides systems, both involved in the regulation of feeding and metabolism. Both systems branched off from their common ancestor early in evolution, prior to the split of the deuterostome and protostome lineages. Therefore, NPF and sNPF signaling systems are a prime example to ask questions on the sustained evolutionary selection for both systems in protostomes in contrast to the deuterostomian chordate lineage where only the NPF orthologs (NPYs) have been conserved and even have been duplicated multiple times, but where the sNPF system seems to have been lost or evolved into the prolactin-releasing peptide system.



THE (LONG) NPF SIGNALING SYSTEM AND ITS ROLE IN FEEDING AND METABOLISM

Figure 3 shows an alignment of representative NPF neuropeptides that have been biochemically isolated

or identified by genome sequencing (for a broader overview of protostomian sNPF and NPF peptides, see Supplementary Tables 1, 2). NPFs generally consist of more than 28 amino acids, apart from the shorter predicted *C. elegans* orthologs and some truncated insect NPFs,



Schme_NPY	----SGLTKQKYSLFSGPEDLRNYLRQLNEYIALSSRP	RYa
Drome_NPF	----SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVR	Fa
Aplca_NPF	DNSEMLAPPRPPEEFTSAQQLRQYLAALNEYYSIMGRPR	Fa
Caeel_FLP-27	-----EASAFGDIIGELKG----	KGLGGRMRFa
Caeel_FLP-34_1	-----ADISTFASA----	INNAGRLRYa
Caeel_FLP-34_2	-----ALNRDSLVS----	LNNAERLRFa
Ampfi_NPY	-----ATTGDKALDAILSG----	QYRHHRLRYa
Ophvi_NPY	-----ALDAILSG----	QYRSHRLRYa
Ophar_NPY	-----ATTGDKALDAILSG----	QYRSHRLRYa

FIGURE 3 | Amino acid sequence alignment of representative NPFs from different invertebrate phyla. Genus and species abbreviations used in the alignment are: Schme, *Schmidtea mediterranea*; Drome, *Drosophila melanogaster*; Aplca, *Aplysia californica*; Caeel, *Caenorhabditis elegans*; Ampfi, *Amphipura filiformis*; Ophvi, *Ophionotus victoriae*; Ophar, *Ophiopsila aranea*. Identical residues are highlighted in black and conserved residues in gray.

and share the common RXRF/Yamide carboxyterminal motif (Figure 1).

NPF Signaling in *D. melanogaster*

Most studies on the regulation of feeding and metabolism by NPF were conducted in the genetically tractable model organism, *D. melanogaster*. In 1992, cloning and functional expression of two distinct *D. melanogaster* NPY-like receptors revealed that they could be activated by mammalian NPY and peptide YY (27). The first insect NPY-like peptide was also identified in *D. melanogaster*, and consisted of 36 amino acids with a characteristic RVRFa carboxyterminal sequence (35). The C-terminal F residue, instead of the vertebrate NPY-defining Y residue, prompted the name conversion from NPY to NPF, previously adopted in other protostomes as well (15). In 2002, *Drosophila* NPF was shown to dose-dependently activate the *Drosophila* NPY-like receptor NPFR when expressed in CHO cells (26). By means of immunocytochemistry and *in situ* hybridization, NPF was localized in the midgut and brain of *D. melanogaster*, suggesting a role in feeding, digestion and/or metabolism (35).

The first experimental evidence for a role of *Drosophila* NPF in the regulation of metabolism was provided by analysis of *npf* transcription levels following sugar exposure. A sugar-rich diet fed to *D. melanogaster* larvae evoked *npf* expression in two distinct neurons of the suboesophageal ganglion. Additional experiments with mutant flies deficient in sugar sensing, highlighted that not sugar ingestion, but taste perception of sugar was essential for *npf* expression (41). Subsequent studies showed that *npf* expression is high in young, foraging larvae and low in older larvae that display food aversion and burrowing. Experimentally induced overexpression and downregulation of *npf* transcript levels shifted these stage-specific feeding-related phenotypes (42).

In-depth characterization of the NPF signaling pathway revealed that NPF functions downstream of insulin signaling to regulate feeding in *Drosophila* larvae. NPF does not specifically influence total food intake, but may rather regulate food choice behavior (43, 44). NPF neurons are hypothesized to modulate the reward circuit to acquire lower-quality foods upon food deprivation (43). NPF signaling through its NPFR receptor

promotes the intake of noxious food in starved flies and inhibits the aversive response that is normally elicited. In satiated flies, however, activation of the insulin signaling pathway results in the inhibition of the NPF-induced feeding response toward noxious food (44). NPF thus regulates a feeding response, that integrates both food attractiveness and hunger state (45). Together, these studies show that lower quality food or noxious food can evoke an NPF-mediated feeding response in starved flies, while in metabolically satiated flies, NPF is inhibited resulting in the intake of higher quality food (44, 45).

NPF signaling is also required at the intersection of feeding and stress, namely for the regulation of cold-resistant feeding behavior (46). NPFR1 is expressed in fructose-responsive sensory neurons in the thorax, suggesting that NPF may modulate these neurons directly (47). NPF could modulate the activity of the transient receptor potential ion channel A (TRPA) called PAIN and inhibits the regular avoidance response to aversive stimuli.

NPFR1 colocalizes with the majority of dopaminergic neurons in the larval *D. melanogaster* CNS, suggesting extensive interplay between these two signaling pathways (48, 49). Firstly, NPF expression in the brain of *D. melanogaster* may represent the food-deprived state, in which dopaminergic neurons in the mushroom bodies (MBs) promote appetitive memory formation (48). Secondly, NPF signaling modulates dopaminergic transmission in *D. melanogaster* in appetitive olfaction. NPFR1-expressing dopaminergic neurons display projections toward DL2-lateral horn (LH) neurons that receive olfactory inputs, and modulation of these neurons by NPF is instrumental to stimulate odor-induced appetitive feeding (49). NPF thus can stimulate (MBs) or inhibit (DL2-LH neurons) dopaminergic signaling in functionally distinct neurons even if it does not appear to be implicated in octopaminergic regulation of feeding motivation (50). In relation to this, NPF has recently been proposed to modulate odor-aroused appetitive behavior through a newly characterized DA/NPF-mediated circuit (51). When an appetitive odorant is perceived, the information is integrated in the DL2 neurons and transmitted to Dop1R1 neurons that express NPF. The dorsomedial pair of NPF neurons are essential for the proper manifestation of odor-aroused appetitive behavior (51). NPF signaling also seems to be implicated in odorant detection and is required for sensitization and correct odorant

perception by a subset of olfactory neurons named antennal basiconics (ab)3A that besides NPFR also express the olfactory receptor (OR)22a, which responds to different fruity odorants (52). Related to motivation, sucralose can cause stimulation of a gustatory receptor that signals via dopamine and octopamine to activate a reward pathway in which NPF is also implicated. Supplementation of sucralose to the fly's diet caused an imbalance between energy and food sweetness that is signaled by the sweet taste receptor *Gr64a*. A mechanistic analysis of this sucralose response identified the NPF system as a critical downstream component in this pathway, confirming a conserved role for NPF in sucralose-sweetened food intake stimulation (53).

Since NPF is involved in the regulation of feeding and metabolism on multiple levels in *D. melanogaster*, its effect on obesity has also been studied. Leptin is a neuropeptide involved in the regulation of food attraction, food intake and body weight and its *Drosophila* homolog is Upd-1. Interestingly, the Upd-1 receptor *Domeless* is expressed in the brain's NPF-neurons. The odor-activated food response that is normally elicited by NPF and regulated by internal metabolic status (45), is perturbed in flies lacking Upd-1 in the NPF neurons. When the upstream regulation by *Upd-1* and *Domeless* is absent, NPF-neurons do not register the satiety status of the flies and always display odor responses on the same level as starved flies, leading to overconsumption of food and obesity (54). The enzymatic cofactor tetrahydrobiopterin (BH4) is another compound that can affect the activity of NPF neurons in satiety. BH4, synthesized by the adipose tissue, inhibits NPF signaling by blocking its release and thus induces satiety (55).

In *Drosophila*, NPF modulates both food intake and wakefulness (56). In particular, NPF signaling plays a fundamental role in wake extension during deprived feeding conditions, in order to facilitate the search of new food sources. However, the neuron clusters for wake and feeding phenotypes were completely unrelated, suggesting an independent regulation of the two behaviors (56).

NPF Signaling in Other Insects

Confusion between NPF and sNPF partly results from insect research where a clear distinction between both neuropeptide systems remained difficult for a long time, due to the identification of so-called "NPY-like" neuropeptides that differed in length from vertebrate NPY. Studies in the insects, *Locusta migratoria*, *Schistocerca gregaria* (57), and *Helicoverpa zea* (58), in which only a C-terminal fragment of the "full-length" NPF was isolated and identified by mass spectrometry, contributed to this confusion. Identification of the respective neuropeptide precursor sequences by RNAseq and genome sequencing has clarified this issue. Comparison of the coding sequences of these peptides shows that they are truncated forms of larger NPF neuropeptides and that the shorter C-terminal fragments may thus either be artifacts from the extraction procedure or may be created by extensive posttranslational processing *in vivo* (57, 58).

The earliest indications of NPF being involved in feeding and metabolic regulation in various insect species were based on immunocytochemical localization studies. Its expression in the digestive system or its temporal regulation of expression in

response to food intake suggested a regulatory role of NPF in the control of feeding and metabolism. In the yellow fever mosquito, *Aedes aegypti*, NPF-IR was detected in the midgut and in the subesophageal ganglion where the regulation of food intake resides. Analysis and sequencing of the immunoreactive peptides from head and midgut extracts confirmed the presence of NPF. *A. aegypti* NPF inhibits transepithelial ion transport in the anterior stomach, suggesting a function in the digestive system (59). The titer of *A. aegypti* NPF in the haemolymph is influenced by feeding and decreases drastically after a blood meal (60). Recent studies indicate that genetic and pharmacological disruption of the mosquito NPF pathway results in abnormal host-seeking behavior and blood-feeding (61).

In *S. gregaria*, however, injection of NPF increases food intake, while RNA interference of *npf* transcripts decreases food intake. These treatments resulted in weight gain for the peptide-injected group and stunted weight gain for the knockdown group, which implies a stimulatory role of NPF in feeding (62). *S. gregaria* NPF transcription is spatiotemporally regulated in response to feeding, with high levels in starved animals that drop in the brain, optic lobes, and midgut upon feeding, but significantly rise in the subesophageal ganglion (62). Also in *Bombyx mori*, knockdown of NPFR resulted in a reduction of food intake and growth, pointing toward a role for NPF as a positive regulator of feeding (63).

A typical long NPF as well as its truncated form have been found in *Rhodnius prolixus* (64). NPF-like-IR is present in the stomatogastric nervous system of *Rhodnius*, which regulates feeding. In addition, radioimmunoassay quantification showed a decrease in the intensity of NPF-like IR material in the cell bodies and axons after feeding, suggesting a release into the haemolymph (65).

In the honey bee *Apis mellifera* (66) and in the wasp *Nasonia vitripennis* (67), the NPF sequence does not retain the canonical RPRF/Yamide C-terminal sequence, displaying instead a C-terminal KARYamide motif. Hymenopteran NPF protein precursor sequences show nevertheless clear similarities with NPF/Y precursors of other invertebrate phyla, having YY residues in position 30–31 as well as other conserved amino acids. Interestingly, genomes of hymenopteran species do not seem to encode a clear ortholog of the NPF receptor. It is therefore not unthinkable that evolutionary pressure drastically shaped the so far unknown receptor in hymenopterans into a GPCR with unrecognizable orthology and that receptor-neuropeptide co-evolution resulted in a modification of its ligand.

In *A. mellifera*, NPF levels differ according to the age and tasks of the worker bees. Younger workers providing brood care display low NPF intensities in *in situ* hybridization and qPCR analysis, while older workers that are responsible for foraging display higher expression levels (68). This may suggest a stimulatory role for NPF in food searching and foraging behavior of worker bees, but experiments providing a causal relationship are currently lacking.

NPF Signaling in Other Arthropods

Information about NPF orthologs in arthropods outside the insect orders is currently increasing. Bioinformatic analysis of

genomes and transcriptomes revealed the presence of NPF and NPF receptors in chelicerates, including spiders, scorpions and mites (69). An *in silico* analysis using the *D. melanogaster npf* transcript as a query against the crustacean expressed sequence tags (ESTs) database revealed putative hits for the shrimp *Marsupenaeus japonicus* and the water flea *Daphnia magna* (70). The predicted pro-peptides, respectively, encode a 32 and 38 amino acid NPF peptide, which both display the typical RPRFamide carboxy terminus. A similar analysis also revealed an NPF ortholog in *Daphnia pulex* (71). In another study, *npf* transcripts have been isolated from mixed eyestalk ganglia of *Litopenaeus vannamei* and from the brains of *Melicertus marginatus* (72). Both penaeid shrimp species have two identical transcript sequences and one transcript differs from the other by an in-frame 37-amino acid insertion in the middle of the coding sequence of NPF. Both transcripts are broadly distributed in the nervous system of the animals and the short one is also expressed in some of the midgut samples. A diet supplemented with the shorter NPF induced a significant increase in food intake and growth in juvenile *L. vannamei* suggesting an orexigenic action for the NPF (72).

While a neuropeptide from the crab *Pugettia producta* has been suggested to resemble vertebrate NPY because of its SQRYamide carboxyterminal sequence (73), recent phylogenetic analysis indicates that the RYamide neuropeptide family, which is widespread in arthropods (74, 75), is evolutionarily distinct from the NPF/NPY peptide family (1).

NPF Signaling in Nematodes

In nematodes, neuropeptide signaling systems suggested to be orthologous to NPY/NPF were initially discovered in *C. elegans* (76, 77). Neuropeptide receptor 1 (NPR-1) was cloned from a solitary wild-type strain and firstly assigned to the NPYR family based on its sequence similarity with the vertebrate NPYR groups (78). In a following whole-genome analysis study, using the *L. stagnalis* lyminokinin GPCR as a query, *C. elegans* NPR-1, NPR-2, NPR-3, NPR-4, NPR-5, NPR-6, NPR-7, NPR-8, NPR-10, NPR-11, NPR-12, and NPR-13 were all annotated as NPY-like receptors (76, 79). Two of these NPRs, NPR-11, and NPR-12 cluster closest with the *Drosophila* NPFR as shown by phylogenetic tree analysis (76) and thus appear to be true NPF homologs in *C. elegans*. These NPFR orthologs are also encoded in the genomes of all other nematodes investigated (76, 80).

A specific role in feeding or metabolism has not been investigated for the nematode NPF system. The only functional study that has been carried out is on the NPFR ortholog, NPR-11, which is involved in local search behavior of *C. elegans* when the animal is removed from its bacterial food source (81). In this work, the neuropeptide-like protein 1 (NLP-1), although not containing a peptide with a typical NPF-motif, was reported to act upon NPR-11 in the AIA interneuron to modulate local search behavior.

NPF Signaling in Platyhelminths

The first evidence for NPY orthologs in platyhelminths was found in the central and peripheral nervous system of the cestode *M. expansa*, from which a 39 amino acid peptide was identified by

plasma desorption mass spectrometry (PDMS) upon isolation monitored by pancreatic polypeptide (PP) antiserum (15). The primary structure of this *M. expansa* NPF neuropeptide displays strong sequence similarities with vertebrate neuropeptide Y (15). Subsequently, immunochemical analysis using antibodies against *M. expansa* NPF and against vertebrate PP, Peptide tyrosine tyrosine (PYY) and Substance P (SP) revealed the presence of immunoreactive substances in other cestodes (82–85) and other flatworm classes, including turbellarians (16, 86, 87), monogeneans (88–91), and trematodes (17, 92–94). So far, the immunopositive material has only been identified in *Arthurdendyus triangulatus* (16), *Schistosoma mansoni* and in *Schistosoma japonicum* (17). All three platyhelminth peptides have a length between 36 and 39 amino acids and display the NPF-typical RPRF carboxyterminal sequence. In addition, phylogenetic tree analysis identified several GPCRs similar to NPF/Y receptors in the *S. mansoni* GPCR repertoire (95).

Studies on a putative role of NPF in feeding and metabolism have as yet not been performed in platyhelminths, but PP-, PYY-, and *M. expansa* NPF-IR is present in neurons innervating the oral and ventral suckers of *Schistosoma mansoni* (94), in the pharynx musculature of *Procerodes littoralis* (87) and in fibers and cells of the intestinal wall of *Microstomum lineare* (86), suggesting a possible role in the modulation of food intake and digestion. However, these results should be interpreted with caution as cross immunoreactivity with other neuropeptides cannot be excluded.

NPF Signaling in Molluscs

One year after the identification of NPF in platyhelminths, several independent studies demonstrated the presence of NPF-like peptides in different species of molluscs. Following the detection of PP-IR in circumoesophageal ganglia extracts of the garden snail *Helix aspersa* (18), PMDS and automated Edman degradation led to the identification of an of 39 amino acid peptide that displays pronounced sequence similarities with NPF (18). Two additional NPF orthologs were discovered in the sea slug *Aplysia californica* (19) and in the cephalopod *Loligo vulgaris* (20). All display the C-terminal RPRF motif that typifies invertebrate NPF (19). Interestingly, gel permeation chromatography of the squid extract resolved two peaks of PP-IR with different molecular weights. One was suggested to contain (long) NPF and the second one contained a nine amino acid peptide harboring an N-terminal tyrosine and the NPF-typical RPRF C-terminus, but lacking the PP-fold structure (PYF) (20). This short peptide is either the result of an extraction artifact or the product from another as yet unknown neuropeptide precursor.

The only NPY/NPF receptor homolog known in molluscs to date has been cloned in the snail *L. stagnalis* (25). It is broadly expressed in the CNS. The 39-amino acid *L. stagnalis* NPF, which displays the typical RPRF C-terminal sequence and the N-terminal proline functionally activates this receptor, when expressed Chinese Hamster Ovary (CHO) cells (25).

A role for NPF in feeding and metabolism has been reported in various molluscan species. Injection of *A. californica* NPY (Aplca-NPY or better Aplca-NPF) into the hemocoel of the

animal resulted in a dose-dependent reduction of food intake (96). In *A. californica*, the feeding process consists of an initial ingestion program triggered by the presence of food and initiated by the cerebral ganglion that is progressively converted to an egestion program when the esophagus and the gut signal satiation. The change in feeding state depends on the activity of the feeding central pattern generator (CPG) that receives signals derived from cerebral and buccal ganglia. In particular, the cerebral-buccal interneuron 2, located in the cerebral ganglion, initiates the ingestion program promoting the activity of the CPG interneuron B40. When the animal approaches satiation, the release of the Aplca-NPF enhances the egestion program by reducing the activity of B40 and promoting the activation of the egestion-promoting neuron B20, also located in the CPG. Aplca-NPF thus acts as a satiety signal that balances the activity of the antagonistic CPG interneurons B40 and B20 to potentiate the egestion program (96). Although the role of NPY as an orexigenic agent has been widely demonstrated in vertebrates (97–99) and in *D. melanogaster* (35, 41), the *A. californica* ortholog seems to display the opposite function. In humans and rodents, the gut released peptide YY_{3–36} isoform (PYY_{3–36}) has been reported to inhibit food intake (100). It is produced postprandially by the intestinal L-cells in proportion to the calories ingested and, upon release in the blood circulation, it reaches the arcuate nucleus of the hypothalamus (101). Here, PYY_{3–36} acts on NPY_{2R}, which is an inhibitory presynaptic receptor expressed in NPY neurons, modulating the activity of the NPY orexigenic pathway and leading to decrease in appetite (100, 101). Therefore, the localization and the functional activity of Aplca-NPF seems to be more closely related to the vertebrate PYY_{3–36} than to NPY (96). However, administration of *L. stagnalis* NPY (Lymst-NPY or better Lymst-NPF) in the snail led to a reduction in growth and reproduction without clear short-term effects on food intake (102). Thus, NPF regulation of energy flows appears to be conserved in *L. stagnalis*, while the regulation of food intake seems to be controlled by a leptin-like factor named *L. stagnalis* storage feedback factor (Lymst-SFF). This peptide is released from the glycogen cells of the mantle edge lining the shell of the animal that represents the only energy reserve in *L. stagnalis*.

A recent study on filter feeding has reported a “vertebrate-like” action of *Ruditapes philippinarum* NPF (Rudph-NPF) (103). Bivalves actively control food uptake by adjusting the filtration rate according to internal metabolic signaling. In accordance with this, injection of Rudph-NPF in the hemocoel resulted in a dose-dependent increase of 23% of filtration rate. In addition, a qPCR analysis demonstrated a large increase of Rudph-NPF mRNA levels in the visceral ganglion during starvation that rapidly declined after feeding, resembling the NPY expression pattern in vertebrates (104–107). Rudph-NPF injection was also associated with an increase in insulin and monoamine (serotonin and dopamine) levels, suggesting a coordinated regulation of filter feeding by multiple internal signaling pathways. In conclusion, a controversial role of mollusc NPF in the control of feeding has emerged from the works reviewed here: Aplca-NPF has an anorexigenic effect and seems to be functionally related to the vertebrate PYY_{3–36} isoform, while the Rudph-NPF showed an orexigenic effect more related to the vertebrate

NPY. More interestingly, the Lymst-NPF does not exhibit any clear regulation of food intake, but modulates growth and reproduction. A possible explanation for this diversity could be related to the differential tissue expression of NPF receptors that are present in the different mollusc species. Since only the NPF receptor of *L. stagnalis* has been characterized so far, additional receptor identification studies are needed to elucidate the role of NPF in the regulation of feeding in molluscs.

NPF Signaling in Annelids

The presence of NPF(Y) orthologs in annelids has been assessed by bioinformatics in the genomes of the earthworm *Lumbricus rubellus*, the polychaete worms *Alvinella pompejana* and *Capitella telata*, and the leech *Helobdella robusta* (108, 109). In all species the predicted NPFs range from 29 to 43 amino acids in length and in the oligo- and polychaetes they display the NPF-typical RPRFamide carboxy terminus. In addition, a large transcriptome analysis of *Platynereis dumerilii* identified four NPF paralogous named NPY-1 to 4 of 38 to 48 amino acids in length (110). All the four genes show the typical RPRFamide carboxyterminal sequence and a partial sequence of the NPY-4 pro-neuropeptides has been confirmed by mass spectrometry (110). Although the NPY-4 receptor 1 does not seem to cluster with vertebrate NPY receptors, screening of *P. dumerilii* orphan GPCRs against *Platynereis* peptide mixtures revealed that this receptor is activated by three of NPF paralogues (NPY-1, NPY-3 and NPY-4, all displaying the NPF-typical RPRFamide carboxyterminal motif) in a calcium-based cellular receptor assay (111).

NPF encoding genes seem to have expanded in annelids. In *Capitella*, three predicted paralogous genes encode NPF (109), and in *Helobdella* five paralogues have been predicted, of which one is probably a pseudogene. Interestingly, three of these five paralogous sequences contain an NPY-typical RPRYamide C-terminal motif instead of the canonical invertebrate RPRFamide sequence (109). It has been shown that leech salivary gland neuropeptides, including NPY, aid in suppressing inflammation in their hosts from which they suck blood (112) and therefore, some of the diversified NPF(Y)s in leeches might be the result of convergent evolution because of their ectoparasitic lifestyle. An alternative explanation resides in the genome organization, gene structure, and functional content of the Spiralia (annelids and molluscs), which appear to be more similar to those of some invertebrate deuterostome genomes (*Amphioxus* and sea urchin) as well as non-bilaterian metazoans (such as cnidarians, sponges, and placozoans) than to those of ecdysozoan and platyhelminth protostomes that have been sequenced to date (113, 114). On the other hand, similarity-based clustering for neuropeptides and GPCRs of metazoans revealed that the predicted NPF peptides and receptors of annelids strongly cluster with the ones of arthropods, platyhelminths, and molluscs (2). Evidently, for a comprehensive genomic understanding of the metazoan radiation, a far larger sampling of genomes will be needed. Since only bioinformatic and phylogenetic analyses have been performed on annelids NPFs, their function remains elusive as yet.

NPF Signaling in Echinoderms

One of the first investigations on the presence of evolutionarily conserved neuropeptides in echinoderms has been carried out in the starfish *Marthasterias glacialis* (115). Among the tested antisera, porcine PYY and human PP-like IR was found to be present in the endocrine cells and the basoepithelial plexus of the digestive tract. Analysis of the sea urchin *Strongylocentrotus purpuratus* genome revealed the existence of NPF(Y) receptors (116, 117). In ambulacrarians that comprise both echinoderms and hemichordates both NPY receptors and NPY neuropeptides (in *Saccoglossus kowalevskii*) have been predicted (2) (1, 118). In addition, a bioinformatic study focussing on echinoderms has recently shown the presence of NPY orthologs in *Ophiopsila aranea*, *Asterias rubens* and *Amphiura filiformis* (119). The aligned peptides lack the RQRYamide canonical consensus sequence of vertebrate NPYs but do contain a conserved RYamide carboxy terminus as well as other key amino acids (Figure 3). Functional studies on echinoderm NPY are currently lacking.

THE SHORT NPF SIGNALING SYSTEM AND ITS ROLE IN FEEDING AND METABOLISM

Short NPFs are short neuropeptides of 8 to 12 amino acids in length and display the typical C-terminal consensus sequence M/T/L/FRFa (Figures 2, 4). As mentioned above short NPF and long NPF (NPY) are evolutionarily distant from one another. It has long been assumed that sNPF neuropeptides are confined to arthropods (<http://www.neurostresspep.eu>), but it has now become clear that both sNPF and NPF systems already originated in the common ancestor of protostomes and deuterostomes. Whereas, the long NPF (NPY) signaling system has been retained in both lineages, the short NPF signaling system appears to have been highly conserved across protostomes, and possibly in echinoderms (deuterostomes) as well. This raises important questions as to whether the regulatory functions of sNPF and NPF systems can be correlated with the differential lifestyles and environments of respective species. Although the sNPF system seems to have been lost in vertebrates, sNPF receptors have been shown to cluster with vertebrate prolactin-releasing peptide receptors (2), which also have a prominent role in the regulation of feeding behavior. Further research is needed to clarify this issue.

sNPF Signaling in *D. melanogaster*

In *Drosophila*, four sNPF neuropeptides were predicted from the genome sequence (120), and later on identified by mass spectrometry-based peptidomics (121). Their cognate receptor was identified by means of a calcium-based receptor assay in CHO cells (30).

The use of *snpf* mutant flies showed that sNPF is involved in the regulation of food intake and body size in *D. melanogaster*. sNPF increases food intake in larval and adult flies, yet does not prolong the feeding period in larvae or modulate food preferences, as opposed to NPF (122). Pathway analysis

Caeel_FLP-15	--GGPQGPLRFa
Caeel_FLP-18	---EMPGVLRFa
Caeel_FLP-21	--GLGPRPLRFa
Cragi_sNPF	-----GSLFRFa
Aplca_sNPF	----GGALFRFa
Drome_sNPF_1	ASRSPSLRLRFa
Drome_sNPF_2	-----PQRLRwa
Aedae_sNPF_1	---APQLRLRFa
Aedae_sNPF_2	---APSQRLRwa
Capte_LFRWa_1	----DSPVFRwa
Capte_LFRWa_2	-SSPKPMRLRwa
Plasp_RYa_1	-----GTLMRYa
Plasp_RYa_2	-----VFRYa

FIGURE 4 | Amino acid sequence alignment of representatives of sNPFs from different invertebrate phyla. Genus and species abbreviations used in the alignment are: Caeel, *Caenorhabditis elegans*; Cragi, *Crassostrea gigas*; Aplca, *Aplysia californica*; Drome, *Drosophila melanogaster*; Aedae, *Aedes aegypti*; Capte, *Capitella telata*; Plasp, *Platynereis* species. Identical residues are highlighted in black and conserved residues in gray.

of the sNPF signaling system revealed an interaction with insulin signaling regulating growth. sNPF activates extracellular-activated receptor kinases (ERKs) in insulin-producing cells (IPCs), which in turn modulate the expression of insulin (123, 124). In addition, insulin signaling is implicated in a negative feedback loop controlling sNPF expression and inhibiting food intake (124–127). In starved flies where insulin levels are low, sNPF1 expression is upregulated resulting in facilitation of food search behavior (125). A microarray study revealed additional interaction partners of the sNPF system in *D. melanogaster*. The most pronounced and confirmed upregulated gene after sNPF administration is *mbn*, a Mbn/Dyrk1a kinase that activates the FOXO transcription factor through Sir2/Sirt1 deacetylase action (126). FOXO then in turn activates *snpf* transcription, providing a positive feedback loop. Mbn/Dyrk1a was localized in sNPF1-expressing neurons, further evidencing an interaction with the sNPF signaling system (126). Genetic experiments using a combination of RNAi and overexpression lines of sNPF1 and putative interaction partners revealed that activation of *mbn* transcription is attained through $G_{\alpha s}$, PKA and CREB (126). In addition, there is a positive feedback loop from CREB to sNPF in the regulation of energy homeostasis. CREB can dimerize with cAMP-regulated transcription coactivator (Crtc) to stimulate the expression of sNPF, resulting in an attenuation of the immune response and an increased starvation resistance. When this CREB/Crtc-dependent activation of sNPF is absent, the immune response is stimulated while depleting energy reserves (128).

MicroRNA (miRNA) regulation was also reported to be involved in the sNPF/Dilp signaling pathway in *D. melanogaster* IPCs. The conserved miRNA *miR-9a* is able to bind to the 3'-UTR of *sNPF1* mRNA and downregulates translation leading

to a decreased production of sNPFR-1 in the IPCs (129). This inhibition of the sNPF signaling system results in a growth reduction. The NPF and sNPF system in *D. melanogaster* thus modulate feeding behavior in distinct manners but are both clearly essential for proper control of food intake and metabolism. The importance of the influence of both these systems on this delicate balance was demonstrated in a study investigating resistance to amino acid starvation. Reducing or increasing the expression of either *npf* or *snpf* drastically decreases the resistance to amino acid starvation and reduces lifespan on culture media deprived of amino acids (130).

sNPF Signaling in Other Insects

Four peptides, termed “head peptides” were isolated from *A. aegyptii* head extracts using an FMRFamide-directed antiserum (131). These head peptides have long been assumed to be the sNPFs of *A. aegyptii*. However, they could not be detected in the completed *A. aegyptii* genome by bioinformatics, nor in any tissue by mass spectrometric analyses. In contrast, the mature peptides encoded by the *snpf* gene of *A. aegyptii* were found to be abundantly present in different parts of the CNS and also in the gut (132). Furthermore, *A. aegyptii* NPYR1 is activated by sNPF-3, which indicates that this receptor is an sNPF receptor, and not an NPY receptor ortholog as stated in the study (133).

The intricate involvement of sNPF in feeding and metabolism has been demonstrated in many insects but depending on the species sNPF can act as a stimulating or inhibiting factor.

In *A. aegyptii*, multiple sNPFs inhibit both serotonin-induced peristaltic contractions and ion transport of the anterior stomach using *in vitro* preparations, thus showing a negative modulation of serotonin-induced digestive action (59). *Aedes* sNPF receptor expression is significantly upregulated for 3 days post blood-feeding with a peak 48 h after the blood meal (133). In contrast to sNPFR expression, the amount of sNPF significantly drops in the antennal lobes following a blood meal. This drop in sNPF coincides with an inhibition of odor-mediated host-seeking behavior. Injection of sNPF is sufficient to mimic this inhibition in host-seeking behavior (134). In the mosquito *Culex quinquefasciatus*, sNPF precursor and receptor expression drops 27 h post sugar feeding, while a significant increase in sNPFR could be observed 27 h post blood-feeding (135). Thus, there seems to be a specific difference in the regulation of sNPF signaling according to the meal type. Taken together, these studies show that tissue-specific changes in components of the sNPF signaling pathway are instrumental in subtle modulation of feeding, food choice and food searching-related behaviors in mosquitoes.

In *S. gregaria*, sNPF transcription is inhibited in starved animals, while there is a temporal increase of sNPF transcription immediately after feeding (136). sNPF injection inhibits food intake and knockdown of sNPFR or sNPF precursor significantly increases food intake in *S. gregaria* (136, 137). These results prompted the idea that sNPF functions as a satiety factor that inhibits food intake in *S. gregaria*. Further characterization of the sNPF signaling pathway in *S. gregaria* revealed that the nutrient

content in the haemolymph regulates sNPF transcription through the insulin signaling pathway (138).

In the cockroach *Periplaneta americana*, an upregulation of sNPF-IR cells in the midgut was shown upon starvation, while expression of digestive enzymes was drastically downregulated. In addition, refeeding significantly decreased sNPF IR within 3 h, suggesting an inhibitory function of sNPF on digestion (139). However, the possibility that the sNPF antiserum also recognizes NPF cannot be dismissed. *Ex vivo* incubation experiments of *P. americana* showed that sNPF directly inhibits the release of proteases, amylases, and lipases when co-incubated with midguts actively producing these digestive enzymes (139). sNPF injection in fed cockroaches increases locomotion to a level comparable to that of starved animals (140). This suggests that sNPF modulates locomotion in starved animals and that increasing circulating sNPF levels in fed animals override satiety and evoke food searching behavior.

In the silkworm *B. mori*, starvation caused decreased transcriptional levels of sNPFR, which again links sNPF to a crucial role in fed animals. In addition, mass spectrometric analysis revealed that sNPF levels in the brain decrease during starvation and increase upon refeeding (141). Injection of Bommo-sNPF-2 reduced the latency to feed (142). All these observations suggest that sNPF positively regulates food searching and feeding in this insect species.

A differential peptidomics study in the Colorado potato beetle, *Leptinotarsa decemlineata*, reveals that sNPF is absent in diapausing adults, but present in active beetles (143). This might indicate that sNPF is important in actively feeding animals having increased metabolic activity, while unnecessary in diapausing animals that are metabolically inactive.

In the honey bee, *A. mellifera*, food deprivation causes a significant upregulation of sNPF receptor transcription, pointing to a role of sNPF in starvation-resistance or the stimulation of foraging (68, 125). In another hymenopteran species, *S. invicta*, a downregulation of sNPFR transcription was observed in mated queens that were starved, compared to well-fed congeners (31), again suggesting the importance of sNPF signaling during feeding or metabolically active states.

Although sNPF has several other described functions, it seems to be mainly involved in the regulation of feeding.

sNPF Signaling in Other Arthropods

The first crustacean sNPF was discovered in the giant freshwater prawn *Macrobrachium rosenbergii* two decades ago (144). A recent *in silico* study in this shrimp revealed the presence in the eyestalk and CNS of sNPF transcripts encoding four sNPF peptides (145). Another transcriptome study in ice krill *Euphausia crystallorophias* lead to the discovery of two sNPF precursors that cleave into several active peptides (146). A unique crustacean sNPF, containing an Asp residue in position 2, was found in *Daphnia pulex*. This makes Dappu-sNPF more similar to insect sNPFs than other crustacean sNPFs (147). Bioinformatic analysis of genomes and transcriptomes revealed the presence of sNPF and sNPF receptors in chelicerates (69). So far, no functional studies on crustacean or chelicerate sNPFs have been performed.

sNPF Signaling in Nematodes

C. elegans sNPF neuropeptides display more sequence variation compared to other invertebrate sNPFs, which may be attributed to the extensive expansion and diversification of the sNPF signaling system in nematodes. **Figure 4** shows that peptides derived from three distinct *C. elegans* sNPF neuropeptide precursors, FLP-15, FLP-18 and FLP-21, display the canonical motif XLRFa in accordance with the XXR(F/Y/W)amide C-terminal of sNPF in other protostomes.

A large scale phylogenetic analysis on bilaterian neuropeptide receptors indicated that also sNPF receptors underwent a large expansion in nematodes (1). Phylogenetic analysis showed that several *C. elegans* NPRs cluster with the *Drosophila* sNPF receptor, with NPR-6 being the closest (76). Also, other *C. elegans* receptors, including NPR-1,2,3,4,5, NPR-10, and NPR-13 cluster with the *Drosophila* sNPF receptor in agreement with the large expansion of sNPF receptors as postulated by Mirabeau and Joly (1). FLP-21 derived sNPFs have been shown to activate two candidate sNPF receptors, NPR-1, and NPR-2 in cell-based receptor assays (148–150). Similarly, FLP-18 derived peptides have been identified as a ligand for the NPR-1, NPR-4, and NPR-5 candidate sNPF receptors. FLP-15 has been shown to interact with the NPR-3 sNPF receptor (148, 149, 151, 152).

The role of nematode sNPF receptors in feeding has been examined for NPR-1, NPR-4, NPR-5, and their ligands. Although above-mentioned candidate sNPF receptors have been found in genomes of other nematodes as well, all the feeding-related functional data regarding sNPF in nematodes have been almost exclusively obtained in *C. elegans* (76, 80).

The sNPF receptor NPR-1 is a suppressor of food-dependent aggregation behavior in *C. elegans* (78). When food is present, some wild-type *C. elegans* strains, including the standard laboratory strain N2 Bristol, slow down their movement and disperse as solitary animals across a bacterial lawn. Other strains move faster and aggregate at the border of the food lawn (78). The difference between solitary and aggregation behavior comes down to a single amino acid change in NPR-1. Solitary worms have a gain-of-function allele of the neuropeptide receptor NPR-1, NPR-1 215V (valine at position 215), whereas aggregating animals have the natural isoform NPR-1 215F (phenylalanine at position 215). Worms with disturbed *npr-1* expression display solitary behavior (78). Bacterial odor influences the aggregation of *npr-1* animals, as does population density, although not when food is absent (153). Aggregation behavior is mainly driven by ambient oxygen levels. *C. elegans* escapes atmospheric levels of 21% oxygen, which signals exposure at the surface, by aggregating in groups of animals at the border of a bacterial lawn, where local oxygen levels are reduced.

The functions of NPR-1 in food-dependent aggregation behavior are mediated by two short NPF encoding genes, *flp-18* and *flp-21* (149). Deletion of *flp-21* increases the food-dependent aggregation behavior in NPR-1 215V and 215F worms, yet not to the level of the *npr-1* null mutant (149).

Mutants of *flp-18* have an altered metabolism, higher fat accumulation in the intestine and reduced oxygen consumption. Both *npr-4* and *npr-5* mutants display the same phenotypes

suggesting that *flp-18*-mediated fat accumulation is executed by both receptors (151). Another effect of FLP-18 was observed in local search behavior. When wild type worms are removed from their food source, they increase their turning and reversing movements and explore the local area. When this withdrawal from food continues for a longer period, they start to search for food in bigger areas by inhibiting their turning and reversing behavior. Animals lacking *flp-18* fail to make this behavioral switch (151). This switch is regulated by AIY interneurons via NPR-4 signaling (154–156). AIY release of FLP-18 is also involved in dauer formation, a transition state aiding in survival when food is scarce. This effect of FLP-18 is controlled by NPR-5 in ASJ neurons (151).

Deletion of *npr-2* and *npr-7* have been associated with an increase in the intestinal fat storage, but the underlying mechanism seems different from the one observed in *npr-4* and *npr-5* mutants, since FLP-18, the ligand acting upon these last two receptors, is not active on NPR-2 and NPR-7 (151). The ligand of NPR-2 has been identified as FLP-21. NPR-2, along with NPR-1, seems to increase the adaptation to noxious stimuli in the absence of food. However, neither FLP-21 or FLP-18 are involved in this process (150). Although the same peptide is involved in the regulation of aggregation behavior, the action of FLP-21 upon NPR-2 in the modulation of fat storage has never been assessed.

sNPF Signaling in Molluscs

A recent study in the Pacific oyster *Crassostrea gigas* pointed toward the homology of *C. gigas* LFRFamide neuropeptides and arthropod sNPFs (157). Yet, it is important to note that LFRFamide peptides lack the Arg residue at the fourth position from the C-terminus and have a Phe instead of a Leu residue at the third position from the C-terminus, which typified all known sNPFs at that time (158). Using *in silico* techniques, (157) found a Cragi-sNPF-like receptor and showed that three Cragi-LFRFamide peptides from the same precursor activate this sNPF receptor in a dose-dependent manner (157). They further evidenced that the receptor is differentially expressed in males and females and is upregulated in starved oysters. These results may suggest a role in energy metabolism and reproduction (157) and make the system a convincing functional sNPF ortholog.

Even though the Cragi-sNPF-like system is the first one that was functionally characterized in molluscs, it is not the first LFRFamide (or sNPF) neuropeptide that was discovered in this phylum. Initially, LFRFamides or sNPFs were discovered in gastropods (159) and since then also in cephalopods and oysters (160, 161). In *A. californica*, LFRFamide peptides or sNPFs have an inhibitory effect in buccal neurons (162). In *L. stagnalis*, the sNPF-encoding gene is upregulated in response to *Trichobilharzia ocellata* infection, indicating a role in energy metabolism and reproduction (163). Another example is the involvement of sNPF peptides in feeding along with learning and memory in the cuttlefish *Sepia officinalis* (164, 165). For a detailed review see Bigot et al. (157) and Zatylny-Gaudin and Favrel (161).

sNPF Signaling in Annelids

A bioinformatic study on *C. telata* predicts the presence of an LFRWamide neuropeptide encoding gene reported to be closely related to the mollusk gene (109) that encodes sNPF receptor activating LFRFamides or sNPFs (157). The genome of *C. telata* also encodes an sNPF receptor for which the activating ligand is currently unknown (114). In *Platynereis* the RYamide gene (110), encoding LFRWamides and XXRYamides, displays high sequence similarities with the LFRFamide (sNPF) precursor of mollusks (Figure 4). The *Platynereis* NKY receptor appears to be a candidate sNPF receptor. It was found to be activated *in vitro* (although at a high EC50 of 120 nM) by KAFWQPMGGPLPVETRLASFGSRIEP-DRTEPGSGPNGIKAMRYamide (111). This neuropeptide does, however, not belong to the NPF, nor the sNPF family. *In vivo* studies will be needed to demonstrate the cognate ligand of the annelid sNPF receptor. Knowledge on the function of sNPF signaling in annelids remains so far elusive.

sNPF in Echinoderms

Information of sNPF in echinoderms is almost non-existing. Only in *A. rubens*, a GPCR that resembles an sNPF-type receptor rather than an NPF receptor has recently been identified (166).

CONCLUSIONS

Despite their early evolutionary origin and subsequent evolutionary separation, NPF and sNPF neuropeptidergic signaling systems both control similar feeding aspects. In the species investigated, both of them converge to up- or downregulation of insulin signaling depending on the internal feeding state of the animal. It is remarkable to conclude that almost all invertebrate phyla retained both systems, even if their function in feeding is similar. It is, however, clear from the protostomian species investigated that both systems are

needed for optimal regulation of feeding. This may suggest that both systems are probably controlling slightly different pathways underlying feeding behaviors. In vertebrates, sNPF signaling seems to have been lost during evolution, or may have evolved into the prolactin releasing peptide signaling system, which also regulates feeding and has been suggested to be orthologous to sNPF. Vertebrate long NPFs such as NPY, PPY and PP neuropeptide genes show an evolutionary expansion, either to compensate for the possible loss of sNPF, or to adapt to vertebrate-specific life styles and feeding.

AUTHOR CONTRIBUTIONS

LS and SZ conceptually designed the study. MF and IH equally contributed in writing the first draft of the manuscript. LS, SZ, LT, and IB wrote sections of the manuscript and critically revised it. All authors contributed to the final draft, have read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2019.00064/full#supplementary-material>

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Mechanisms for Temperature Modulation of Feeding in Goldfish and Implications on Seasonal Changes in Feeding Behavior and Food Intake

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In fish models, seasonal change in feeding is under the influence of water temperature. However, the effects of temperature on appetite control can vary among fish species and the mechanisms involved have not been fully characterized. Using goldfish (*Carassius auratus*) as a model, seasonal changes in feeding behavior and food intake were examined in cyprinid species. In our study, foraging activity and food consumption in goldfish were found to be reduced with positive correlation to the gradual drop in water temperature occurring during the transition from summer ($28.4 \pm 2.2^\circ\text{C}$) to winter ($15.1 \pm 2.6^\circ\text{C}$). In goldfish with a 4-week acclimation at 28°C , their foraging activity and food consumption were notably higher than their counterparts with similar acclimation at 15°C . When compared to the group at 28°C during summer, the attenuation in feeding responses at 15°C during the winter also occurred with parallel rises of leptin I and II mRNA levels in the liver. Meanwhile, a drop in orexin mRNA along with concurrent elevations of CCK, MCH, POMC, CART, and leptin receptor (LepR) transcript expression could be noted in brain areas involved in feeding control. In short-term study, goldfish acclimated at 28°C were exposed to 15°C for 24 h and the treatment was effective in reducing foraging activity and food intake. The opposite was true in reciprocal experiment with a rise in water temperature to 28°C for goldfish acclimated at 15°C . In parallel time-course study with lowering of water temperature from 28 to 15°C , short-term exposure (6–12 h) of goldfish to 15°C could also increase leptin I and II mRNA levels in the liver. Similar to our seasonality study, transcript level of orexin was reduced along with up-regulation of CCK, MCH, POMC, CART, and LepR gene expression in different brain areas. Our results, as a whole, suggest that temperature-driven regulation of leptin output from the liver in conjunction with parallel modulations of orexigenic/anorexigenic signals and leptin responsiveness in the brain may contribute to the seasonal changes of feeding behavior and food intake observed in goldfish.

Keywords: appetite control, feeding behavior, temperature change, leptin and leptin receptor, orexigenic factors, anorexigenic factors, goldfish

INTRODUCTION

Temperature change in the environment is a key factor known to affect energy metabolism (1) and body growth in animals (2), and these modulatory effects are partly mediated via regulation of food intake (3). In fish models, circannual rhythm of feeding pattern and food intake has been reported, which is under the influence of environmental cues including seasonal change in water temperature (4). However, the effects of temperature on feeding can be quite variable in different fish species. In general, a rise in water temperature tends to increase food intake, e.g., in salmon (*Salmo salar*) (5), cod (*Gadus morhua*) (6), and flounder (*Pleuronectes americanus*) (7), which can be attributed to the metabolic demand of enhanced body growth caused by activation of the GH/IGF-I axis observed at high temperature (especially during summer) (8–10). Nevertheless, an increase in water temperature can also induce voluntary anorexia in fish species, e.g., in Atlantic salmon (*Salmo salar*), and the phenomenon may be caused by a drop in the peripheral stimulator for feeding, namely ghrelin, in systemic circulation (11). Although central expression of orexigenic/anorexigenic signals modified by temperature change has been documented in fish models, e.g., up-regulation of ghrelin in the brain of Chinese perch (*Siniperca chuatsi*) by temperature rise (12) and elevation of CART expression in the hypothalamus of Atlantic cod (*Gadus morhua*) by low temperature (6), a recent study in Arctic charr (*Salvelinus alpinus*) has revealed that the seasonal changes of NPY, AgRP, POMC, CART, and leptin expressed in brain areas involved in feeding control did not correlate with the annual cycle of feeding reported in the species (13). To date, no consensus has been reached regarding the functional role of orexigenic/anorexigenic signals within the central nervous system (CNS) in the circannual rhythm of feeding observed in fish species.

To unveil the mechanisms underlying temperature modulation of feeding in fish models and their functional implications in seasonal variations in feeding behavior and food intake, goldfish was used as the animal model for our study as (i) it is a representative of cyprinid species, the members of which are commercial fish with high market values in Asian countries, and (ii) the background information for feeding behaviors and appetite control are well-documented in the species (7). In the present study, we sought to address the questions on: (i) Whether the goldfish displays a seasonal change in feeding dependent on water temperature which can be reflected by alterations in feeding behavior and food intake? (ii) Can these feeding responses be induced by short-term and/or long-term manipulation of water temperature? (iii) Can the feeding responses caused by temperature change be explained by parallel modifications of orexigenic/anorexigenic signals expressed in the CNS or in periphery tissues (e.g., in the

liver)? Using goldfish adapted to water temperature at different times of the year but maintained under a constant photoperiod, different types of feeding behaviors and food consumption were monitored over an 8-month period covering the transition from summer to winter and correlated to the corresponding change in water temperature. To confirm that the differences in feeding responses observed between the summer and winter months indeed were caused by temperature change, long-term and short-term exposure of goldfish to the “summer temperature” (28°C) and “winter temperature” (15°C) were performed to test if the treatment could mimic the seasonal changes in feeding. To elucidate the mechanisms for feeding control by temperature, parallel measurements of leptin I and II mRNA expression in the liver and transcript levels of NPY, AgRP, orexin, CART, POMC, CCK, MCH, and leptin receptor (LepR) in selected brain areas were also conducted. The results of our study have provided new information on the mechanisms for feeding control by temperature change in the environment which may contribute to the seasonal cycle of food intake observed in goldfish.

MATERIALS AND METHODS

Animal Maintenance and Preparation Prior to Experiments

Goldfish (*Carassius auratus*) with body weight of 28–34 g were acquired from local pet stores and maintained at $20 \pm 2^\circ\text{C}$ in well-aerated 700 L tanks (a total of ~300 fish with 50–60 fish/tank in $\times 5$ circular tanks with a diameter of 120 cm and water depth of 60 cm) under a 12-h dark:12-h light photoperiod with regular replacement of water at a rate of ~10% total volume every 48 h using a Gardena® C1060 automatic irrigation system (Gardena, Ulm, Germany). To minimize the influence of reproductive status on feeding, mixed sexes of goldfish during sexual regression were used in our studies. For seasonal change in feeding responses (including food consumption and different types of feeding behaviors), the fish were housed in 200 L tanks (with 20–25 fish/tank in $\times 4$ replica tanks with the dimension of $73 \times 60 \times 60$ cm) in a separate room open to the outside via ventilation vents (to allow for seasonal change in water temperature) but maintained under the same photoperiod setting. For monitoring of feeding responses in our seasonality study and long-term/short-term acclimation experiments to summer (28°C)/winter temperature (15°C), the fish were housed in 20 L “observation tanks” (2 fish/tank with the dimension of $35 \times 25 \times 20$ cm and up to $\times 8$ replica tanks per group for individual experiments) with temperature maintained ($\pm 1^\circ\text{C}$) by a submerged heater and cooling coil linked with a PolyScience® thermal controller (Preston Industries Inc., Niles, IL). To study the feeding responses after temperature acclimation, goldfish were entrained for 14 days with a “one-meal-per-day” feeding schedule (14) (with food provision of 2% BW/fish at 10:00 a.m. using an automatic feeder) prior to the scoring of feeding behaviors/measurement of food intake. For the studies on target gene expression, goldfish were sacrificed by anesthesia with 50 mg/L MS222 (Sigma-Aldrich, St. Louis, MO) followed by spinosectomy

Abbreviations: AgRP, Agouti-Related Peptide; α -MSH, Alpha Melanocyte-Stimulating Hormone; CART, Cocaine- and Amphetamine-Regulated Transcript; CCK, Cholecystokinin; MCH, Melanin-Concentrating Hormone; NPY, Neuropeptide Y; POMC, Proopiomelanocortin; LepR, Leptin Receptor; CNS, Central Nervous System.

according to the procedures approved by the Committee for Animal Use for Research and Teaching at the University of the Hong Kong.

Seasonal Change in Feeding and Its Correlation With Water Temperature

Seasonal changes in feeding behavior and food consumption were monitored in goldfish over a period of 8 months (Jul, 2016–Feb, 2017) covering the transition from summer to winter under a 12-h dark:12-h light photoperiod. The period from July

to Sept of 2016 covered the summer months with an average water temperature at $28.4 \pm 2.2^{\circ}\text{C}$, from Sept to Nov of 2016 covered the autumn months with water temperature at $24.0 \pm 1.7^{\circ}\text{C}$, from Nov to Dec of 2016 covered the early-mid phase of winter with water temperature at $20.4 \pm 3.5^{\circ}\text{C}$, and Jan–Feb of 2017 covered the peak phase of winter with water temperature at $15.1 \pm 2.6^{\circ}\text{C}$. After a 14-day entrainment of “one-meal-per-day” feeding schedule, feeding behaviors and food consumption were measured as described previously (14). In this case, different patterns of feeding behaviors were recorded

TABLE 1 | Primer sequences and PCR conditions for real-time PCR assays.

Gene target/GenBank accession no	PCR condition:				Cycle	Tm	Product size
	Denaturing	Annealing	Extension	Detection			
Upstream & downstream primer sequences							
<i>β</i>-ACTIN/AB039726							
5'-CTGGTATCGTGATGGACTCT-3'	94°C	56°C	72°C	87°C	×35	91°C	285 bp
5'-AGCTCATAGCTCTTCTCCAG-3'	30 s	30 s	30 s	20 s			
EF-1α/AB056104							
5'-GATTGTTGCTGGTGGTGTG-3'	94°C	52°C	72°C	87°C	×35	89°C	216 bp
5'-GCAGGGTTGTAGCCGATTT-3'	30 s	30 s	30 s	20 s			
LEPTIN I/FJ534535							
5'-TCCAAAGCTCCTCATAGG-3'	94°C	50°C	72°C	86°C	×45	89°C	270 bp
5'-TGGTGGGTGGCGTTTTCC-3'	30 s	30 s	30 s	20 s			
LEPTIN II/FJ854572							
5'-TATCGTGGACACCCTAACTAC-3'	94°C	50°C	72°C	85°C	×45	89°C	224 bp
5'-GGTCTAAAGCCAAGAACCCTAA-3'	30 s	30 s	30 s	20 s			
LEPTIN RECEPTOR/EU911005							
5'-CTGGCTTGAAGGTGAACGGAC-3'	94°C	65°C	72°C	78°C	×45	87°C	156 bp
5'-TTGGGTGACAGTGACAGTAGTC-3'	30 s	30 s	30 s	20 s			
CART/AF288810							
5'-CCAAAGGACCCGAATCTGA-3'	94°C	64°C	72°C	72°C	×35	90°C	171 bp
5'-TTTGCCGATTCTTGACCT-3'	30 s	30 s	30 s	20 s			
CCK/CAU70865							
5'-CCGCAGTCTCAGAAGATGGG-3'	94°C	64°C	72°C	87°C	×35	91°C	197 bp
5'-GGAGGGGCTTCTGCGATA-3'	30 s	30 s	30 s	20 s			
MCH/AM403730							
5'-AGGCTTGAGCGAGAACTTGG-3'	94°C	64°C	72°C	86°C	×35	91°C	272 bp
5'-CCCAGAAGACCTACACCTCCC-3'	30 s	30 s	30 s	20 s			
POMC/AJ431209							
5'-AAGCGCTCCTACTCCATGGA-3'	94°C	60°C	72°C	83°C	×35	85°C	282 bp
5'-CTCGTCCCAGGACTTCATGAA-3'	30 s	30 s	30 s	20 s			
NPY/M87297							
5'-GTAGTGTTGCGGGTAGCGA-3'	94°C	64°C	72°C	88°C	×35	92°C	234 bp
5'-CAGACACCCCGACCCAAG-3'	30 s	30 s	30 s	20 s			
OREXIN/DQ923590							
5'-GCAGAGCTGCTCATTGTTGACGTT-3'	94°C	64°C	72°C	84°C	×35	82°C	286 bp
5'-AACCTTGTGATTACCTCAGGAGT-3'	30 s	30 s	30 s	20 s			
AGRP/AJ555492							
5'-TGGCATCACATCCAAACCT-3'	94°C	64°C	72°C	82°C	×35	88°C	230 bp
5'-CAGGTGATGACCCAAGCAG-3'	30 s	30 s	30 s	20 s			
APELIN/FJ755698							
5'-GAGCATAGCAAAGAGCTGGA-3'	94°C	64°C	72°C	89°C	×35	94°C	340 bp
5'-GCTGAGGATGAGTGGCTTGT-3'	30 s	30 s	30 s	20 s			

for 2 h using a KPsec VD 714 Surveillance System (Avtech) after introduction of food pellets (TetraMin GmbH, Germany; with ~47% crude protein, ~10% crude oils & fat, ~6% crude fiber and supplements of vitamins and minerals). By the end of the 2-h period, the remains of unconsumed pellets were collected and routinely dried in a 45°C oven for 3–4 days until a constant mass had been acquired. After correction for the loss of soluble components in food pellets (~0.15% by weight), the mass difference of the remains vs. the amount added at the beginning (~2% BW/fish) was used as an index for food intake. Based on the video recorded, the cumulative counts of three types of feeding behaviors observed in goldfish, namely complete feeding/surface foraging, incomplete feeding/food spitting and bottom feeding/bottom foraging, were scored manually in a single-blind manner by the parameters defined by Volkoff and Peter (15). Briefly, complete feeding was defined as the feeding act of engulfing food pellets on water surface in a single foraging movement. Incomplete feeding, in contrast, referred to the “food rejection act” of regurgitation/spitting of food pellet without swallowing. Unlike complete feeding occurred on the water surface, bottom feeding was defined as the feeding act to pick up food pellets/debris sunk to the bottom. In the present study, the data for food intake were also correlated with water temperature in individual experiments conducted at different times of the year using Pearson product-moment correlation analysis.

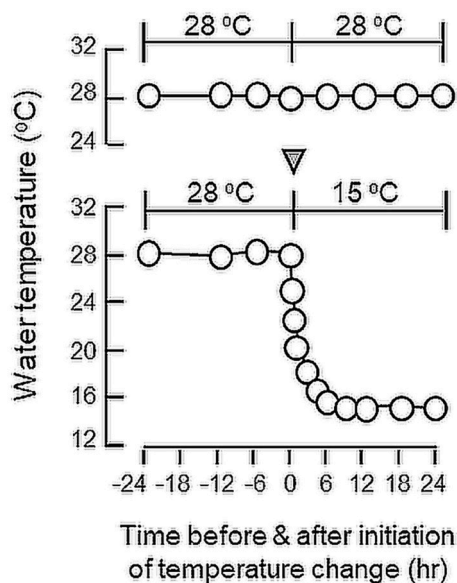


FIGURE 1 | Profile of temperature change during the short-term acclimation of goldfish from summer temperature (28°C) to winter temperature (15°C). Goldfish were maintained in 28°C water for 4 weeks during the summer (Jun–July, 2017) prior to the activation of the cooling system linked to the water tank to gradually reduce the water temperature to 15°C (as indicated by the inverted triangle). The cooling system could allow for a gradual drop in water temperature from 28 to 15°C within 6 h without the need of transferring the fish during the experiment. Without activating the cooling system (as shown in upper panel), water temperature was maintained at 28°C without noticeable change over a 24-h period.

Feeding Changes With Long-Term Acclimation to Summer and Winter Temperature

To confirm that seasonal variations in feeding observed were caused by temperature change in the environment, goldfish maintained at 20°C during the autumn period (Sep–Oct, 2017) were divided into two groups and subjected to long-term acclimation for 4 weeks in water tanks maintained at summer temperature (28°C) and winter temperature (15°C), respectively. During the period, the fish were trained with “one-meal-per-day” feeding at 28/15°C and used for the scoring of feeding behaviors and food consumption as described in the preceding section. To examine the mechanisms involved in temperature regulation of feeding behavior and food intake, parallel experiments were also performed to study the effects of a 4-week acclimation at 28°C during the summer (July–Aug, 2016) and 15°C during the winter (Jan–Feb, 2017) on transcript expression of feeding regulators identified in the liver and brain areas involved in feeding control in fish models, including the telencephalon, hypothalamus and optic tectum (7). The long-term acclimation at respective temperatures for the two seasons was conducted to minimize the effect of daily fluctuations of water temperature on target gene expression. After acclimation to the respective temperature, the liver and target brain areas were excised and total RNA and genomic DNA were extracted with Trizol (Invitrogen) according to the instructions of the manufacturer. DNA contents in individual samples were quantified by OD_{260/280} reading and the data obtained were then used for subsequent data normalization for target gene expression. The RNA samples prepared were digested with DNase I, reversely transcribed by Superscript II (Invitrogen), and subjected to real-time PCR for transcript measurement of target regulators for feeding in goldfish using a RotorGene-Q qPCR System (Qiagen) with a Lightcycler® 480 SYBR Green I Master Kit (Roche) (16). PCR reactions were conducted with primers and PCR conditions for different gene targets as shown in Table 1. In our study, parallel measurements of β actin and elongation factor 1 α (EF-1 α) gene expression were also conducted to serve as internal controls.

Feeding Responses and Gene Expression Induced by Short-Term Temperature Change

To study the short-term responses induced by temperature change, goldfish trained with “one-meal-per-day” feeding and acclimated at 28°C were transferred to water tanks at 15°C for 24 h. Parallel transfer of goldfish to water tanks at 28°C was used as a control treatment. After 24-h exposure to temperature drop, feeding experiment was initiated (at 28°C for control and 15°C for treatment) to monitor the effects of acute temperature change on feeding behaviors and food consumption as described previously. To test for the reversibility of temperature effect, reciprocal experiment was also performed by transferring goldfish acclimated at 15°C to water tanks at 28°C for 24 h. In this case, parallel transfer to water tanks at 15°C was used as the control group. To establish the time-course of target gene expression for feeding regulators associated with the

short-term temperature change, goldfish acclimated at 28°C was housed in water tanks linked with the thermal controlling unit to allow for a gradual drop of water temperature to 15°C in 6 h without disturbing the fish (Figure 1). After that, the fish was maintained at 15°C until the end of the 24-h period. For the control treatment, fish were housed in water tanks at 28°C with no temperature change over the same period. In our study, the liver as well as selected brain areas including the telencephalon, hypothalamus and optic tectum were harvested from individual fish at 24 and 12 h before and at 0, 6, 12, and 24 h after the initiation of temperature drop to 15°C. Total RNA and DNA were extracted from these samples with Trizol and RT samples prepared were then used for real-time PCR measurement of target gene expression as described in the preceding section.

Data Transformation and Statistical Analysis

For measurement of feeding behaviors, cumulative counts for different types of feeding behaviors were scored every 10 min continuously over a period of 2 h. Food consumption over the 2-h period was normalized as the mass of food pellets taken by the fish over 60 min and used as an index for food intake after thermal acclimation. For real-time PCR of target gene expression, standard curves constructed with serial dilutions of plasmid DNA carrying the ORF/amplicons for target genes with a dynamic range of $\geq 10^5$, amplification efficiency $\geq 98\%$ and correlation coefficient ≥ 0.95 were used for data calibration with RotorGene Q-Rex software (Qiagen). To adjust for variations in the amount of tissues used in RNA extraction, raw data

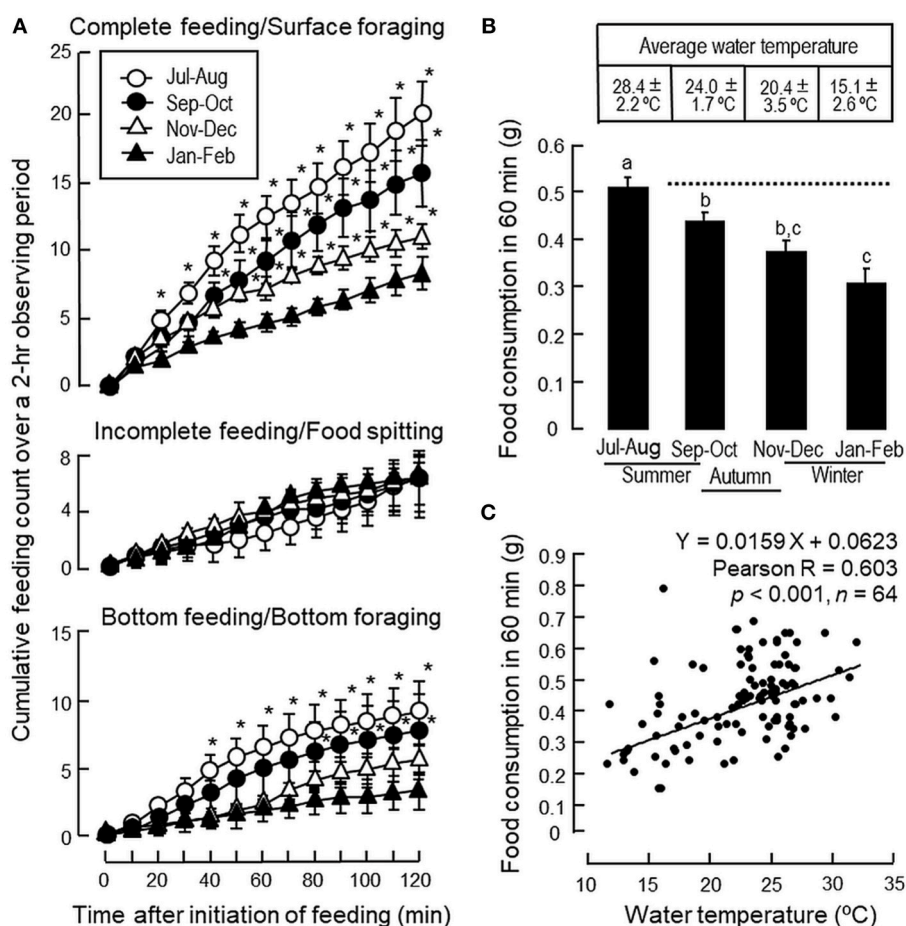


FIGURE 2 | Seasonal changes of feeding behaviors and food intake in goldfish during the transition from summer to winter. **(A)** Seasonal changes of complete feeding/surface foraging, incomplete feeding/food spitting and bottom feeding/bottom foraging during the transition from the summer (Jul–Aug, 2016), autumn (Sep–Oct, 2016), early-mid phase of the winter (Nov–Dec, 2016) to the peak phase of the winter (Jan–Feb, 2017). **(B)** Seasonal change of food consumption related to the temperature drop in the environment during the same period. **(C)** Positive correlation of the gradual decline in food intake observed during the transition from summer to winter months as shown in **(B)** with the parallel drop in water temperature as revealed by Pearson product-moment regression analysis. Data presented, including feeding behaviors, food consumption and water temperature are expressed as mean $\pm$ SEM ($n = 14$ –16). Feeding behaviors were scored over a period of 2 h and the data of feeding counts obtained during the summer, autumn and early-mid phase of the winter were compared with the corresponding data of the same time point from the group scored during the peak phase of the winter using Student's *t*-test. For food intake occurred during the same period, the data for food consumption from different groups were analyzed by one-way ANOVA followed by Tukey *post-hoc* test. Differences between treatment groups were considered as significant at $p < 0.05$.

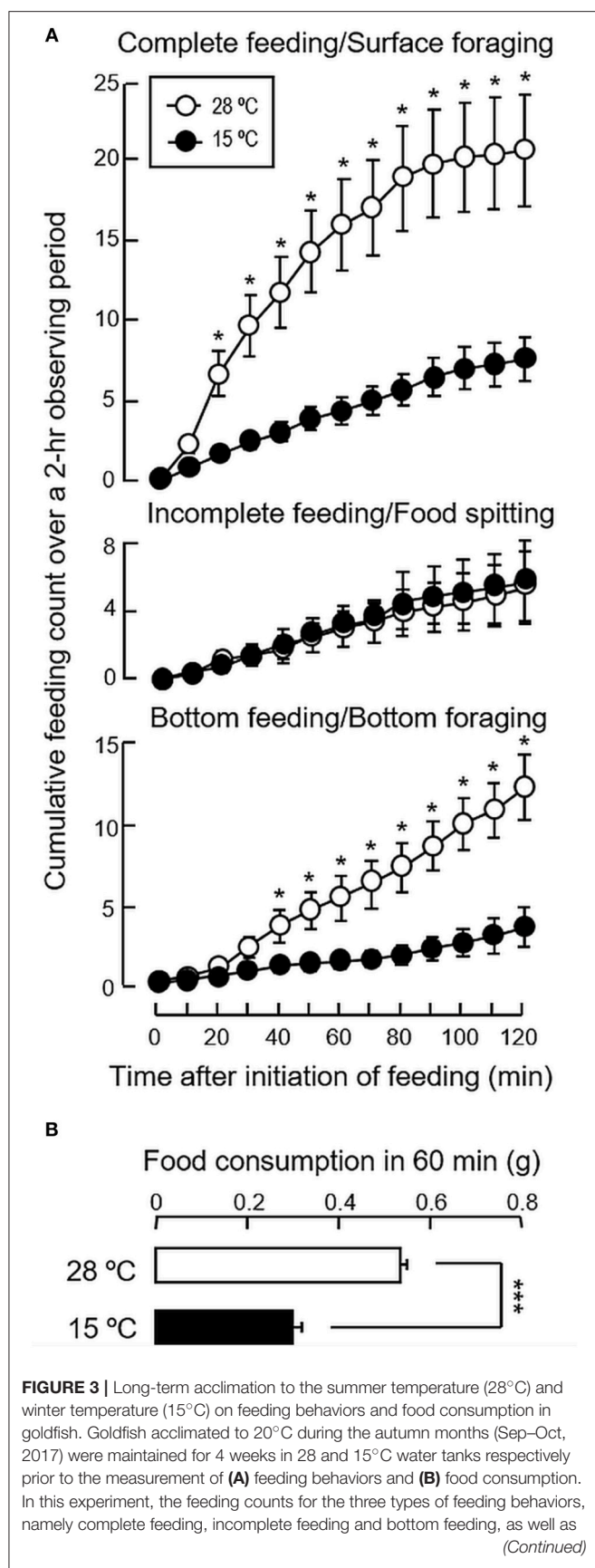


FIGURE 3 | the food intake occurred during the same period were compared between the two groups using Student's *t*-test. Data presented are expressed as mean $\pm$ SEM ($n = 12$) and the difference between the two groups was considered as significant at $p < 0.05$ (* $p < 0.05$ and *** $p < 0.001$).

for transcript expression (in femtomole mRNA detected) were expressed as a ratio of genomic DNA (per μg DNA) detected in the same sample. Since the internal controls for β actin and EF-1 α did not show significant difference after long-term/short-term acclimation, the normalized data were presented directly or transformed as a percentage of mean values in the reference control. For the data obtained from seasonality study or experiments with 4-week/24-h acclimation to summer/ winter temperature (with temperature change as the variable), statistical analysis with Student's *t*-test or one-way ANOVA followed by Tukey *post-hoc* test was performed. For the time-course study on gene expression with temperature drop from 28 to 15°C (with time and temperature change as two variables), the data were analyzed by two-way ANOVA prior to Tukey test. In both cases, data presented are expressed as mean $\pm$ SEM ($n = 10$ –16) and differences between treatment groups were considered as significant at $p < 0.05$.

RESULTS

Seasonal Change in Feeding and Its Correlation With Water Temperature

In goldfish subjected to seasonal change in temperature during the transition from summer to winter, except for a lack in response for incomplete feeding/food spitting activity, the cumulative counts for feeding behaviors, including complete feeding/surface foraging and bottom feeding/ bottom foraging, were found to be reduced gradually from the summer (Jul–Aug, 2016), autumn (Sept–Oct, 2016), early-mid phase of the winter (Nov–Dec, 2016) to the peak phase of winter (Jan–Feb, 2017) (Figure 2A). During the same period, water temperature was reduced from $28.4 \pm 2.2^\circ\text{C}$ in summer to $15.1 \pm 2.6^\circ\text{C}$ during the peak phase of winter with a gradual drop in food consumption (Figure 2B). In the same study, Pearson's analysis also revealed a positive correlation between the drop in water temperature and the gradual decline in food consumption during the progression from summer to winter period (Figure 2C).

Long-Term Thermal Acclimation on Feeding and Gene Expression of Feeding Regulators

To test if temperature change can serve as the cause for seasonal variations in feeding, long-term acclimation of goldfish for 4 weeks to either summer (28°C) or winter temperature (15°C) were performed. In this case, the cumulative counts for complete feeding/surface foraging and bottom feeding/bottom foraging in the group acclimated at 28°C were found to be notably higher than the group maintained at 15°C (Figure 3A). Similar to the results of seasonal change in feeding behaviors, the counts for

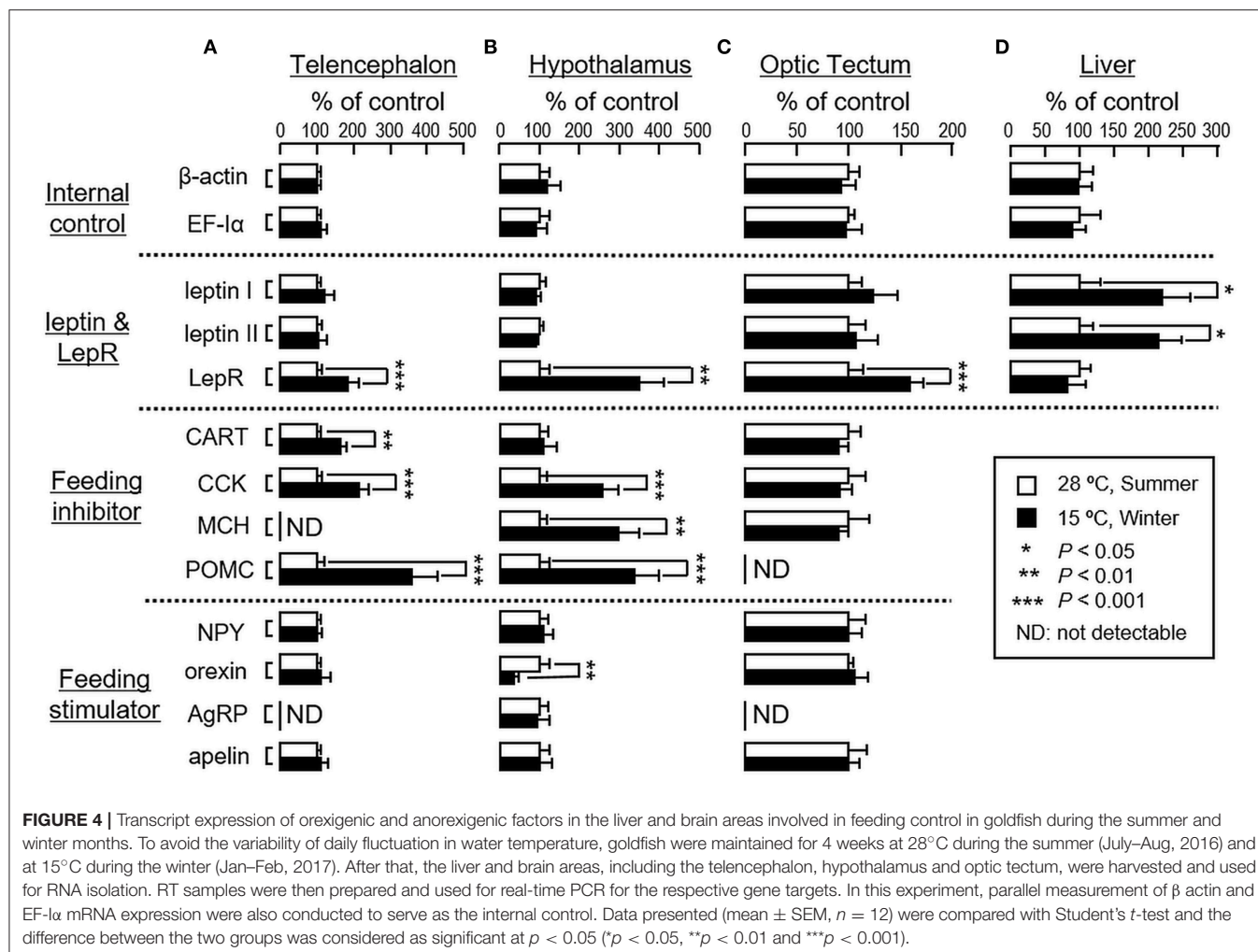


FIGURE 4 | Transcript expression of orexigenic and anorexigenic factors in the liver and brain areas involved in feeding control in goldfish during the summer and winter months. To avoid the variability of daily fluctuation in water temperature, goldfish were maintained for 4 weeks at 28°C during the summer (July–Aug, 2016) and at 15°C during the winter (Jan–Feb, 2017). After that, the liver and brain areas, including the telencephalon, hypothalamus and optic tectum, were harvested and used for RNA isolation. RT samples were then prepared and used for real-time PCR for the respective gene targets. In this experiment, parallel measurement of β actin and EF-1 α mRNA expression were also conducted to serve as the internal control. Data presented (mean $\pm$ SEM, $n = 12$) were compared with Student's t -test and the difference between the two groups was considered as significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

incomplete feeding/food spitting were not affected by variation in water temperature. When compared to the group at 28°C, a parallel drop in food consumption was also noted with thermal acclimation to 15°C (Figure 3B), which was in agreement with the decline in foraging activity occurring both at the surface and bottom levels.

In parallel study using goldfish acclimated at 28°C during the summer as a reference control, acclimation of the fish to 15°C during the winter did not alter transcript expression of β actin and EF-1 α in the liver as well as in brain areas including the telencephalon, hypothalamus and optic tectum (Figure 4). In the telencephalon, however, parallel rises in LepR, CART, CCK and POMC mRNA levels were noted with no significant changes in transcript expression for leptin I, leptin II, NPY, orexin and apelin (Figure 4A). A similar pattern of transcript expression was also observed in the hypothalamus except that 15°C acclimation during winter did not alter CART expression but induced an elevation in MCH with a concurrent drop in orexin mRNA level (Figure 4B). In the optic tectum, unlike the responses in telencephalon/hypothalamus, except for the rise in LepR mRNA, significant changes in transcript expression for the other target genes examined were not apparent (Figure 4C). In the same

study, interestingly, acclimation at 15°C during the winter was effective in increasing leptin I and II mRNA levels in the liver but with no concurrent change in LepR gene expression at the hepatic level (Figure 4D).

Short-Term Thermal Acclimation on Feeding and Gene Expression of Feeding Regulators

As shown in Figure 5A, a notable reduction in the counts for complete feeding/surface foraging and bottom feeding/bottom foraging was observed following a 24-h exposure to 15°C water in goldfish previously acclimated at 28°C, while the opposite was true with parallel transfer of goldfish acclimated at 15°C to 28°C water for 24 h in the reciprocal experiment. Consistent with the results for long-term acclimation, short-term changes in water temperature (from 28 to 15°C/from 15 to 28°C for 24 h) were not effective in altering incomplete feeding/food spitting activity. Of note, modifications in foraging activity were also reflected by corresponding changes in food intake. In this case, food consumption was reduced in 28°C fish after transfer to 15°C water but increased in 15°C fish after transfer to 28°C water (Figure 5B). In contrast, parallel transfer of goldfish to

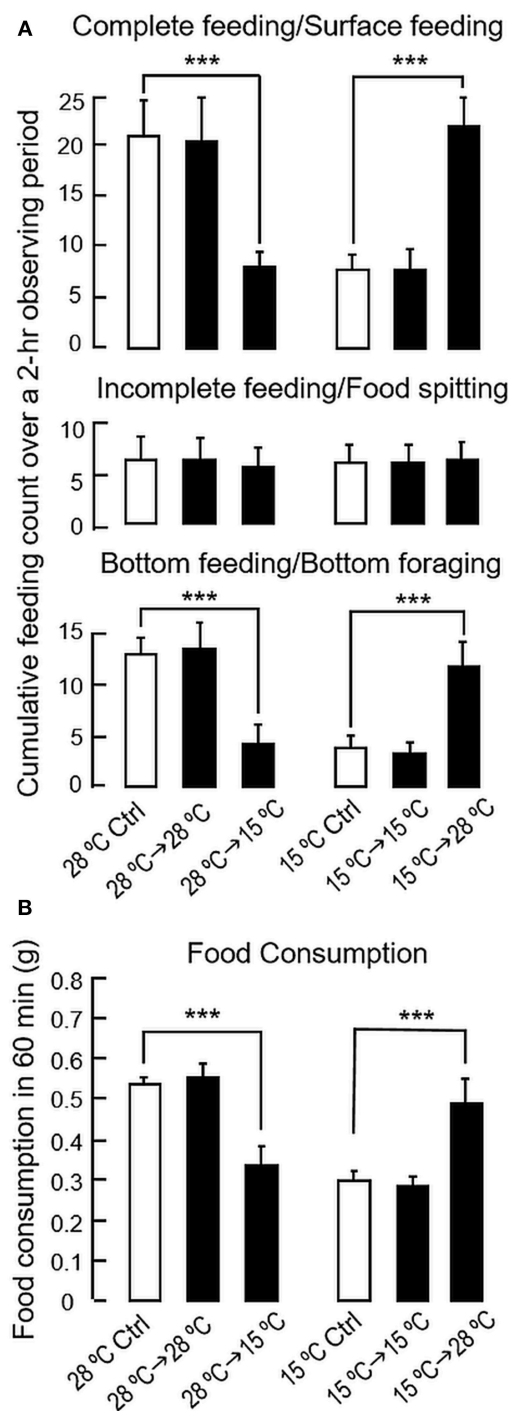


FIGURE 5 | Short-term acclimation to the summer temperature (28°C) and winter temperature (15°C) on feeding behaviors and food consumption in goldfish. Goldfish acclimated to 20°C during the autumn months (Sep–Oct, 2017) were maintained for 4 weeks in 28 and 15°C water tanks, respectively. After that, the fish acclimated to 28°C were transferred to water tanks at 15°C for 24 h. In reciprocal experiment, the fish acclimated to 15°C were transferred to water tanks at 28°C during the same period. As control treatment, parallel experiments without transferring the fish or with parallel transfer into water tanks with the same acclimation temperature (i.e., from 28 to 28°C/from 15 to 15°C) were also conducted. Following the short-term exposure to temperature change, measurement of different types of feeding behaviors (A) and food intake (B) were performed according to the standard protocols. The data obtained (mean ± SEM, $n = 10$ –12) were analyzed with one-way ANOVA followed by Tukey *post-hoc* test. Difference between groups was considered as significant at $p < 0.05$ (***) ($p < 0.001$).

FIGURE 5 | 15°C) were also conducted. Following the short-term exposure to temperature change, measurement of different types of feeding behaviors (A) and food intake (B) were performed according to the standard protocols. The data obtained (mean ± SEM, $n = 10$ –12) were analyzed with one-way ANOVA followed by Tukey *post-hoc* test. Difference between groups was considered as significant at $p < 0.05$ (***) ($p < 0.001$).

water tanks with “acclimated temperature” (i.e., 28°C to 28°C and 15°C to 15°C) did not trigger any noticeable changes in feeding behaviors/food intake, indicating that the feeding responses observed were not caused by handling stress during the experiment.

To shed light on the mechanisms for feeding control by short-term temperature change, a time-course experiment was conducted in goldfish acclimated at 28°C with a gradual drop of water temperature from 28°C to 15°C. In our system, water temperature could be reduced to 15°C within the first 6 h after initiation of temperature change (Figure 1). Similar to our seasonality study, short-term exposure to 15°C could trigger differential changes in transcript expression of feeding regulators in the liver as well as in different brain areas. In the telencephalon, CART, CCK, POMC and LepR mRNA levels were found to be elevated in a time-dependent manner with no significant changes in β actin, NPY, orexin, leptin I and leptin II gene expression (Figure 6). The pattern of transcript expression in the hypothalamus, including the rises in CCK, POMC, and LepR gene expression, was comparable with that of the telencephalon. Interestingly, a drop in orexin mRNA with a parallel rise in MCH transcript level were also noted, which were absent in the telencephalon (Figure 7). In the optic tectum, except for the rise in LepR mRNA, no significant changes were observed regarding the gene expression for β actin, NPY, orexin, CART, CCK, MCH, leptin I, leptin II, and LepR (Figure 8). In the same study, however, leptin I and II mRNA levels were found to be elevated in the liver but without parallel change in β actin and LepR gene expression (Figure 9).

DISCUSSION

In poikilotherms, especially in fish species, body functions including somatic growth (8, 9, 17), reproduction (18, 19), metabolism (20), locomotor activity (21), stress responses (22), embryonic development (23), and immune functions (24) are known to be sensitive to temperature change. In fish models, circannual cycle in feeding pattern/food intake has been reported and can be associated with seasonal changes in water temperature and photoperiod (4). In general, elevation in feeding can be noted in fish species during the spring/summer months with higher temperature (25). This is at variance with the case in non-hibernating homeotherms, e.g., domesticated cats, with increased feeding in the late autumn/winter (26), which may be related to the elevated metabolic demand for thermogenesis at low temperature. The seasonal change in feeding observed in fish species is also in agreement with the results of previous studies showing that food intake can be reduced by low temperature,

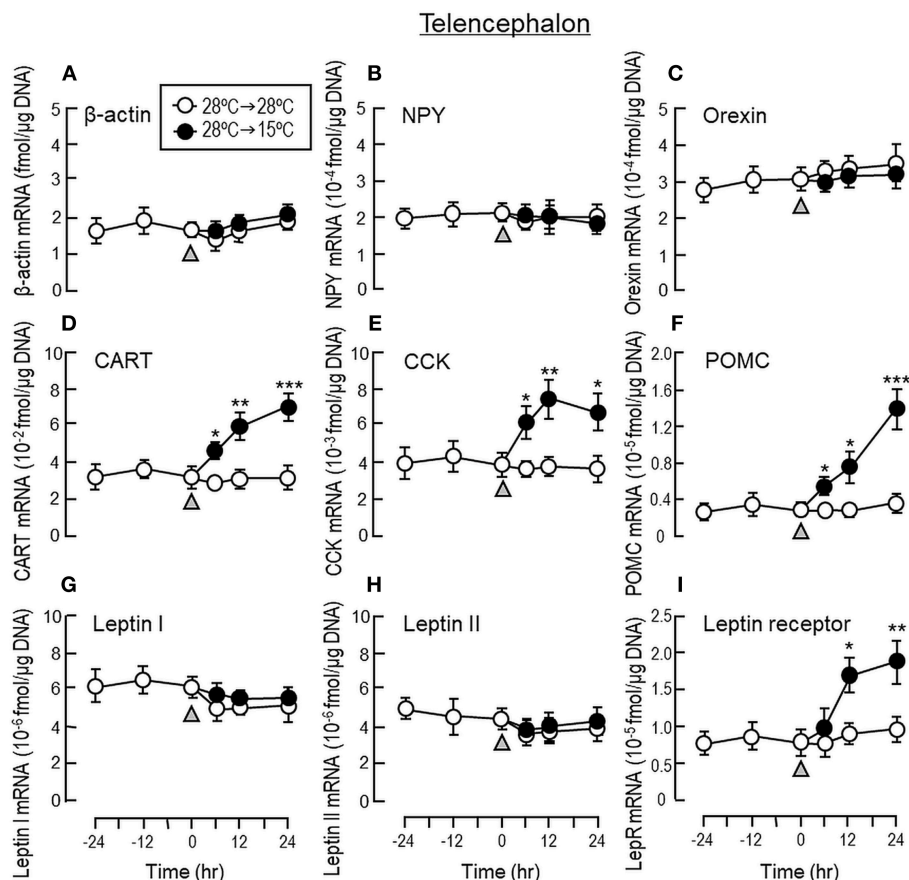


FIGURE 6 | Transcript expression of orexigenic and anorexigenic factors within the telencephalon of goldfish with short-term exposure to winter temperature (15°C). Water temperature for goldfish acclimated at 28°C was gradually reduced to 15°C over a 24-h period using a cooling system linked with the water tank. The telencephalon was harvested from individual fish at different time points before and after the activation of the cooling system (as indicated by gray triangle). Total RNA was isolated, reversely transcribed and used for real-time PCR for respective gene targets, including (A) β actin, (B) NPY, (C) Orexin, (D) CART, (E) CCK, (F) POMC, (G) leptin I, and (H) leptin II and (I) leptin receptor. Parallel experiment with goldfish maintained at 28°C water without activation of the cooling system was used as the control treatment. Similar to the previous study on seasonality of orexigenic/anorexigenic signals, transcript expression of β actin was used as the internal control. For our time course study, the data obtained (mean $\pm$ SEM, $n = 12$) were analyzed using two-way ANOVA followed by Tukey test. Difference between groups was considered as significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

e.g., in catfish (*Ictalurus punctatus*) (27), halibut (*Hippoglossus hippoglossus*) (28), sickleback (*Gasterosteus aculeatus*) (29), turbot (*Scophthalmus maximus*) (30), and tench (*Tinca tinca*) (31). However, species-specific variations in feeding responses do exist in fish models. For examples, high temperature is known to induce voluntary anorexia in Atlantic salmon (*Salmo salar*) (11) and summer fasting can also be observed in some cold water fish, e.g., in cunner (*Tautoglabrus adspersus*) (32), suggesting that the “temperature effect” on feeding can be quite different between warm water and cold water species.

To confirm that seasonal change in feeding do exist in goldfish, a cyprinid species known to be well-adapted to a wide range of water temperature, its feeding behavior and food consumption were monitored over a period of 8 months covering the transition from summer to winter. In our study, a gradual decline in foraging behavior (both surface and bottom foraging) was noted during the progression from summer to winter with a parallel

drop in water temperature. The decline in foraging activity also occurred with parallel reduction in food intake, which was found to have a positive correlation with the attenuation in water temperature during the same period, suggesting that the seasonal change in environmental temperature may contribute to the observed differences in feeding responses between the summer and winter months. In goldfish, regulation of food consumption can be achieved by alteration of foraging activity in water surface/at bottom level with concurrent modification in food spitting activity, e.g., after treatment with NPY (33) or spexin (14). However, food spitting activity did not exhibit significant changes in our seasonality study or parallel experiments with long-term/short-term acclimation to different temperatures and the involvement of this food rejection behavior in the seasonal cycle of feeding is rather unlikely. In our study, using the fish acclimated to summer temperature (28°C) as a reference, long-term and short-term acclimation to winter temperature

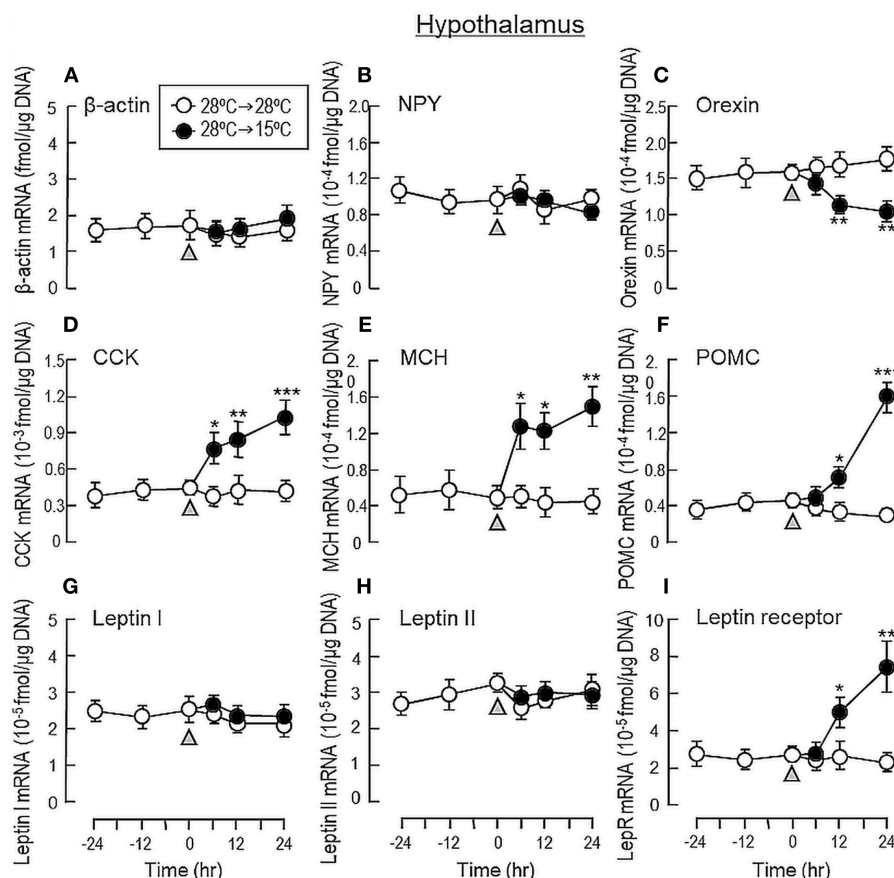


FIGURE 7 | Transcript expression of orexigenic and anorexigenic factors within the hypothalamus of goldfish with short-term exposure to winter temperature (15°C). Water temperature for goldfish acclimated at 28°C was gradually reduced to 15°C over a 24-h period using a cooling system linked with the water tank. The hypothalamus was harvested from individual fish at different time points before and after the activation of the cooling system (as indicated by gray triangle). Total RNA was isolated, reversely transcribed and used for real-time PCR for respective gene targets, including (A) β actin, (B) NPY, (C) Orexin, (D) CCK, (E) MCH, (F) POMC, (G) leptin I, and (H) leptin II and (I) leptin receptor. Parallel experiment with fish maintained at 28°C water without activation of the cooling system was used as the control treatment. For our time course study, the data obtained (mean $\pm$ SEM, $n = 12$) were analyzed with two-way ANOVA followed by Tukey test. Difference between groups was considered as significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

(15°C) were both effective in mimicking the decrease in foraging activity and food intake observed during the seasonal change from summer to winter. The results of short-term acclimation (from 28 to 15°C and from 15 to 28°C) also reveal that the changes in feeding responses were highly reversible and rapid modifications in feeding behavior/food intake could be noted within 24 h exposure to temperature change. Our findings are highly comparable with the previous study in salmon parr showing that a short-term cold stress (>4 h) was sufficient to induce a rapid drop in food intake (34) and provide evidence that temperature change in the environment can trigger the seasonal cycle of feeding in goldfish, presumably via a rapid modulation in feeding behavior/foraging activity.

In homeotherms, including birds and mammals, modification of food intake by thermal stress (1, 35) is typically associated with corresponding changes in orexigenic/anorexigenic signals in the brain as well as in peripheral tissues (e.g., GI tract and adipose tissue) (2, 3, 36). In mammals (e.g., rat), the

central effects of thermal regulation are commonly accepted to be mediated by the temperature-sensitive neurons within the hypothalamus (37), presumably via activation of thermo-TRP ion channels (38). In bony fish, the functional roles of orexigenic factors including NPY (33), orexin (39), AgRP (40), apelin (41), and ghrelin (42) and anorexigenic factors including CCK (43), CART (44), α MSH (45), MCH (46), and leptin (47) in appetite control are well-documented, but not much information is available for their regulation by temperature change. At present, only four studies have been reported on this topic in fish models. These include the previous studies showing up-regulation of CART in the hypothalamus of Atlantic cod (*Gadus morhua*) at low temperature (6) and reduction in hypothalamic levels of ghrelin receptor and NPY in salmon (*Salmo salar*) with parallel drops in plasma ghrelin at high temperature (11). Recently, two other reports have been published demonstrating that ghrelin and CCK expression in the brain could be elevated by high temperature in perch

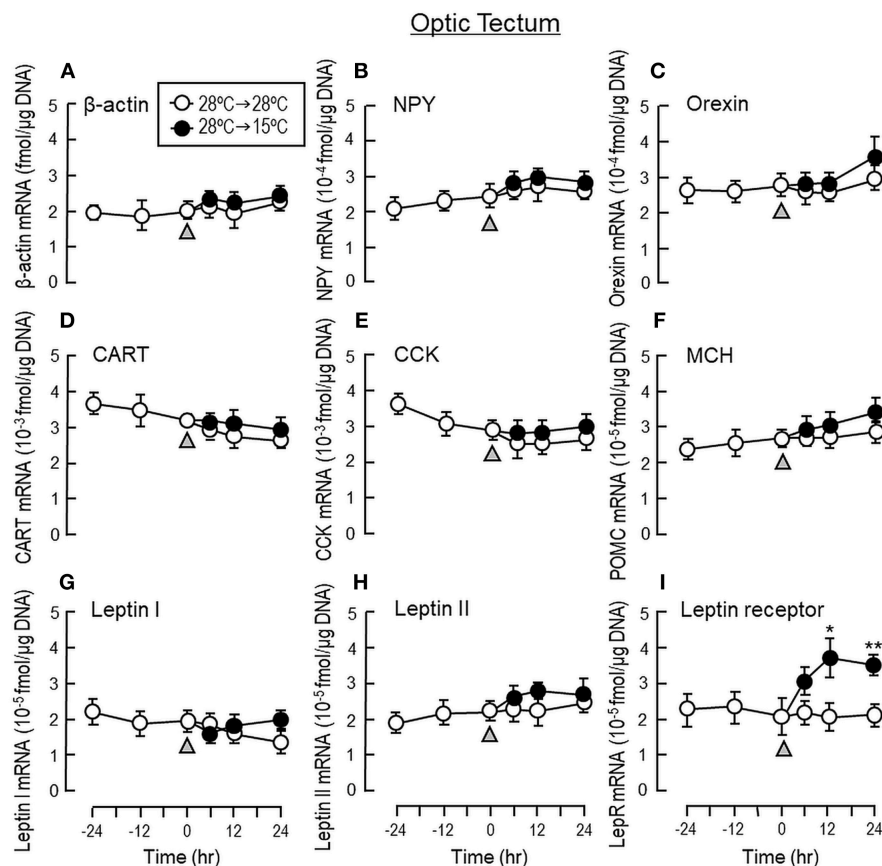


FIGURE 8 | Transcript expression of orexigenic and anorexigenic factors within the optic tectum of goldfish with short-term exposure to winter temperature (15°C). Water temperature for goldfish acclimated at 28°C was gradually reduced to 15°C over a 24-h period using a cooling system linked with the water tank. The optic tectum was harvested from individual fish at different time points before and after the activation of the cooling system (as indicated by gray triangle). Total RNA was isolated, reversely transcribed and used for real-time PCR for respective gene targets, including (A) β actin, (B) NPY, (C) Orexin, (D) CART, (E) CCK, (F) MCH, (G) leptin I, and (H) leptin II and (I) leptin receptor. Parallel experiment with goldfish maintained at 28°C water without activation of the cooling system was used as the control treatment. For our time course study, the data obtained (mean $\pm$ SEM, $n = 12$) were analyzed with two-way ANOVA followed by Tukey test. Difference between groups was considered as significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

(*Siniperca chuatsi*) (12) and seahorse (*Hippocampus erectus*) (48), respectively. Unfortunately, the results from these studies are still limited and a common consensus has not been reached for temperature control of feeding based on the feeding regulators examined. In fish models, seasonal variations in central expression of orexigenic/anorexigenic signals has been reported, e.g., for ghrelin (49), leptin (50), CCK (51), and NPY (52). Therefore, it would be tempting to speculate that their regulation by temperature can mediate the circannual cycle of food intake. However, the idea was not supported by the recent study in Arctic charr (*Salvelinus alpinus*), in which the seasonal patterns of NPY, AgRP, POMC, CART, and leptin expression in brain areas involved in appetite control did not match with its circannual rhythm of feeding (13). To date, the functional link between seasonal cycle of feeding and thermal regulation of orexigenic/anorexigenic signals in the fish brain remains unclear and further studies are highly warranted.

To shed light on the role of orexigenic/anorexigenic signals in seasonal change of feeding in cyprinid species, long-term acclimation of goldfish during the summer at 28°C and during the winter at 15°C were also conducted. In fish models, e.g., salmon (*Salmo salar*) (53), common carp (*Cyprinus carpio*) (54), and more recently in goldfish (*Carassius auratus*) (47), two forms of leptin, namely leptin I and II, have been identified, which are believed to be the result of fish-specific/3R whole genome duplication (55). Unlike mammals with leptin expressed mainly in adipose tissue, leptin is expressed at high levels in the liver of fish species (54–56) and exerts its effect as a satiety factor by regulating central expression of NPY, POMC and/or CCK, e.g., in goldfish (*Carassius auratus*) (57) and trout (*Oncorhynchus mykiss*) (58). When compared with its “summer counterpart” at 28°C, goldfish at 15°C during the winter was found to have notable elevations in leptin I and II mRNA levels in the liver with parallel rises of LepR gene expression in the telencephalon, hypothalamus and optic tectum, which are the

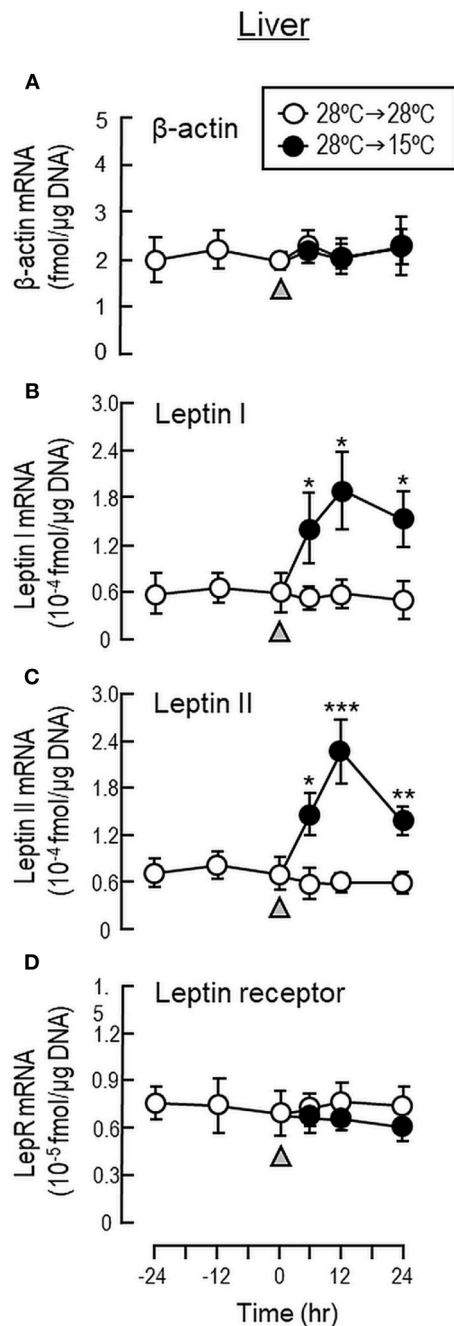


FIGURE 9 | Transcript expression of leptin and leptin receptor in the liver of goldfish with short-term exposure to winter temperature (15°C). Water temperature for goldfish acclimated at 28°C was reduced to 15°C over a 24-h period using a cooling system linked with the water tank. The liver was harvested from individual fish at different time points before and after the activation of the cooling system (as indicated by gray triangle). Total RNA was isolated, reversely transcribed and used for real-time PCR for respective gene targets, including (A) β actin, (B) leptin I, (C) leptin II and (D) leptin receptor. Parallel experiment with goldfish maintained at 28°C water without activation of the cooling system was used as the control treatment. For our time course study, the data obtained (mean $\pm$ SEM, $n = 12$) were analyzed with two-way ANOVA followed by Tukey test. Difference between groups was considered as significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

major brain areas in goldfish involved in appetite control (7). Although the functional roles of NPY, AgRP, orexin, and apelin as orexigenic factors in fish models are well-documented (59) and their stimulatory effects on feeding have also been confirmed in goldfish (33, 41, 60), except for the drop in orexin mRNA occurring in the hypothalamus at 15°C, noticeable changes in gene expression for these feeding stimulators were not observed in the brain areas examined. In the same study, 15°C acclimation during the winter was found to up-regulate central expression of anorexigenic factors, including the transcript expression of CCK, CART, and POMC in the telencephalon and CCK, MCH, and POMC in the hypothalamus. In contrast, significant changes of leptin I, leptin II, CCK, CART, MCH, and POMC signals were not apparent in the optic tectum. A similar pattern of transcript expression observed in our seasonality study was also noted in our time-course experiment with a gradual drop of water temperature to 15°C within 6 h in goldfish acclimated at 28°C. In this case, similar to the rapid responses of foraging/food intake with short-term thermal acclimation, notable changes of transcript expression for leptin I and II in the liver as well as LepR and other feeding regulators expressed in different brain areas were also observed within 6–12 h exposure to temperature change and maintained up to 24 h during the course of the experiment. These results, as a whole, suggest that the reduction in foraging activity and food intake in goldfish caused by the seasonal change in water temperature may be mediated by the rises of leptin I and II signals in the liver with parallel enhancement in leptin sensitivity via LepR up-regulation in brain areas involved in feeding control. Meanwhile, central regulation of orexigenic/anorexigenic signals can also occur, with a down-regulation of orexin in the hypothalamus along with parallel rises of CCK, CART, MCH, and POMC expression in the telencephalon/hypothalamus. In our study, the orexigenic/anorexigenic factors expressed in the optic tectum did not exhibit seasonal change/noticeable responses to temperature drop. Presumably, this brain area is not a major site within the CNS for temperature sensing or thermal responses in goldfish.

In summary, we have confirmed that seasonal change of feeding with a parallel reduction in foraging activity and food intake do exist in goldfish as a result of temperature drop during the transition from summer to winter period. These feeding responses can occur rapidly and are highly reversible with respect to temperature change, and may involve the leptin output from the liver with differential modifications of orexigenic/anorexigenic signals and leptin responsiveness in brain areas for appetite control. To our knowledge, our study represents the first report on (i) thermal regulation of leptin expression in the liver and (ii) involvement of leptin/LepR system in seasonal change of feeding induced by temperature drop in a fish model. Although our studies have provided new insights on the mechanisms for seasonal change of feeding in fish species, the functional components for thermal detection, e.g., through the thermal sensing neurons within the hypothalamus (37) or vagus nerve network (61), have yet to be determined. Of note, POMC expression induced by CCK (43) and CART expression

induced by leptin (62) and MCH (63) have been reported in goldfish, especially in brain areas responsible for feeding control, and the possibility for functional interactions among different feeding regulators in the seasonal cycle of feeding cannot be excluded. Besides temperature change, photoperiod is another environmental cue known to affect feeding in fish species (4). Given that melatonin has been shown to inhibit food intake in goldfish (64) and stimulate leptin expression in the liver (65) with parallel changes of orexigenic/anorexigenic signals in the brain of zebrafish (*Danio rerio*) (66), the functional interplay between photoperiod and temperature via a “crosstalk” of melatonin with other feeding regulators for sure can be an interesting topic to follow up for the seasonal change of feeding in fish models.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study was carried out in accordance with the recommended guidelines for the care and use of laboratory animals for research and teaching at the University of Hong Kong (Hong Kong). The protocol used in our study (CULATR 4608-18) was approved

by the Committee on the Use of Live Animal for Teaching and Research, University of Hong Kong.

AUTHOR CONTRIBUTIONS

AW was the PI and grant holder. AW and TC were responsible for project planning and data analysis. TC and MW were involved in the experiments for seasonal studies and thermal acclimation. Manuscript preparation was done by AW and BC.

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Bombyxin (*Bombyx* Insulin-Like Peptide) Increases the Respiration Rate Through Facilitation of Carbohydrate Catabolism in *Bombyx mori*

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Bombyxin-II, an insulin-like peptide of the silkworm *Bombyx mori*, has been shown to reduce both the trehalose concentration in the hemolymph and the glycogen content in some tissues of *B. mori* larvae. However, little is known about how these storage carbohydrates are utilized. To address this question, the effects of bombyxin-II injection into *Bombyx* larvae on the tissue lipid level, respiration rate, and glycolytic activity of tissues were investigated. Bombyxin-II did not affect lipid accumulation in the hemolymph and fat body, while it increased the rate of oxygen consumption and increased the content of fructose 2, 6-bisphosphate, a potent activator of glycolysis, in the gonads, imaginal discs, and midgut. These results suggest that bombyxin facilitates cellular energy production thereby supporting the tissue growth of insects.

Keywords: insect, insulin-like peptide, bombyxin, *bombyx mori*, respiration rate, glycolysis, fructose 2, 6-bisphosphate, carbohydrate metabolism

INTRODUCTION

Recent studies on insect insulin-like peptides (ILPs) have revealed that these peptides play important roles in the regulation of growth and development, metabolism, reproduction, as well as aging (1, 4, 5). The first study demonstrating the actions of insect ILPs on sugar metabolism was performed with bombyxin, the first identified ILP in invertebrates (6). Bombyxin is a family of ILPs mainly produced by the brain of the silkworm *Bombyx mori*, and nearly 40 genes encoding bombyxin have so far been identified in the *B. mori* genome (7). Bombyxin is produced by 4 pairs of large neurosecretory cells in the dorsomedial part of the brain, axonally transported to and secreted from the corpora allata (8). The secretion of bombyxin is stimulated by feeding and inhibited by fasting (9), as in the case for insulin in mammals. However, when bombyxin was discovered, nothing was known about its physiological function. This peptide was purified from *B. mori* as a hormone that stimulates the prothoracic glands of the saturniid moth *Samia cynthia ricini* but a later study found that it had no prothoracicotrophic activity in *B. mori*, from which it was derived (10). It is well known that in mammals insulin is secreted after meals to promote glucose uptake into the liver and muscle and regulates carbohydrate metabolism. The effects of insulin on carbohydrate metabolism include the promotion of glycogen synthesis, inhibition of gluconeogenesis, and conversion of excess sugar into lipid in specific tissues (2, 11). Therefore, as a first step to explore the physiological function of bombyxin, its effects on the sugar

concentration in the hemolymph and glycogen content in the fat body and muscle were investigated (12). As expected, bombyxin injection into *Bombyx* larvae resulted in a dose-dependent decrease in the trehalose concentration in the hemolymph. Trehalose is a major blood sugar in most insects, circulating at high concentrations to serve as a readily available storage carbohydrate for peripheral tissues. This non-reducing disaccharide is catabolized into glucose by trehalase (EC 3.2.1.28) present in the hemolymph (in a soluble form) or in the plasma membrane of tissues (in a membrane-bound form) and then taken up into cells (13). Therefore, the observed decrease in the hemolymph trehalose suggested its incorporation into and utilization by some tissues. Unexpectedly, however, bombyxin injection did not increase the glycogen content in the fat body and muscle but decreased it in the fat body, in contrast to the effects created by insulin in mammals.

Subsequent studies on the metabolic effects of ILPs in other insects consistently demonstrated their hypoglycemic effect, but their effects on glycogen accumulation differed between insects. In *Drosophila melanogaster*, the genetic ablation of insulin-producing cells in the brain or deletion of *Drosophila* ILP genes caused hyperglycemia and an increase in glycogen content (5, 14, 15), suggesting a role for ILPs in reducing both hemolymph sugar and tissue glycogen content, consistent with the results in *B. mori*. However, in the mosquito *Aedes aegypti*, although injection of ILP3, one of *Aedes* ILPs, into decapitated insects reduced circulating trehalose levels, such a treatment led to an increase in the glycogen content of the insects (16). In the blood-sucking bug *Rhodnius prolixus*, Rhopr-ILP transcript knockdown led to an increase in the hemolymph carbohydrate level in both unfed and recently fed insects, but the response of the fat body carbohydrate content to this treatment differed between the two insect conditions: it was decreased in unfed insects, but was unchanged in the recently fed insects (17).

The effect of ILP manipulation on the lipid level has also been examined in some insects. In *D. melanogaster* and *R. prolixus*, the reduction of ILP expression by means of above-mentioned techniques induced an increase in lipid levels in specific tissues or in the whole body (15), suggesting that ILP acts on tissues to decrease lipid levels. In contrast, however, ILP injection in *A. aegypti* resulted in an increase in the lipid level of the body (17). Thus, the effect of ILPs on lipid metabolism seems to differ between insect species.

These results suggested that insect ILPs regulate carbohydrate metabolism as does insulin, but the mechanisms and implications of the metabolic regulation by insulin/ILP may differ between mammals and insects, and even among various insect species. In the current study, we investigate how the storage carbohydrates are utilized under the control of bombyxin in *B. mori* larvae with the aim of understanding the significance of metabolic effects of ILPs in insects.

MATERIALS AND METHODS

Animals

A racial hybrid of the silkworm *Bombyx mori*, Kinshu × Showa, was used. Larvae were fed the artificial diet

“SilkMate 2S”(Nihon Nosan Kogyo, Yokohama, Japan) and reared under a 12-h light/12-h dark photoperiod at $25 \pm 1^\circ\text{C}$. Fifth instar larvae were used for biochemical analyses in accordance with the previous study (12), but fourth instar larvae were used for the measurement of the respiration rate, because the fifth instar larva was too large to fit into a reaction chamber for respiration rate measurement.

Hormone Injection

Day-3 fourth instar larvae or day-3 fifth instar larvae were ligated between the thorax and abdomen, and the anterior part of the body including the head and thorax was cut off to produce isolated abdomens. For the respiration assay, the isolated abdomens were weighed before use. Day-8 fifth instar larvae, which were used for collecting wing discs, were ligated between the head and thorax (neck-ligation). Three hours after the ligation, 20 μl of synthetic bombyxin-II (18) solution was injected into the dorsolateral part of the abdomen. The dose of bombyxin-II was varied between experiments. The control isolated abdomens or neck-ligated larvae were injected with the same volume of vehicle (phosphate-buffered saline containing 0.5% bovine serum albumin).

Determination of Lipid Content in the Hemolymph and Fat Body

Hemolymph and fat body were sampled 3 and 6 h after bombyxin injection. Hemolymph (150–200 μl) was collected in an ice-cold microcentrifuge tube containing 2 μl of 500 mM sodium diethyldithiocarbamate, a phenoloxidase inhibitor, from a tiny hole made on the first proleg and centrifuged at $6,000 \times g$ for 10 min to remove hemocytes. After hemolymph collection, fat body was dissected from the fifth segment of the same larva, rinsed with 0.9% NaCl, and collected into an ice-cold microcentrifuge tube. Both collected samples were frozen at -30°C until use. Lipids in these samples were extracted with chloroform/methanol following Bligh and Dyer (3) with some modifications. The fat body was homogenized in 300 μl of 0.9% NaCl by sonication, and 50 μl of the homogenate was used for lipid extraction. The homogenate was diluted with the same volume of 0.2 M acetic acid and blended with 250 μl of methanol and 125 μl of chloroform in a glass tube using a vortex mixer. After 10 min, the mixture was successively blended with 125 μl of chloroform and 125 μl of 0.1 M acetic acid, followed by centrifugation at $1,500 \times g$ for 10 min. An aliquot from the chloroform layer at the bottom of the tube was transferred to a fresh tube and evaporated using a vacuum centrifuge. For lipid extraction from the hemolymph, 40 μl of the hemolymph was mixed with 60 μl of 0.17 M acetic acid and processed in the same way. Lipids in the evaporated samples were quantified by the vanillin phosphoric acid method using a Lipid Quantification Kit (Cell Biolabs, San Diego, CA). The absorbance at 540 nm was measured on a plate reader. A lipid standard included in the kit was used to generate a standard curve for lipid determination.

Determination of Protein Content in the Fat Body

An aliquot of the same fat body homogenate used for lipid determination was diluted with the same volume of distilled water and centrifuged at $14,000 \times g$ for 10 min. The protein content in the supernatant was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and a microtiter plate. The absorbance at 540 nm was measured on a plate reader. The protein content was determined with bovine serum albumin as a standard.

Determination of Total Sugar Concentration in the Hemolymph

The hemolymph sample (20 μ l) was diluted 1:10 with 0.9% NaCl and heated in boiling water for 10 min. After centrifugation at $14,000 \times g$ for 10 min, 20 μ l of the supernatant was mixed with 1 ml of ice-cold anthrone reagent using a vortex mixer. The mixture was heated at 95°C for 10 min, followed by cooling

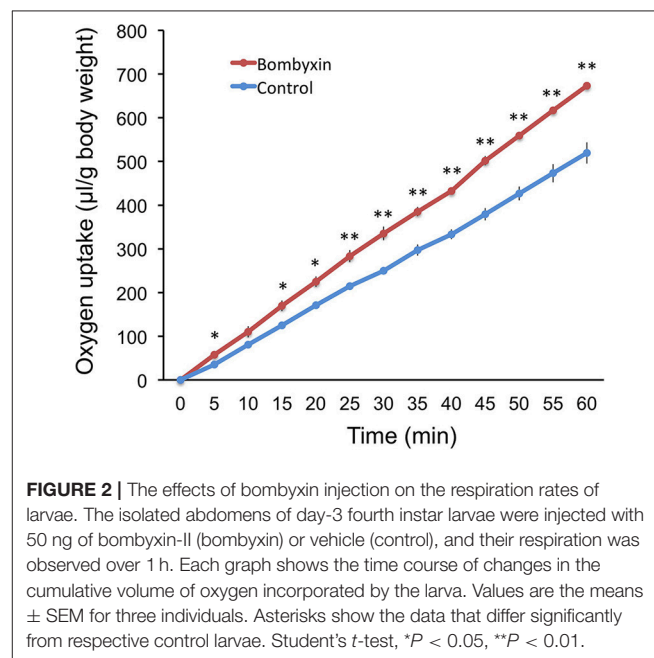
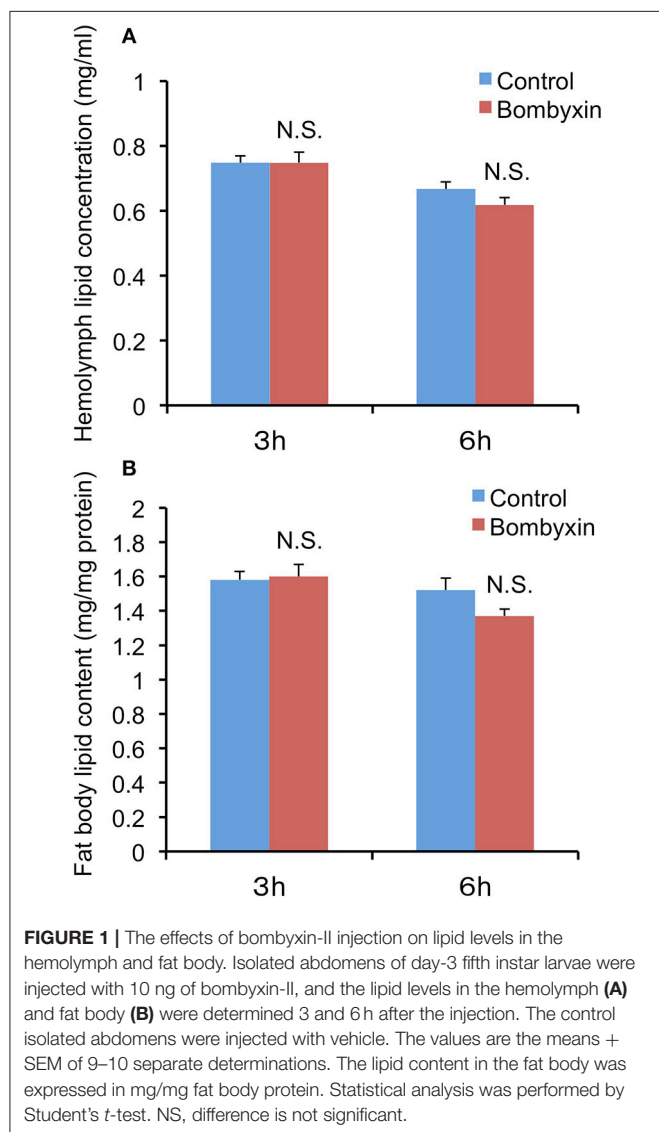
in water and vortexing. One hundred microliter of the reaction mixture was used for the measurement of absorbance at 620 nm on a plate reader. The sugar concentration was determined using glucose as a standard.

Measurement of Respiration Rate

The respiration rate of animals was measured using the O₂ UP TESTER (TAITEC, Tokyo, Japan). Briefly, the reaction chamber containing the isolated abdomen and a specific volume of 20% KOH was connected with a capillary, and the capillary was then horizontally sunk in a water bath set at 25°C. When the animal consumes oxygen and releases carbon dioxide by respiration, the latter gas is absorbed by KOH solution, resulting in the movement of water into the capillary. Thus, the respiration rate of the animal can be estimated by measuring the movement rate of water along the capillary. The position of the forefront of water in the capillary was recorded every 5 min. Groups of three isolated abdomens each of bombyxin- and vehicle-injected animals were assayed at the same time to obtain the mean respiration rate of each group. The same experiment was repeated four times.

Quantification of Fructose-2,6-Bisphosphate (Fru-2,6-P₂) in Tissues

The ovaries, testes, and small pieces of the fat body, muscle, and midgut were dissected from the isolated abdomen of day-3 fifth instar larvae. The wing discs were dissected from neck-ligated day-8 fifth instar larvae. The collected tissues were rinsed twice in 0.75% NaCl, blotted on filter paper, and frozen in liquid nitrogen. The frozen tissues were weighed and then homogenized with a glass homogenizer in 10 volumes of 100 mM NaOH, followed by heating at 80°C for 5 min. After centrifugation at $12,000 \times g$ for 15 min, the supernatant was mixed with 6 volumes of 20 mM HEPES buffer containing 1 mM acetic acid to adjust pH to 7–8.



The mixture was centrifuged again and the supernatant used as a sample for quantification of Fru-2,6-P₂.

Fru-2,6-P₂ was quantified according to the method of Van Schaftingen et al. (19), where its content in the sample is determined as its ability to activate pyrophosphate-dependent phosphofructokinase (PPi-PFK). PPi-PFK was purified from the potato tuber following Van Schaftingen et al. (19) with some modifications. The grated potato tuber (500 g) was mixed with two volumes of ice-cold 20 mM HEPES, pH 8.2, containing 20 mM potassium acetate and 2 mM dithiothreitol (DTT), followed by filtration. Sodium pyrophosphate and magnesium chloride were added to the filtrate to give final concentrations of 2 mM and pH was adjusted to 8.2 at 0°C. The filtrate was heated at 59°C for 5 min with stirring and then cooled to 0°C. Polyethylene glycol 6,000 was added to the filtrate (6 g/100 ml), and the mixture was stirred for 15 min and then left to stand for 10 min. After centrifugation at 4,000 × g and 0°C for 10 min, the supernatant was collected and mixed with polyethylene glycol 6,000 (8 g/100 ml). After incubation as described above, the mixture was centrifuged to collect the precipitate. The precipitate was dissolved in 20 mM Tris-HCl buffer, pH 8.2, containing 20 mM KCl and 2 mM DTT. This solution was loaded onto the DEAE-cellulose column and the adsorbed materials were eluted with the same buffer containing increasing concentrations of KCl (20–400 mM). The eluate was fractionated and the fraction with highest PPi-PFK activity was stored at –20°C after mixing with the same volume of glycerol.

An assay mixture was produced following the procedure of Van Schaftingen et al. (19) with some modifications. In brief, 250 µl of Tris/Mg²⁺/fructose-6-phosphate/NADH mixture, 50 µl of enzyme cocktail (aldolase, glycerol-3-phosphate dehydrogenase, and triose phosphate isomerase), 10 µl of PPi-PFK, and sample or standard Fru-2,6-P₂ were mixed, and the volume was made up to 500 µl with distilled water. After incubation at 25°C for 5 min, 25 µl of sodium pyrophosphate solution was added and the absorbance at 340 nm of the reaction mixture was read with a spectrophotometer every minute for 10 min.

All the samples from 3 to 6 bombyxin-injected and control larvae were assayed at the same time, and the means of Fru-2,6-P₂ content of each tissue were compared between bombyxin-injected and control groups. Fru-2,6-P₂ content was expressed as nmol/g for the midgut, fat body, and muscle, or as nmol/pair or set for the gonads and wing discs. The same experiment was repeated 2–5 times.

Statistical Analysis

Statistical analysis was performed using the Student's *t*-test. For the analysis in some experiments, a special treatment of data was necessary prior to the analysis for the following reasons. In the experiment to determine the respiration rate of animals (Figure 3) or the Fru-2,6-P₂ content of tissues (Figure 4), the number of animals or tissues to be assayed in one set of experiments was too small to analyze the significant difference between the control and experimental groups due to the limitation in the capacity of an equipment or sampling schedule. Therefore, the same set of experiments was repeated several times. However, it was impossible to simply gather

the data obtained in several sets of experiments for statistical analysis, because the measured values were varied considerably from experiment to experiment due to unknown differences of experimental conditions. Thus, we decided to calculate first the average of measured values for control and experimental groups in each set of experiments and then calculated the value for the experimental group relative to that for the control group, with the control value as 1. The same experiment (measurement) was repeated 3–5 times and the relative values were calculated in the same way. Finally, statistical analysis was performed using the data sets obtained in these experiments.

RESULTS AND DISCUSSION

Effects of Bombyxin Injection on the Lipid Levels in the Hemolymph and Fat Body

In mammals, insulin promotes the conversion of excess carbohydrates into lipids. Therefore, we first examined the possibility that the previously observed reduction in trehalose and glycogen levels after bombyxin-II injection into *Bombyx* larvae reflects their conversion into lipids. When lipid levels in the hemolymph and fat body, a major lipid storage tissue, were determined 3 and 6 h after injection of 10 ng bombyxin-II into the isolated abdomens of day-3 fifth instar larvae, no significant changes in the lipid levels, when compared with controls, were detected in either tissue (Figure 1). In parallel with this experiment, the total sugar level in the hemolymph at 6 h after bombyxin-II injection was also determined to confirm the effect of bombyxin-II on sugar metabolism. The total sugar concentration in control larvae (isolated abdomens) was 2.78 ± 0.26 mg/ml, whereas that in bombyxin-injected larvae was 1.89

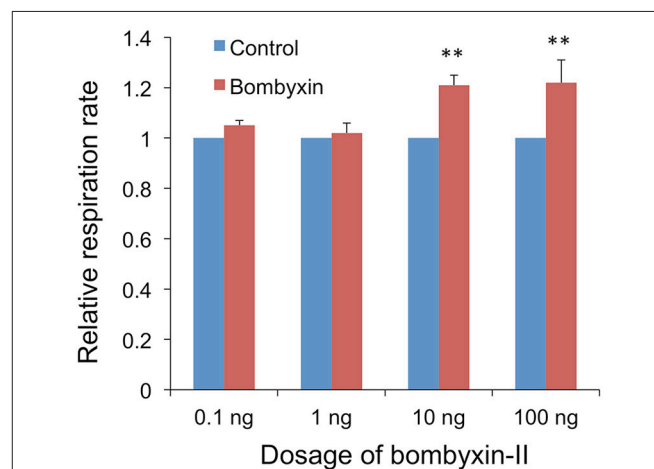


FIGURE 3 | Dose-dependent effects of bombyxin injection on the respiration rate of larvae. Three isolated abdomens were injected with bombyxin, and another three isolated abdomens were injected with vehicle, and their respiration was observed over 1 h to determine the respiration rate (µl/min). The average value for the respiration rates of bombyxin-injected larvae was divided by that of control larvae to calculate a relative respiration rate. The same test was repeated 4 times for each dose of bombyxin-II. The graph shows the means + SEM of the relative respiration rates for each dose of bombyxin-II. Student's *t*-test, ***P* < 0.01.

± 0.11 mg/ml, showing a significant decrease (t -test, $p < 0.01$) in the sugar level in bombyxin-injected larvae. These results suggest that bombyxin-II do not promote lipid synthesis, at least within 6 h after injection.

The Effect of Bombyxin-II Injection on the Respiration rate of *B. mori* Larvae

Given that storage carbohydrates were not converted into lipids, it was conceivable that those were used for cellular respiration. To examine this possibility, 50 ng of bombyxin-II was injected into the isolated abdomen of day-3 fourth instar larvae, and the amount of consumed oxygen was recorded every 5 min over 1 h beginning at 15 min after injection (Figure 2). This dose (50 ng), and not 10 ng, was selected in this experiment, because we were not aware of the minimum dose of bombyxin-II necessary to elicit a detectable change in the respiration rate. The cumulative volume of oxygen consumption in bombyxin-injected larvae was significantly larger at all time points of measurement except for 10 min after the onset of observation. The respiration rate of bombyxin-injected larvae was fairly constant over 1 h and was approximately 20 % higher than that of controls.

Next, to examine the dose-dependency of the effect of the hormone, 0.1, 1, 10, or 100 ng of bombyxin-II were injected. No effect was detected with 0.1 and 1 ng, while an increase of approximately 20% in the respiration rate was observed with 10 and 100 ng of bombyxin-II (Figure 3). This result indicates that 10 ng of bombyxin-II is sufficient to maximally increase the respiration rate of the larvae.

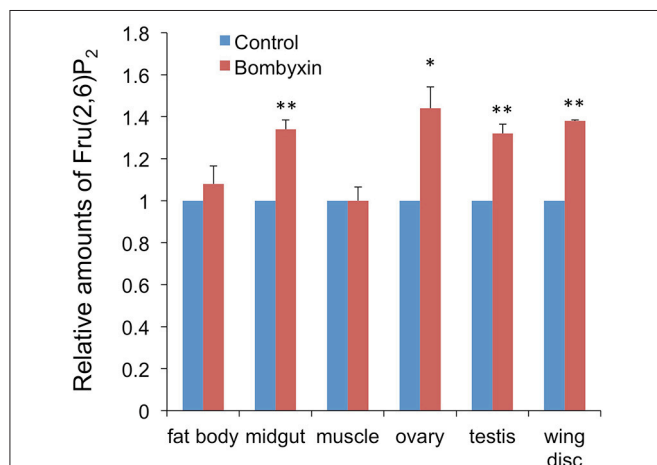


FIGURE 4 | The effects of bombyxin injection on the Fru-2,6-P₂ content in tissues. The isolated abdomens of day-3 fifth instar larvae were injected with 10 ng of bombyxin-II or vehicle, and various tissues were dissected 1 h after the injection to determine the Fru-2,6-P₂ content in each tissue. Only the wing discs were dissected from neck-ligated day-8 fifth instar larvae. In one experiment, 3–6 samples for each tissue were assayed to calculate an average Fru-2,6-P₂ amount relative to the control. The same experiment was repeated 2–5 times and the means $\pm$ SEM of the relative Fru-2,6-P₂ amounts are shown in the graph. Student's t -test, * $P < 0.05$, ** $P < 0.01$.

Effects of Bombyxin-II Injection on the Fru-2,6-P₂ Content in Larval Tissues

An elevated respiration rate after bombyxin-II injection suggested that the previously observed reductions in the trehalose concentration in the hemolymph and glycogen content in some tissues after bombyxin injection were the results of an increased consumption of these storage carbohydrates for cellular energy production. To test this hypothesis, we next examined the effect of bombyxin injection on the tissue content of Fru-2,6-P₂, known as a potent activator of phosphofructokinase, a rate-limiting enzyme of glycolysis (20). We used the tissue content of this sugar phosphate as an index of glycolytic activity, because we did not have any assay method to directly determine the glycolytic activity of tissues. Ten nanogram of bombyxin-II was injected into the isolated abdomens of day-3 fifth instar larvae or neck-ligated day-8 fifth instar larvae, and 1 h later the tissue content of Fru-2,6-P₂ was measured. The midgut, fat body, muscle, testes, and ovaries were dissected from the day-3 larvae. The wing discs were collected from the day-8 neck-ligated larvae because this tissue was too small on day-3 and resides in the thorax. A significant increase in the Fru-2,6-P₂ content was observed in the midgut (34%), ovaries (44%) testes (32%), and wing discs (38%), whereas no increase was detected in the fat body and muscle (Figure 4).

An Implication of the Metabolic Effects of ILPs In Insects

In mammals, insulin regulates many aspects of carbohydrate metabolism, lipid metabolism, and protein metabolism. The major effects of insulin on carbohydrate metabolism include (i) increases in the rate of glucose transport across the cell membrane in adipose tissue and muscle, (ii) in the rate of glycolysis in these tissues, (iii) in the rate of glycogen synthesis in a number of tissues including liver and muscle, (iv) in the rate of glucose oxidation by the pentose phosphate pathway in the liver and adipose tissue, (v) a decrease in the rate of glycogen breakdown in the muscle and liver, and (vi) inhibition of both glycogenolysis and gluconeogenesis in the liver (2). In insects, however, direct targets of ILPs in their metabolic regulation have not yet been determined in most cases, although their effects on hemolymph sugar concentration, on glycogen content in some tissues, and on lipid levels in specific tissue or whole body were demonstrated in several insects. The only study getting at the direct target of insect ILP was the study on the effects of bombyxin-II on the enzymatic activity of trehalase and glycogen phosphorylase in *Bombyx* larvae (12). In this study, Satake et al. demonstrated that bombyxin increased trehalase activity in the midgut and muscle and the glycogen phosphorylase activity in the fat body, consistent with the bombyxin-induced decreases in the hemolymph trehalose and in the glycogen content in the fat body, respectively. These results strongly suggested that the major storage carbohydrates in *B. mori* are somehow consumed in response to bombyxin-II. However, the fate of the consumed storage carbohydrates was a riddle. The results of the current study may provide an answer to this question, at least in part. It seems that the consumed carbohydrates are not converted

to lipid, another form of energy reserves, because bombyxin-II injection did not change the lipid levels in the hemolymph and fat body. The observed effects of bombyxin-II injection on the respiration rate and Fru-2,6-P₂ content in some tissues suggest that the consumed carbohydrates were used for energy production, although direct evidence has not been obtained. The effects of ILP to reduce trehalose and glycogen have also been shown in *D. melanogaster*, suggesting that such effects of ILP could be generalized to other insects. In *D. melanogaster*, it was also reported that deletion of ILP genes led to the reduction in heat production of larvae, indicative of decreased overall metabolic rates (21). The promotion of glycolysis is one of the major actions of insulin in mammals (2, 22). Therefore, activation of energy metabolism might be a common and essential role of ILPs in animals.

The best-known activity of insect ILPs is the promotion of tissue and systemic growth (4, 14, 23, 24). Interestingly, the increase in the Fru-2,6-P₂ content after bombyxin injection was observed in the wing discs and gonads, both of which have been shown to grow in response to bombyxin (23) or *Aedes* ILP (16). Therefore, it seems that ILPs simultaneously stimulate cellular growth and energy metabolism so that the enhanced production of energy can support cellular growth including protein synthesis, DNA duplication, and proliferation.

It is evident, however, that further studies are necessary to establish an energy metabolism-promoting action of ILPs and to understand the cellular and molecular mechanisms underlying this action of ILPs.

The Fru-2,6-P₂ content of the midgut was also increased in response to bombyxin-II. This increase might meet an increased

energy demand of the midgut for digestion and absorption of food, because bombyxin is secreted soon after food intake (9). In contrast, no bombyxin-stimulated increases in the Fru-2,6-P₂ content were observed in the muscle and fat body. Glycolytic activity of these tissues might be regulated in a bombyxin-independent manner.

In the female adult mosquito, ILP injection induced increases in glycogen and lipid levels of the insects (16). Also, in the blood-sucking bug, knocking down ILP expression reduced the carbohydrate content in the fat body and leg muscle, indicative of the effect of ILP to increase tissue glycogen content (17). Why do the effects of ILP differ between species? It is interesting to note that the adult mosquitos and blood-sucking bugs feed on blood meal with long intervals, differing from *Bombyx* and *Drosophila* larvae, which feed on food continually. Insects having long periods of starvation between meals may have evolved the ILP-dependent mechanisms by which excess carbohydrates ingested in a big meal are converted to energy reserves such as glycogen and lipid to prepare for subsequent starvation, like humans.

AUTHOR CONTRIBUTIONS

AM conceived the research. YK, HW, and AM conducted the experiments and analyzed the data. YK and AM wrote the manuscript.

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Insulin-Like Peptide Signaling in Mosquitoes: The Road Behind and the Road Ahead

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Insulin signaling is a conserved pathway in all metazoans. This pathway contributed toward primordial metazoans responding to a greater diversity of environmental signals by modulating nutritional storage, reproduction, and longevity. Most of our knowledge of insulin signaling in insects comes from the vinegar fly, *Drosophila melanogaster*, where it has been extensively studied and shown to control several physiological processes. Mosquitoes are the most important vectors of human disease in the world and their control constitutes a significant area of research. Recent studies have shown the importance of insulin signaling in multiple physiological processes such as reproduction, innate immunity, lifespan, and vectorial capacity in mosquitoes. Although insulin-like peptides have been identified and functionally characterized from many mosquito species, a comprehensive review of this pathway in mosquitoes is needed. To fill this gap, our review provides up-to-date knowledge of this subfield.

Keywords: insulin signaling, insulin-like peptides, mosquitoes, insulin receptor, aedes, anopheles, culex

INTRODUCTION

Insulin-like peptides (ILPs) are broadly conserved among metazoans and are the most studied peptide hormones because of their important regulatory roles in metabolism, growth, and development. All ILPs are 6–8 kDa, share a common structural motif called the insulin fold, and are processed from precursors with similar domain structure (Pre, B, C, A) (1). Among arthropods, insulin signaling is most well-understood in the model insect *Drosophila melanogaster*. The *D. melanogaster* genome has eight ILPs (dILPs), each with specific tissue expression. For instance, some dILPs originate from the brain and ventral nerve cord, while others are expressed in the midgut, fat body, or imaginal discs (2–4).

Mosquitoes have varying numbers of ILPs (Table 1) ranging from five to eight, and, similar to the situation in *D. melanogaster*, expression has been detected in the nervous system, fat body, midgut, ovaries, and other tissues. Each mosquito species has a distinct set of ILPs that are of neural origin, while others are expressed in multiple tissues (5, 7–9).

Similar to other metazoans, the mosquito insulin receptor (MIR) is a transmembrane receptor tyrosine kinase (RTK) and consists of a dimer of α and β -monomers. The α -subunits define ILP ligand binding specificity, whereas the β -subunits mediate the downstream signal to cellular components. The MIR uses insulin receptor substrate (IRS) as an adaptor molecule to initiate signaling (10). Upon binding of the ligand to its receptor, the β -subunits undergo auto-phosphorylation at specific tyrosine residues. The activated RTK subsequently phosphorylates specific tyrosine residues of the IRS (11). IRS then recruits

TABLE 1 | Number of insulin-like peptides identified in different mosquito genera and species.

Mosquito species	Number of ILPs	References
<i>Aedes aegypti</i>	Eight: Aa ILP1-8	(5)
<i>Anopheles gambiae</i>	Five: AgILP1/7, 2, 3/6, 4, 5	(6)
<i>Anopheles stephensi</i>	Five: AsILP1-5	(7)
<i>Culex pipiens</i>	Three: (more likely)	(8)

downstream factors to the receptor-IRS complex. The phosphorylated tyrosine residues of the receptor-IRS complex interact with phosphatidylinositol-3-kinase (PI3K) proteins (12, 13). Recruitment of PI3K results in the formation of the IRS-PI3K complex. Subsequently, PI3K catalyzes synthesis of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2).

Phosphatase and Tensin homolog (PTEN) is a negative regulator and can reverse this conversion from PIP3 to PIP2 and decrease the level of PIP3 in the cell. The phosphoinositide-dependent protein kinase (PDK) responds to the high PIP3 levels by recruiting Akt (13, 14). Akt is considered the master regulator kinase because the phosphorylation of Akt affects a number of downstream protein substrates including the target of rapamycin (TOR) (15, 16). TOR activation occurs both as a direct downstream event of insulin signaling activation or, independent of Akt, by the availability of amino acids.

TOR and ILP signaling pathways are considered nutritional sensors at the cellular and systemic level, respectively. Akt-mediated phosphorylation of forkhead-related FOXO proteins prevents the FOXO transcription factor from being translocated to the nucleus (17–20). FOXO proteins are indispensable in an organism's response to starvation since they promote conservation of energy or even catabolism (21). There has been some work exploring TOR and FOXO signaling in mosquitoes (22–28), but a detailed review is outside the scope of this article.

IDENTIFICATION AND STRUCTURE OF ILPS

Prior to the identification of mosquito ILPs and MIR, it was well-known that shortly after blood feeding, neurohormones are released from the brain neurosecretory system that stimulate the ovaries to secrete ecdysteroids, which are necessary for vitellogenesis by the fat body. The silkworm *Bombyx mori* ILP, bombyxin, was demonstrated to stimulate ecdysteroidogenesis in prothoracic glands in silkworm larvae. This led to hypothesis that insulins are involved in regulation of the ecdysteroid pathway in mosquitoes and commercially available porcine and bovine insulin were tested on unfed mosquito ovaries to test this hypothesis. This led to the discovery of the MIR in *Ae. aegypti* (29) and the discovery of other components of the insulin signaling pathway followed shortly (30). However, ILP identification in *Ae. aegypti* lagged by over half a decade (5). The publication of the *Anopheles gambiae* genome was seminal

in the identification of ILPs in mosquitoes. Seven ILP genes corresponding to five unique ILPs (AgILP1-5) and one MIR were identified in the *A. gambiae* genome (9). Two AgILP genes encode identical B and A peptides therefore seven ILP genes produce five peptides. Genes encoding eight unique ILPs were found in the *Ae. aegypti* genome (5). Except for AaILP6, seven other *Ae. aegypti* ILPs had a propeptide structure consistent with that of other invertebrate ILPs. AaILP6 is unique because it has a short C peptide and an extended A peptide, similar to the vertebrate insulin growth factors (IGFs), however, the C peptide of AaILP6 had multiple dibasic proteolytic cleavage sites, in contrast to only one in vertebrate IGFs (5). To date, there is no empirical evidence to confirm whether or not the predicted dibasic sites are actually cleaved/processed in *Ae. aegypti*.

The proximity of AaILP1, 3, and 8 in the genome scaffold suggested that they may form a polycistronic transcription unit controlled by a single promoter. All three of these have independent putative polyadenylation sites and are capped to generate monocistronic mature mRNAs (5). AgILP1/7 and 3/6, the duplicated gene pairs in *A. gambiae*, and AaILP1 and 3 appear to be orthologs. Phylogenetic analysis supported an evolutionary relationship between AaILP1 and AgILP1/7, as well as between AaILP3 and AgILP3/6. A functional relationship between *Anopheles stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus* ILP3 was also demonstrated (31). The third member of the *Ae. aegypti* ILP operon, AaILP8, was not related in sequence to any of the other dipteran ILPs (5).

Sequences encoding full-length transcripts of five ILPs from the *A. stephensi* genome (AsILP 1–5) and three from *Culex pipiens* (CpILP1, CpILP2, and CpILP5) were identified (7, 8). ILP4 of *A. stephensi* was highly similar to *A. gambiae* ILP4 but this ILP does not have an apparent ortholog in either *Ae. aegypti* or *C. pipiens*. *Ae. aegypti* ILP5 and *A. gambiae* ILP5 share up to 81% sequence similarity, uncommon for ILPs, and, together with *Cx. pipiens* and *A. stephensi* ILP5, share the unique feature of an additional amino acid between the second and third cysteine residues in the A chain (5, 7). DmILP7, an ortholog of AaILP5, also shares this feature and is well-conserved with the mosquito sequences [(5); **Figure 1**]. AaILP2 and AgILP2 form another related ILP subgroup (**Figure 1**).

The putative IGF-like ILP, AaILP6, was closely related to another ILP identified as a gene transcript in *Aedes subalbatus* (5). The remaining two AaILPs, AaILP4 and 7, do not appear to have any dipteran orthologs (**Figure 1**). This is not surprising considering that both possess an additional amino acid between the third and fourth cysteine residues in the A peptide, a feature not known for other members of the insulin superfamily (5).

A unifying feature of all ILPs is the presence of six conserved cysteine residues that form disulfide bonds between the B and A chains (**Figure 1**). However, outside of these core residues, amino acid sequence similarity diverges between the different types of ILPs. Some functional forms of ILPs are clearly conserved throughout the mosquito (such as ILP2, ILP3) and even dipteran lineages (such as mosquito ILP5/DILP7), and form distinct groupings when subjected to neighbor-joining analysis (**Figure 2**), but the evolutionary relatedness of different ILP isoforms to one another has poor branch support and remains

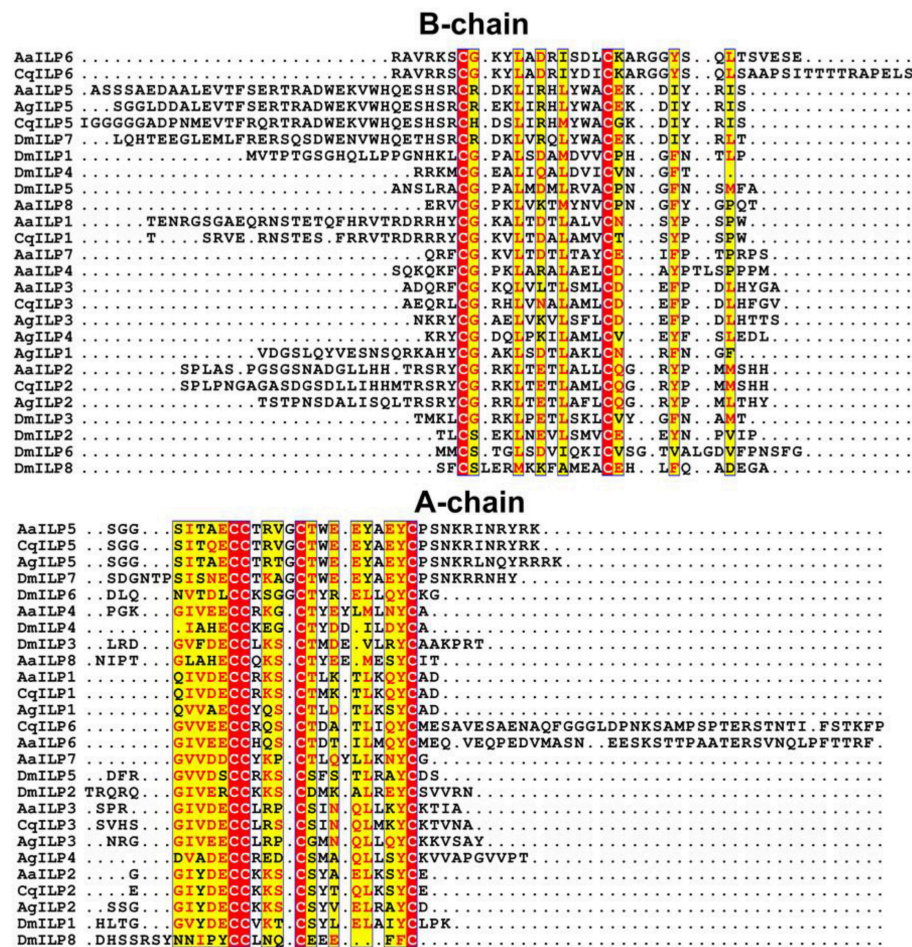


FIGURE 1 | Alignment of mosquito ILP amino acid sequences comparing *Ae. aegypti* (Aa), *C. quinquefasciatus* (Cq), *A. gambiae* (Ag), and *D. melanogaster* (Dm) ILPs by B- or A-chain. Red highlights indicate all identical residues, yellow highlights indicate majority conserved residues (red text indicates conserved residues, black indicates variants). The exceptionally long predicted A-chain of *C. quinquefasciatus* ILP6 is truncated at Pro146 for the sake of space. Alignments were performed in Pôle Rhône-Alpes de Bioinformatique (PRABI) website (32) and ESPrnt 3.0 (33).

unclear. It is likely that the secondary structure imposed by disulfide bonds and as yet undetermined key functional residues are the most critical components for ILP interaction with the MIR, whereas other amino acids may be more important in preserving spacing in the molecule, rather than the identity of their functional group. This limits our ability to predict functions of ILPs in related species based on amino acid sequence alone.

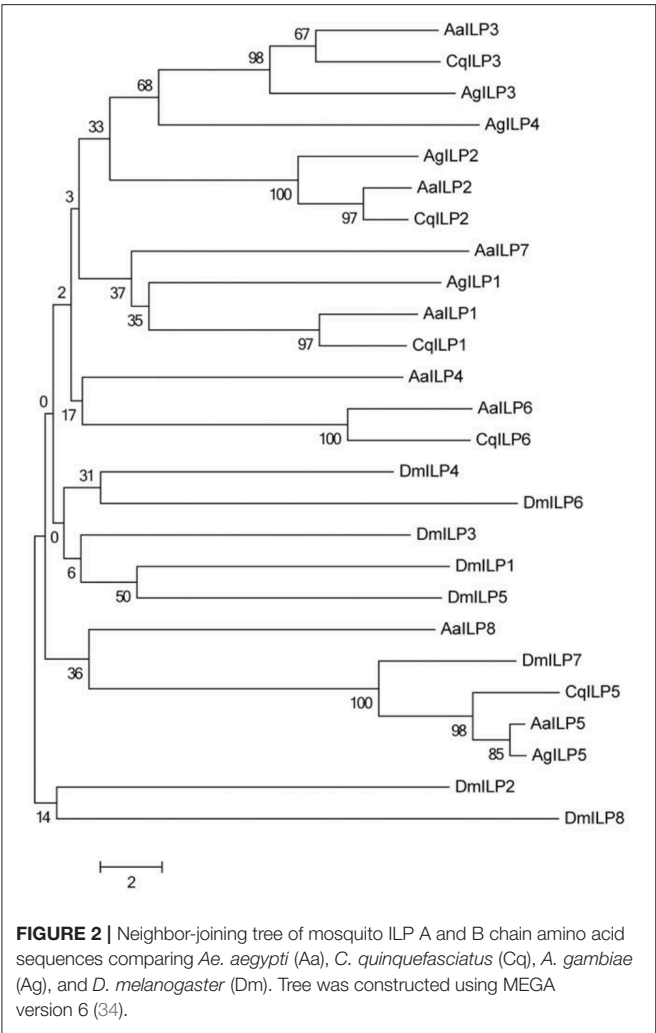
FUNCTIONS OF INSULIN-LIKE PEPTIDES IN MOSQUITOES

Function and signaling of ILPs are best characterized for *Ae. aegypti* [for other reviews see (35–37)]. Unlike *D. melanogaster*, genetic manipulations of ILPs to study pluripotency in mosquitoes is still in its infancy. The only native ILP isolated so far from mosquitoes is from *A. stephensi*, AsILP3 (31). AaILP3, AaILP4, and AaILP8 were chemically synthesized (23, 38, 39) and used to deduce their functions. With the availability of new

CRISPR-Cas9 based gene editing tools, the functions of two additional *Ae. aegypti* ILPs, AaILP7, and AaILP8, were recently investigated (40) (Table 2, Figure 3).

Nutrient Metabolism

Among mosquitoes, the role of ILPs in nutrient allocation is best studied in *Ae. aegypti*. AaILP1, ILP3, and ILP8 are specifically expressed in the brains of adult females (5). Synthetic AaILP3 (sAaILP3) binds to the MIR with high affinity and has been shown as a critical regulator of egg production (23, 38, 39, 41). sAaILP4 and sAaILP8 did not show any competition with sAaILP3 for binding to the MIR (39). Similarly, sAaILP3 (but not sAaILP4 and sAaILP8) injected into mosquitoes decapitated after a sugar meal dose-dependently increased the levels of stored glycogen and lipids and decreased the levels of trehalose (38) in the whole body of females, suggesting its function is analogous to mammalian insulin in vertebrates.



AaILP3 transcript levels were higher in mosquitoes that emerged from the high carbohydrate larval diet (43), further suggesting a role in nutrient metabolism. AaILP3 also stimulated the midgut to express trypsin-like proteases that digested the blood meal while amino acid sensing through the target of rapamycin (TOR) pathway enhanced AaILP3, ovary ecdysteroidogenic hormone (OEH), ecdysteroids, and vitellogenin synthesis (23, 47).

Some evidence exists that microRNAs regulate AaILP expression. For instance, in the absence of miR277, transcript levels of both AaILP7 and AaILP8 increased in head whereas AaILP1 and AaILP3 transcript levels did not change, suggesting that miR277 targets the first member (AaILP8) of the ILP8-ILP1-ILP3 operon (40). CRISPR-Cas9 depletion of AaILP7 and AaILP8 led to metabolic and reproductive defects. A dramatic lipid increase in the fat body in AaILP7 knockouts and a decrease in AaILP8 knockouts suggests a role of these ILPs in modulating lipid deposition and mobilization (40). Glycogen levels exhibited the opposite trends in these mosquitoes, which suggest that both AaILP7 and AaILP8 are involved in lipid and glycogen balance. In

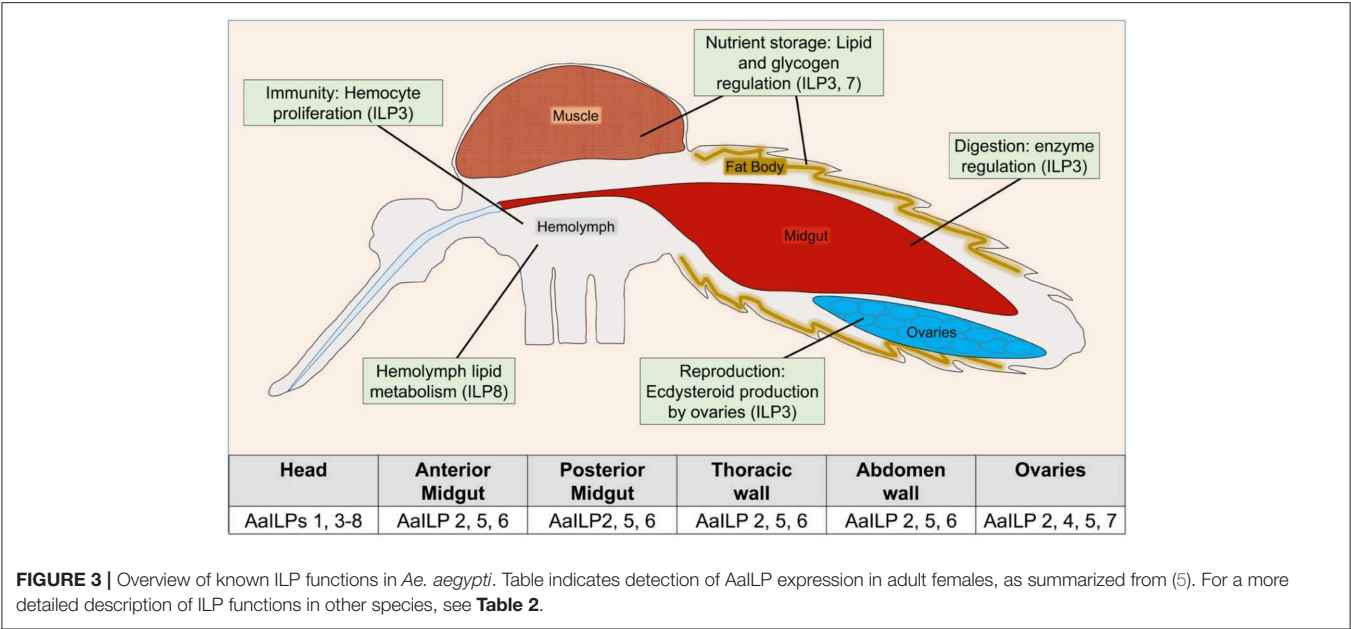
TABLE 2 | Potential functions of mosquito insulin-like peptides.

Insulin-like peptide (ILP)	Potential function	References
<i>Aedes aegypti</i>		
AaILP1	Not yet studied	
AaILP2	Not yet studied	
AaILP3	Nutrient metabolism, regulation of digestive enzymes, Ecdysteroid production from ovaries, immune response	(23, 38, 41, 42)
AaILP4	Nutrient metabolism in males	(43)
AaILP5	Not yet studied	
AaILP6	Not yet studied	
AaILP7	Glycogen metabolism post blood meal, nutrient metabolism post blood meal	(40)
AaILP8	Hemolymph lipid metabolism, larval molt	(40, 43)
<i>Anopheles stephensi</i>		
AsILP1	Not yet studied	
AsILP2	Not yet studied	
AsILP3	Ecdysteroids production by ovaries	(31)
AsILP4	Ecdysteroids production by ovaries; <i>Plasmodium falciparum</i> early infection	(31, 44)
AsILP5	<i>P. falciparum</i> oocyst development	(44)
<i>Anopheles gambiae</i>		
AgILP1/7	Not yet studied	
AgILP2	Not yet studied	
AgILP3/6	<i>P. falciparum</i> infection	(45)
AgILP4	Blood meal nutrients metabolism	(46)
AgILP5	Blood meal nutrients metabolism	(46)
<i>Culex pipiens</i>		
CpILP1	Diapause/overwintering	(8)
CpILP2	Not yet studied	
CpILP5	Higher expression but not associated with diapause	(8)

our study, AaILP8 transcript levels were higher in the late fourth instar larvae suggesting a possible role in larval to pupal molt (43), a function similar to *D. melanogaster* ILP8 (2).

In *A. gambiae*, artificial blood meal (albumin and amino acid mixture) rapidly triggered transcription of two ILPs- AgILP3 and AgILP4, in the brain of starved mosquitoes, and the response was higher compared to sucrose fed mosquitoes (46). In *A. stephensi*, expression of ILPs did not change significantly with age or upon ingestion of a sugar or blood meal (7, 44) suggesting differences in mosquito species. ILP functions in nutrient allocation in *Culex* spp. have not been studied yet.

Insulin receptor knockdown by RNA interference (RNAi) in newly eclosed females and subsequent decapitation within 2 h post blood meal resulted in slow blood digestion in *Ae. aegypti* (23). In mosquitoes decapitated post blood meal, sAaILP3 was able to restore trypsin transcripts and enzyme levels, while sAaILP4 and sAaILP8 had no effect on trypsin expression (23). A similar effect of insulin receptor knockdown on midgut trypsin levels was observed in *C. quinquefasciatus* (48). Whether this phenomenon extends to *Anopheles* spp. remains to be explored.



Reproduction

Ecdysteroid and Vitellogenin Production

The first indication of ILP involvement in insect reproduction was the use of bovine insulin to stimulate ecdysteroid production by *in vitro* ovaries isolated from unfed female *Ae. aegypti* (29). Further evidence that this effect was transduced through the insulin signaling complex was provided by using inhibitors or activators of the insulin receptor, PI3K, and Akt, which altered this response (6, 30, 49). Bovine insulin in combination with 20-hydroxyecdysone activated transcription of the yolk protein precursor gene and vitellogenin (Vg) in fat body culture. RNAi-mediated knockdown of the MIR and Akt inhibited insulin-induced Vg gene expression in *in vitro* fat body culture assays (47). sAaILP3 activated ecdysteroid production in unfed ovaries *in vitro* (23, 38, 50). sAaILP4 also stimulated ovaries to produce ecdysteroids *in vitro*, however, five times higher concentrations of sAaILP4 compared to sAaILP3 were required (39).

Anopheles stephensi sILP3 and sILP4 were both able to stimulate ovaries to produce ecdysteroids *in vitro* across the genera. Both sAsILPs stimulated ecdysteroid production from unfed ovaries in *A. stephensi*, *A. gambiae*, *Ae. aegypti*, and *C. quinquefasciatus* (31) suggesting a conserved role of ILPs in the regulation of ecdysteroid productions in mosquitoes. Insulin receptor knockdown in *C. quinquefasciatus* resulted in low levels ecdysteroids in blood-fed female ovaries (48) further supporting the findings that insulin signaling is required for ecdysteroid production.

Yolk Deposition

As the blood meal is digested by the female mosquito and nutrients are mobilized, the developing eggs uptake these nutrients as the yolk. Insulin receptor knockdown resulted in a decrease in the amount of yolk deposited in *Ae. aegypti* ovarioles (23). Injection of sAaILP3 in decapitated, blood-fed females

stimulated yolk deposition in ~50% ovarioles (23), whereas sAaILP3 injection in unfed females stimulated yolk deposition in a ~2% ovarioles that were later resorbed and never resulted in egg deposition (41, 51). RNAi knockdown of PTEN, a negative regulator of insulin receptor substrate, in *Ae. aegypti* led to an increase in egg production (52) further supporting the role of insulin signaling in reproduction.

CRISPR-Cas9 mutations of AaILP7 and AaILP8 affect ovarian development, but the phenotypes were different. AaILP7 mutant ovaries and their follicles were similar in size to the wild-type at 24 h post blood meal but were only half the size of those in the control by 72h. These mosquitoes also had elevated lipid stores at 72 h. In contrast, AaILP8 mutant ovaries were small and melanized by 24 h post blood meal (40). In *C. quinquefasciatus* females, insulin receptor knockdown and filarial nematode infection resulted in the complete shutdown of egg maturation and deposition (48).

Diapause

Diapause is characterized by an arrest in ovarian development and the sequestration of large amounts of lipid reserves. The short day lengths program the temperate mosquitoes such as *C. pipiens* to enter a reproductive diapause. Insulin signaling and FOXO (forkhead transcription factor), a downstream molecule in the insulin signaling pathway, are shown to mediate the diapause response (22). In non-diapausing mosquitoes, RNAi knockdown of the insulin receptor led to primary follicles arrested in a stage comparable to diapause. Juvenile hormone application reversed this diapause-like state. When dsRNA directed against FOXO was injected into mosquitoes programmed for diapause, fat storage was dramatically reduced and the mosquito's lifespan was shortened, suggesting that a shutdown of insulin signaling activates the downstream gene FOXO, leading to the diapause phenotype (22). Transcript levels of CpILP1 and 5 were

significantly lower in diapausing females than in their non-diapausing counterparts (8). Knocking down CpILP1 with RNAi in non-diapausing mosquitoes resulted in a cessation of ovarian development similar to diapausing female mosquitoes, whereas CpILP5 did not alter ovarian development (8).

Lifespan

The first report of the involvement of insulin signaling in lifespan regulation in invertebrates came from work in *Caenorhabditis elegans*. In *C. elegans*, a hypomorphic mutation in the insulin receptor homolog, Daf-2, resulted in a 300% increase in lifespan (53). In *D. melanogaster*, hypomorphic insulin receptor expressing flies showed an 85% increase in lifespan (54). In mosquitoes, overexpression of a myristoylated and active form of *A. stephensi* and *Ae. aegypti* Akt in the fat body of transgenic mosquitoes after blood feeding significantly increased adult survivorship relative to non-transgenic sibling controls (55). Similarly, PTEN overexpression also extended mosquito lifespan (56). Therefore, the effect on lifespan in these experiments with mosquitoes seems to be opposite of that seen in *C. elegans* and *D. melanogaster*, however, the direct effect of insulin receptor knockdown on lifespan has not yet been studied in mosquitoes. The lack of research is partly due to a lack of easily available genetic tools to make hypomorphic insulin receptor expressing mosquito lines. Most work in mosquitoes is done by RNAi, the effect of which lasts only for 7–10 days. In *A. stephensi*, high doses of ingested human insulin with blood meal were shown to reduce lifespan (57–59). In contrast, ingested human IGF1 extended lifespan in this species (60).

MOSQUITO IMMUNITY/ MOSQUITO-PATHOGEN INTERACTIONS

Mosquito hemocytes serve as the most important constitutive defense element against pathogens that enter the hemocoel (61, 62) and can produce phagocytic and melanotic immune responses (63, 64), effector molecules (65–69), and enhanced defense associated with immune priming (70). Decapitation of *A. aegypti* mosquitoes after blood feeding inhibited hemocyte proliferation and a single dose of sAaILP3 rescued hemocyte proliferation. Knockdown of the insulin receptor by RNAi inhibited ILP3 rescue activity. This suggests another role of ILPs in hemocyte proliferation, and thus immunity (42).

Malaria Parasite

The first indication that insulin signaling could play a role in mosquito-pathogen interaction came from a study suggesting human insulin could promote the development of *Plasmodium falciparum* oocysts in the midguts of *A. stephensi* and *A. gambiae*, although the insulin levels used vastly exceeded those in human blood at the physiological levels (71). *P. falciparum* glycosylphosphatidylinositols, a parasite factor that mimics insulin in mammals (72, 73), was later shown to activate insulin receptor, Akt/PKB (Protein kinase B), and the mitogen-activated protein kinase, DSOR 1, in the malaria vector *A. stephensi* (74).

Human IGFs, within a physiological range and higher levels of human insulin, has been shown to induce nitric oxide (NO)

synthesis in mosquito cell culture and in the *A. stephensi* midgut (74, 75). Inducible NO synthesis in *A. stephensi* limits malaria parasite development through the formation of inflammatory levels of reactive NO that likely induce parasite apoptosis in the mosquito midgut lumen (57, 74, 76, 77). Both radioactive human insulin and IGF1 persisted intact in the midgut up to 30 h post ingestion and human insulin could activate mosquito insulin receptor by phosphorylation (60).

P. falciparum-infected blood meal increased expression of AsILP2, 3, 4, and 5 in the head and midgut of *A. stephensi* (7). Similarly, soluble *P. falciparum* products directly induced AsILP expression in immortalized *A. stephensi* cells *in vitro*. Knockdown of AsILP4 by RNAi induced early expression of immune effector genes within 1–6 h after infection, resulting in significantly reduced parasite abundance prior to invasion of the midgut epithelium. In contrast, knockdown of AsILP3 increased expression of the same genes 24 h after infection. These data suggest that *P. falciparum* parasites alter the expression of mosquito ILPs to blunt the immune response and facilitate parasite development in the mosquito vector (44). *P. berghei* infection significantly increased AsILP3, 4, and 5 expression. Simultaneous knockdown of AsILP3, 4, and 5 by RNAi reduced *P. berghei* development, yet the difference was not statistically significant (78), whereas insulin receptor knockdown in *A. stephensi* significantly reduced *P. berghei* development to oocysts (78).

In transgenic *A. stephensi*, overexpression of Akt in the midgut of heterozygous mosquitoes resulted in 60–99% reduction in the numbers of mosquitoes infected with *P. falciparum*, and parasite infection was completely blocked in homozygous transgenic mosquitoes (79). In addition, a single nucleotide polymorphism (SNP) in the AgILP3 gene (Ins34) was reported in field-collected *A. gambiae* mosquitoes from Mali (45). This synonymous SNP in Ins34 in AgILP3 precursor gene resulted in a change from GGC to GGT at nucleotide position 462. The CC genotype at the Ins34 locus in M form mosquitoes was more common in samples that were not infected with *P. falciparum* suggesting a role of this pathway in malaria parasite infection.

Filarial Parasites

Insulin receptor knockdown in *C. quinquefasciatus*, the major vector of *Wuchereria bancrofti* in India, completely blocked the development of filarial nematode parasites to the infective third instar larval stage (48). This is the only study on the role of mosquito insulin signaling in nematode development and the data suggest a conserved role of insulin signaling in parasite development within mosquito vectors.

CONCLUSIONS

Insulin-like peptides are pleiotropic peptide hormones, and owing to this, the structural and functional characterization of ILPs has long been a major interest for insect endocrinologists. The functions of insect ILPs, in general, is in a discovery phase compared to the state of knowledge for insulins and related peptides in vertebrates. A primary action of insulin in mammals is to reduce circulating glucose through increased glycogen and

triglyceride synthesis. This action is conserved in mosquitoes and has been supported by the work in *Ae. aegypti*. Over the past several years, a succession of studies has suggested a central role of ILPs/insulin signaling in regulating growth, development, reproduction, diapause, aging, and pathogen development in mosquitoes. Yet, only a few studies have used genetic tools to dissect out the functions of individual ILPs. Unraveling the individual functions and functional redundancy of ILPs will provide new understanding of this complex pathway.

FUTURE GOALS

It is clear from the review of literature that there are many unanswered questions regarding the roles of insulin signaling in mosquitoes. Only a few mosquito ILPs have been functionally characterized, mostly because of the challenges in peptide purification and synthesis, efficient transcript knockdown, and potentially overlapping functions. So far only one endogenous mosquito ILP has been purified and characterized, limiting our understanding of the post-translational processing of these molecules, and there is no data on ILPs that may fold similarly to IGF-I, without cleavage of the C-peptide (e.g., AaILP6). In addition, cell-based expression systems have so far been unable to produce biologically active mosquito ILPs, and chemical ILP synthesis has been difficult due to complex formation of disulfide bridges between multiple Cys residues and proper cleavage of the C-peptide required for folding. Both *Ae. aegypti* and *D. melanogaster* research has benefitted from a handful synthetic ILPs, albeit at a high synthesis cost, and availability of more synthetic ILPs would inform ligand-receptor interactions as well as ILP functions. Also, a standard HPLC or GC-MS protocol for measuring ILPs titers would also help improve our knowledge of ILP functions, either as circulating hormones or as neurotransmitters.

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Recent advances in gene editing technologies now allow explorations of ILP functions outside of model organisms. Most notably, CRISPR-Cas9 knock-ins or knock-outs will facilitate acquisition of new knowledge on how ILPs control physiologies such as nutrient storage, lifespan, development, fecundity, host seeking (appetite), regulation of proteases, and immune response. Genetic knockouts allow for persistent, lifelong knockouts or overexpression mutants which could not previously be achieved through RNAi or injection of synthetic peptides. Additionally, the use of an epitope tag such as HA coupled with single guide RNA (sgRNA) in a donor construct along with a fluorescent marker could be a tool for simultaneously tracking ILP expression locations and patterns, even when the hormone itself is knocked out. With the improvement of gene editing in mosquitoes, it will be possible to understand the functions of ILPs in more mosquito species in addition to *Ae. aegypti*, *An. gambiae*, and *An. stephensi* in order to understand if ILP functions are conserved or change in different mosquito taxa.

An area of research that is clearly open is the role of insulin signaling in mosquito-pathogen interactions. Therefore, research should focus on understanding the diverse functions of ILPs in mosquitoes including mosquito-pathogen interactions. Insulin mimetics that bind to the insulin receptor and block the downstream processes might be a new avenue to explore for mosquito and mosquito-borne disease control.

AUTHOR CONTRIBUTIONS

AS, AN, and MG-N wrote the draft manuscript. AN and MG-N wrote the final manuscript.

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Analysis of the Role of the Mc4r System in Development, Growth, and Puberty of Medaka

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In mammals the melanocortin 4 receptor (Mc4r) signaling system has been mainly associated with the regulation of appetite and energy homeostasis. In fish of the genus *Xiphophorus* (platyfish and swordtails) puberty onset is genetically determined by a single locus, which encodes the *mc4r*. Wild populations of *Xiphophorus* are polymorphic for early and late-maturing individuals. Copy number variation of different *mc4r* alleles is responsible for the difference in puberty onset. To answer whether this is a special adaptation of the Mc4r signaling system in the lineage of *Xiphophorus* or a more widely conserved mechanism in teleosts, we studied the role of Mc4r in reproductive biology of medaka (*Oryzias latipes*), a close relative to *Xiphophorus* and a well-established model to study gonadal development. To understand the potential role of Mc4r in medaka, we characterized the major features of the Mc4r signaling system (*mc4r*, *mrap2*, *pomc*, *agrp1*). In medaka, all these genes are expressed before hatching. In adults, they are mainly expressed in the brain. The transcript of the receptor accessory protein *mrap2* co-localizes with *mc4r* in the hypothalamus in adult brains indicating a conserved function of modulating Mc4r signaling. Comparing growth and puberty between wild-type and *mc4r* knockout medaka revealed that absence of Mc4r does not change puberty timing but significantly delays hatching. Embryonic development of knockout animals is retarded compared to wild-types. In conclusion, the Mc4r system in medaka is involved in regulation of growth rather than puberty.

Keywords: medaka, Mc4r, knockout, puberty, growth

INTRODUCTION

Puberty is the process through which an individual develops from an immature to the mature stage and obtains first time the capability to reproduce. This process can be affected by both the genetic background and environmental factors (1, 2). Teleost fish of the genus *Xiphophorus* (platyfish and swordtails) show polymorphisms in puberty onset timing, body length and reproduction tactics (3). The *P* (Puberty) locus encoding the melanocortin 4 receptor (Mc4r) is critically involved in establishing these polymorphisms (4, 5). Distinct alleles of *mc4r* and copy number variation of these alleles determine the onset of puberty to be early or late (4).

Mc4r belongs to the class A of G protein-coupled receptor (GPCR) and is a member of the melanocortin receptor family, which has five members, Mc1r to Mc5r (6). These receptors regulate a wide array of functions, for example, pigmentation for Mc1r (7), and energy homeostasis for Mc4r (8). The Mc4r signaling system consists besides the receptor itself and its cognate ligands, which are processed from the agonist precursor pro-opiomelanocortin (Pomc), of the antagonist agouti-related peptide (Agrp), and melanocortin receptor accessory protein 2 (Mrap2) (6). Vertebrate Pomc is cleaved into physiological ligands α -MSH, β -MSH, γ -MSH, ACTH, but teleosts lack γ -MSH (9). All melanocortin receptors bind the four ligands, with the exception of Mc2r, which interacts exclusively with ACTH (10). Ligands have different affinities to each of the receptors (8). β -MSH has the highest affinity to Mc4r, and synthetic ligand NDP-MSH is a highly potent α -MSH analog that also activates Mc4r (11). Agrp can act as an antagonist or inverse agonist, inhibiting the activation of Mc4r by MSH or the constitutive activity of Mc4r by its N-terminus, the intramolecular ligand (12).

Mc4r has been reported to be involved in many physiological processes in several fish species. The most prominent physiological processes affected are food intake and energy balance, as reported in goldfish (13) and rainbow trout (14). In zebrafish, Mrap2 regulating Mc4r signaling controls adult somatic growth and stimulates larval growth during development (15). Zebrafish Mrap2 has two forms (16): Mrap2a is the larval form and blocks Mc4r, and Mrap2b is the adult form and enhances Mc4r function (15).

In mammals, Mc4r is mainly expressed in the central nervous system. In humans, mutations in *MC4R* are connected to early-onset obesity (17), and in mice, *Mc4r* knockout causes hyperphagic obesity (18). These mice have an obese phenotype like the *Agrp* overexpression mice (*A^{vy}*) (19). Mc4r was shown to be involved in energy intake and expenditure and acts as a potential target for pharmacological intervention with obesity (17). Leptin and ghrelin signaling pathways are acting upstream of Mc4r, while kisspeptin is downstream of Mc4r (20). Due to its role in the center of this regulatory network, increasing the knowledge about Mc4r is instrumental for a better understanding of metabolic disorders.

Fish of the genus *Xiphophorus* provide a useful tool to study the genetic basis of puberty regulation and the molecular factors involved in this process (4, 5, 21). However, for functional studies reverse genetics cannot be applied to these fish, because they are livebearing and have internal embryo development. Medaka (*Oryzias latipes*) is a phylogenetically closely related species to *Xiphophorus*. For both species, high-quality genomes are available (22, 23), and medaka is an established genetic model to study development and physiology (24). As an egg-laying fish, comparable to zebrafish, medaka is amenable to gene knockdown, knockout and knockin (25). The embryos of medaka are transparent, and normal embryonic development has

been well-described (26). However, Mc4r and its physiological function have not yet been investigated in medaka.

Although in *Xiphophorus* fish the association of distinct male polymorphisms depending on puberty timing and its regulation by Mc4r have been shown, a similar role of Mc4r in other species remains unclear. To advance our knowledge of fish puberty, we investigated the Mc4r system in medaka fish. Through analysis of the temporal and spatial expression profile and functional studies on *mc4r* mutants, we find that the role of *mc4r* is related to development and growth in medaka, like in zebrafish, but not to controlling puberty timing like in *Xiphophorus* fish.

MATERIALS AND METHODS

Animals

All wild-type medaka used in the study were from the Carbio strain. The knockout *mc4r* medaka mutant “−2/+3” was generated by TALEN technology. For details of the generation of the mutant see (27). Adult medaka (*Oryzias latipes*) were maintained under a standard light/dark cycle of 14/10 h at 26°C in the fish facility of Biocenter at the University of Wuerzburg. Eggs of medaka fish were collected and cultured in Danieau's medium (NaCl 17.4 mM, KCl 0.21 mM, MgSO₄ 0.12 mM, Ca(NO₃)₂ 0.18 mM, HEPES 1.5 mM, methylene blue 0.0001%) until hatching.

All animals were kept and sampled in accordance with the applicable EU and national German legislation governing animal experimentation, in particular, all experimental protocols were approved through an authorization (568/300–1,870/13) of the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law (TierSchG).

Phylogenetic Analysis

The annotated Mc4r, Mrap2, Pomc, Agrp1 sequences from human and 14 fish species were retrieved from either National Center for Biotechnology Information nucleotide sequences database (NCBI)¹ or Ensembl genome browser². Protein sequences were used in the phylogenetic analysis and were aligned by ClustalW from the software package MEGA7 (28). Evolutionary analyses were conducted with MEGA7 based on maximum likelihood method with 1,000 bootstrap replicates.

Eleven Actinopterygians (ray-finned fish) including Amazon molly (*Poecilia formosa*), cave fish (*Astyanax mexicanus*), cod (*Gadus morhua*), fugu (*Takifugu rubripes*), medaka (*Oryzias latipes*), Southern platyfish (*Xiphophorus maculatus*), spotted gar (*Lepisosteus oculatus*), stickleback (*Gasterosteus aculeatus*), tetraodon (*Tetraodon nigroviridis*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*); one Sarcopterygian (lobe-finned fish), coelacanth (*Latimeria chalumnae*); one Chondrichthyan (cartilaginous fish), elephant shark (*Callorhynchus milii*); and one Agnathan (jawless fish), lamprey (*Petromyzon marinus*) were used in the study. Common carp (*Cyprinus carpio*) was additionally used in Mrap2 phylogeny.

Abbreviations: Agrp, agouti-related peptide; dpf, days post fertilization; GPCR, G protein-coupled receptor; KO, knockout; LG, linkage group; Mc4r, melanocortin 4 receptor; Mrap2, melanocortin receptor accessory protein 2; Pomc, pro-opiomelanocortin; SD, standard deviation; WT, wild-type.

¹<https://www.ncbi.nlm.nih.gov/>

²<http://www.ensembl.org/index.html>

These were compared with human (*Homo sapiens*). Accession numbers are listed in **Table S1**.

Quantitative Gene Expression Analysis

To determine the time course of expression of genes from the Mc4r signaling system, three pools of medaka embryos and larvae from 0, 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 days post fertilization (dpf) were collected (0 dpf $n = 100$, 1–4 dpf $n = 50$, 5–8 dpf $n = 30$, 10–20 dpf $n = 15$). The exact amounts of transcripts could not be determined in 0 dpf sample due to lack of proper normalization factors. To determine the tissue expression of Mc4r signaling pathway genes, three pools of male or female adult fish tissues ($n = 3$ –4) of brain, eye, gill, kidney, liver, gonad, skin, and muscle were analyzed. Total RNA was isolated using TRIZOL reagent (life technologies, Carlsbad, California, United States). After DNase treatment, a reverse transcriptase with random hexamer primers was used to synthesize first strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, United States) according to manufacturer's manual. Primers for *mc4r*, *mrap2*, *pomca*, *pomcb*, *agrp1*, and housekeeping gene *ef1a* are listed in **Table S2**. cDNA was applied to Reverse Transcription Quantitative PCR (RT-qPCR) using SYBR Green reagent on a Mastercycler realplex² Eppendorf machine Eppendorf (Eppendorf, Hamburg, Germany). Data were analyzed by the $\Delta\Delta CT$ method and normalized to housekeeping gene *ef1a*. The values were presented as mean $\pm$ standard deviation (SD).

Whole Mount *in situ* Hybridization

The spatial expression patterns of *mc4r* and *mrap2* were analyzed by whole mount RNA *in situ* hybridization following procedures described previously (29–31). To generate riboprobes, cDNA from brain were used to amplify both genes (primers listed in **Table S2**). The amplicons were cloned into a pGEM-T Easy vector and verified by sequencing. Plasmids were linearized, and *in vitro* transcription was performed by T7 or SP6 RNA polymerase (Roche, Basel, Switzerland) with digoxigenin or fluorescein RNA labeling mix (Roche). Whole mount *in situ* hybridization was performed on dissected intact brains fixed in 4% PFA and dehydrated in methanol for storage. Before hybridization brains were rehydrated with PBST and digested with 10 μ g/ml proteinase K for 45 min. Samples were hybridized with riboprobes overnight at 68°C in a humidified chamber and then were subjected to stringent washes of SSC series (0.05X SSC used for washes of high stringency) and by PBST. The samples were embedded in 3% agarose and 100 μ m section were cut by TPI Vibratome Series 1000 Sectioning System (Technical Products International Inc., St. Louis, Missouri, United States). After blocking with 5% sheep serum, anti-digoxigenin-AP (1:5,000) or anti-fluorescein-AP (1:2,000) were applied to the sections overnight at 4°C. NBT/BCIP solution (Roche) or FastRed Tablets (Sigma, St. Louis, Missouri, United States) were used to develop the signals by AP reaction according to manufacturer's instruction. Double *in situ* hybridization was performed by first visualizing fluorescein probes and thereafter digoxigenin probes. Sections were mounted in 80% glycerol for microscopy (Zeiss AxioPhot, using AxioVision Rel 4.8 software,

Oberkochen, Germany). Fluorescence pictures were taken with a Confocal microscope (Nikon Eclipse Ti C1, using NIS-Elements AR software, Minato, Tokyo, Japan). The transcript localization was determined according to the medaka brain atlas (32).

Functional Analysis of Knockout Fish

Medaka wild-type (WT) fish (Carbio strain) and Mc4r knockout (KO) –2/+3 TALEN-KO strain (27) (*mc4r* wt and mutant sequences provided in **Supplementary Data**) were compared with respect to hatching time, puberty onset and growth. Three groups of 60–200 wild-type and knockout embryos were collected, and the hatching time of each embryo was recorded. Embryo development from day 0 to day 7 was compared. Linear growth (forehead to trunk terminus) of larvae was imaged by stereomicroscope (Nikon SMZ1000 microscope, Minato, Tokyo, Japan, with LEICA DFC450C to capture images, using LAS V4.1 software, Wetzlar, Germany) and measured using ImageJ 1.51 (33, 34).

Three groups of 25 wild-type and 22–25 knock-out fish were raised in a two-chamber aquarium (**Figure S3B**) to guarantee identical rearing conditions for two groups. Puberty refers to the stage when fish become sexually mature and acquire for the first time the ability to reproduce (1). Since medaka has obvious secondary sexual characters and a special courtship behavior during the mating process, here puberty onset was determined for females as the day of the first eggs release and for males by the appearance of papillary processes in the anal fin (35). Three independent experiments were conducted. The linear growth of juveniles and adult fish were measured by rulers. The growth of juveniles (the period when males and females are undistinguishable) was measured and compared between KO and WT. The linear length and the age of adults were measured at the time of puberty, and KO and WT fish were compared.

Statistical treatment of the data was done with GraphPad Prism 6 software. Two-way ANOVA was used for comparison of WT and KO females and males for the time of puberty onset and the size at puberty. To compare each sample with every other sample, corrections were made for multiple comparisons by Sidak's test. Multiple *t*-test with correction for multiple comparison by Holm-Sidak method was used to compare the WT and KO growth curves. One-way ANOVA was used for comparison of larval length and to compare KO day7 and KO hatch with WT hatch, corrections were made for multiple comparisons by Dunnett's test. The values were presented as the mean $\pm$ SD. Mann-Whitney test was used to compare WT and KO hatching time. Data are presented in box plots; whiskers are from min to max. In Kaplan-Meier plots for puberty, data were compared with the log-rank (Mantel-Cox) tests. GraphPad Prism 6 was used to display the results.

RESULTS

Mc4r Signaling System Genes in Fish

The phylogenetic trees of the Mc4r signaling system genes show in general a topology that follows the known organismal relationships (**Figure S1A**). Interestingly, *mc4r* of Southern platyfish and blind cavefish have longer branches indicating

faster evolution toward specialized functions in these lineages. Different from all other species, only zebrafish has two copies of *mrap2* (Figure S1B) which have previously been assigned to different functions for growth regulation (15). Thus, this appears to be a special situation in zebrafish, which cannot be generalized. However, search in common carp (*Cyprinus carpio*) genome reveals also two copies of *mrap2*, *mrap2a*, and *mrap2b* (Table S1). Two *pomca* and one *pomcb* sequences were found in several teleosts, including Southern platyfish and medaka (Table S1). More basal teleost groups, on the other hand, like zebrafish and cavefish have only one *pomca*. The *agrp1* gene is present as single copy gene in all analyzed ray-finned fish including medaka (Figure S1C).

An important feature of *mc4r* as a puberty gene determining a sexually selected trait in *Xiphophorus* is sex-chromosome linkage. It is located in Southern platyfish on linkage group (LG) 21, which is the sex chromosome. However, in medaka, *mc4r* is on linkage group LG 20 and not on the sex chromosome (LG 1). None of the other Mc4r signaling system genes are sex-linked, neither in *Xiphophorus* nor in medaka (Table S3).

Developmental and Organ-Specific Expression of Mc4r Signaling System Genes

Analysis of the temporal expression pattern of *mc4r* signaling system genes during embryonic development revealed presence of transcripts of *mc4r* and *mrap2* already at 0 dpf (stage 10–11 blastula stage, developmental stages see Table S4), indicating that these are maternal mRNA contribution (data not shown). Especially for *mrap2*, the maternal contribution appears to be very high. Other genes are expressed exclusively from zygotic transcription. Expression of *mc4r* increases gradually starting from 1 dpf and stabilizes around 5 dpf (Figure 1A). Expression of *mrap2* increases gradually from 1 dpf on (Figure 1B). Expression of *pomca* starts and increases from 3 dpf on (Figure 1C). Expression of *pomcb* initiates at 3 dpf at low levels and increases at 6–8 dpf (Figure 1D). Expression of *agrp1* starts with very low levels at 4–5 dpf and is highly upregulated at 8 dpf (Figure 1E). Expression of *agrp1* shows a dramatic upregulation after the fish start to feed.

Differential expression among tissues and between males and females were analyzed using RT-qPCR. Brains of both sexes highly express *mc4r* and *mrap2* (Figures 2A,B). High expression of *mc4r* in brains of medaka confirms previous analysis (5). The *pomca*, *pomcb* and *agrp1* genes were also highly expressed in brains of both sexes (Figures 2C–E). The *agrp1* gene has a higher (1.5-fold) expression in the female brain (Figure 2E). Besides brain, *mc4r* is also expressed in all tested tissues at low levels; except for skin, all the rest are background expression (Figure 2A). The *mrap2* gene shows remarkably high expression in male kidney (12.6-fold higher than brain) and ovary (2.9-fold) (Figure 2B). No other tissues express *pomca*, but *pomcb* showed low expression in eyes, gills, gonads, skins and muscles (Figures 2C,D). Testis expresses high levels of *agrp1*, while there is only low expression in all other tissues (Figure 2E). In summary, high expression of all *mc4r* signaling system genes

during ontogenesis when the larvae start to feed and in the brain of adult fish is in agreement with functions in appetite regulation and energy homeostasis in medaka. The sexually dimorphic expression pattern of *mrap2* (kidney, gonad) and *agrp1* is intriguing and warrants further studies to elucidate a sex-related function.

Co-localization of *mc4r* and *mrap2* in the Adult Brain

Mrap2 has been described as *Mc4r* accessory protein of important function (15). To exert such function *mrap2* has to be co-expressed with *mc4r* in the same cells. To investigate the localization of *mc4r* and *mrap2* expression, whole mount *in situ* hybridization was performed using intact brains from adult females and males.

While *mc4r* was expressed in the preoptic region and hypothalamus region, *mrap2* was only expressed in the hypothalamus (Figure 3A; Figure S2A). In the hypothalamus, *mc4r* was expressed more anteriorly than *mrap2*. In addition, the expression region of the two genes was also overlapping in the central part of the hypothalamus (Figure 3B; Figure S2B). The anatomical overlap of the expression domains may indicate that both genes are co-expressed on the cellular level. There was no noticeable difference between males and females (Figure 3C). The fact that only a subset of *mc4r* expression domains overlap with that of *mrap2* indicates that in certain areas of the brain the Mc4r signaling system may function without the accessory protein.

Effect of Mc4r Knockout on Hatching Timing and Linear Growth

To investigate if *mc4r* would have a similar function as puberty onset determining gene in medaka like *Xiphophorus* fish, puberty onset was monitored in a medaka *mc4r* knockout line (27). We used a line that has the $-2/+3$ mutation (*mc4r* sequences in Figure S3A, Supplementary Data, these indels in the *mc4r* gene were verified by genotyping and sequencing) and compared to wild-type medaka. In medaka, puberty onset can be determined from the development of secondary sexual characteristics in males (anal fin papillary processes), which can be easily observed under a stereomicroscope. In females, the first egg-laying indicates puberty completion (36). We found no significant difference in puberty timing between KO and WT, neither in male fish nor in female fish (Figure 4A; Figure S3C). Body length at puberty also did not differ between KO and WT in males. However, KO females matured at shorter body length than WT female (Figure 4A). Moreover, the average standard body length of the KO fish was generally shorter than that of WT although the difference is not significant, except at some later time points in trial 3 (Figure 4B), showing that the knock-out fish have a trend of a slower linear growth rate.

We also noticed that hatching time was delayed for KO fish. Compared to zebrafish, medaka fish develop slower and stay in the chorion until much more advanced developmental stages (26, 37). This allowed us to better distinguish changes in the timing of hatching. From three independent experiments, the KO

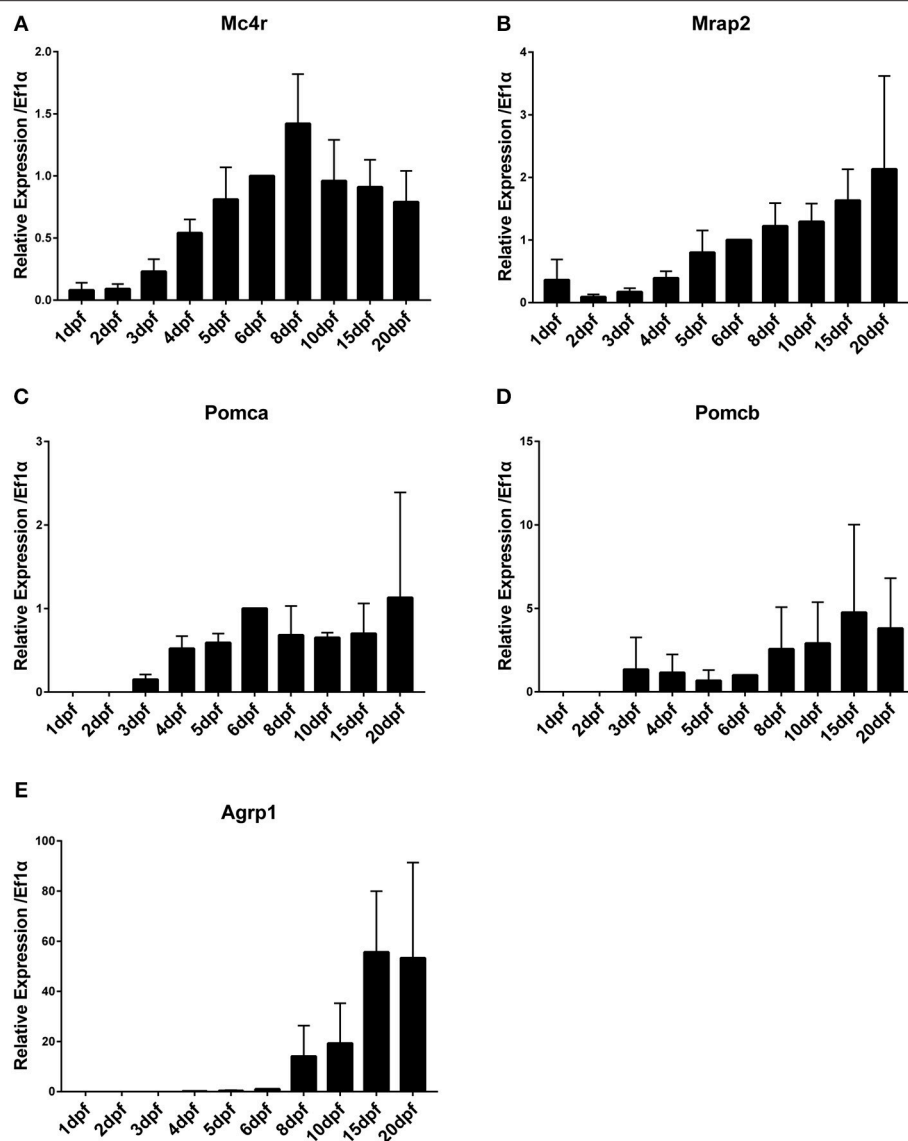


FIGURE 1 | Time course of gene expression during medaka embryonic and larval development. (A) *Mc4r*, (B) *Mrap2*, (C) *Pomca*, (D) *Pomcb*, (E) *Agrp1*. Expression levels are normalized to *ef1a* and relative to expression observed in 6 dpf. Relative expression levels are presented as mean $\pm$ SD.

fish clearly showed significantly delayed hatching, for at least 1 day (Figure 5A). In order to observe if this is due to a possible change in embryo development and growth, we followed the development of WT and KO embryos. Starting at day one the KO embryos were about one stage slower than WT (data not shown). The differences became more obvious for embryos from day three on, where the body of KO fish was covering smaller portions of the yolk sphere than of WT fish. When we compared the standard body length of freshly hatched WT (day 7) and KO fish dechorionated on day 7, KO larvae were significantly shorter than WT (Figures 5B,C). Finally, we compared the body length of freshly hatched KO to WT. The hatched KO had the same size as hatched WT (Figures 5B,C) despite being 1–4 days older. All in all, these results indicate that *mc4r* in

medaka is more involved in regulating growth than the onset of puberty.

DISCUSSION

Characterization of the *Mc4r* Signaling System in Medaka

As the first step toward an investigation of the *Mc4r* signaling system genes in medaka fish, we performed an evolutionary analysis of *Mc4r* signaling system genes in various fish. Contrary to Southern platyfish, in which *mc4r* is present in multiple copies on the sex chromosome (4, 5), the medaka *mc4r* is a single copy gene. This confirms now on the whole genome sequence level

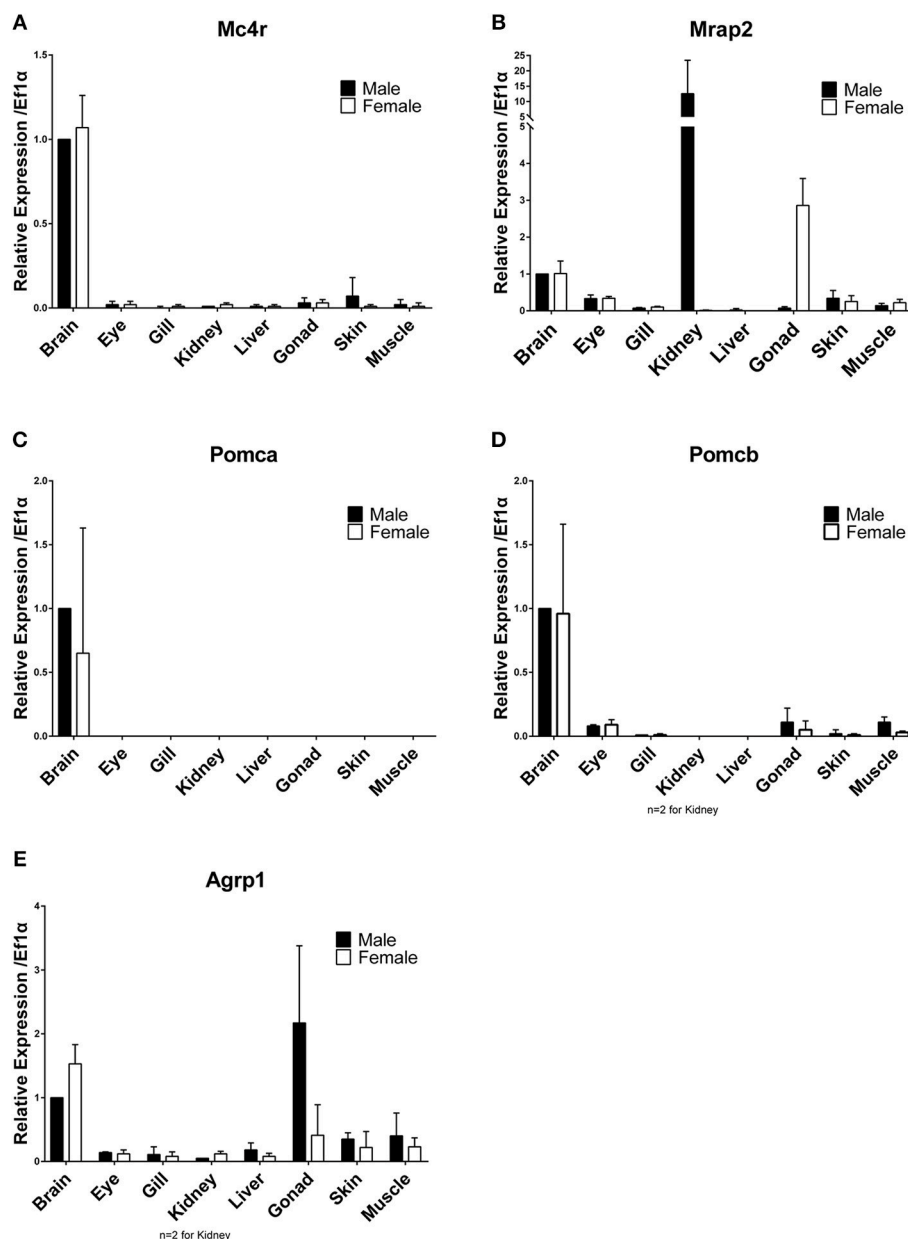
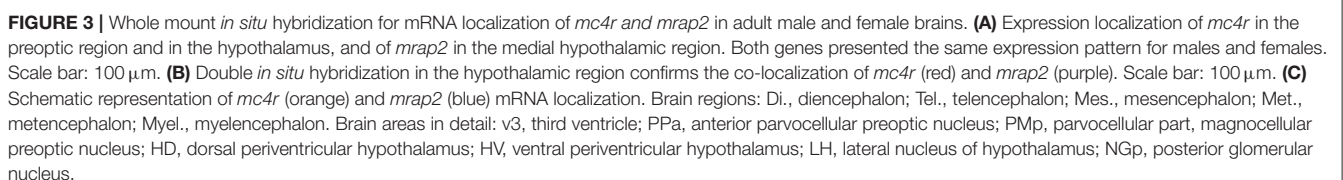


FIGURE 2 | Differential tissues gene expression in adult males and females. **(A)** *Mc4r*, **(B)** *Mrap2*, **(C)** *Pomca*, **(D)** *Pomcb*, **(E)** *Agrp1*. Expression levels are normalized to the expression level of *ef1a* and relative to expression observed in male brains. Relative expression levels are presented as mean $\pm$ SD.

the earlier evidence from Southern blot data (5). Moreover, the medaka gene is not located on the sex chromosome. In the *Mc4r* phylogeny, the long branch for the Southern platyfish indicates considerable protein sequence divergence which we hypothesize as being due to special functions of *Mc4r* in regulating puberty onset that evolved in the *Xiphophorus* fish lineage (4, 5). Similarly, the long branch for cavefish reflects a special adaptation of *Mc4r*, e.g., in control of energy metabolism and adaptation to limited nutrients in the caves (38).

The *mrp2* gene has been reported so far to have two copies only in zebrafish (15). We found also two copies in common carp.

This indicates a lineage-specific duplication in cyprinids. The two *pomc* paralogs (*pomca* and *pomcb*) probably arose during the 3rd round of whole genome duplication of the teleost, since only one copy of *pomc* is present in other vertebrates. However, two sequences of *pomca* (*pomca1* and *pomca2*) were found in most of Percomorpha teleost, indicating a lineage-specific duplication of this gene. The *agrp* gene has been found in some species to exist in two versions, *agrp1* and *agrp2*. *agrp1* (also named *agrp* previously) has been related to obesity (39, 40) and was described as an antagonist and inverse agonist of *Mc4r*. On the other hand, *agrp2* [in some fish named as *asip2b* (41, 42)] is involved in



which code for membrane proteins, are expressed early in development, concurrent with the late neurula stage and early brain development. Thus, *mc4r* and *mrap2* genes are expressed already in early and late embryonic stages, when the brain and integrated functional proteins in the neurons are developing. The *pomc* genes, precursors of Mc4r agonists, start expression 2 days after *mc4r* and *mrap2*. At the hatching stage, all genes

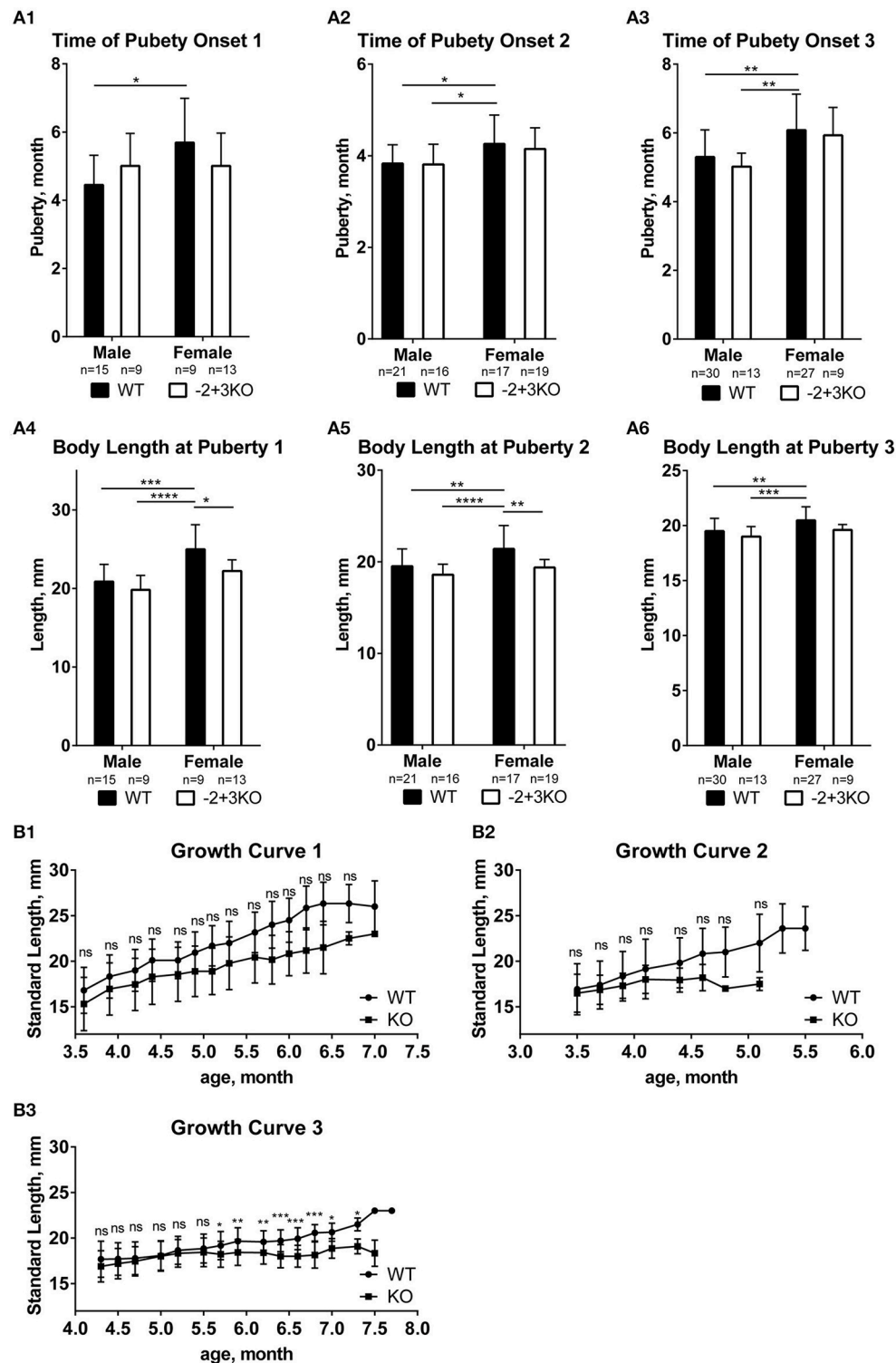


FIGURE 4 | Effect of *mc4r* knockout on medaka growth and puberty. **(A)** No differences in puberty onset timing in the three trials (**A1**, **A2**, **A3**), but some differences in body length at puberty between WT and KO medaka females and males in three replicates (**A4**, **A5**, **A6**). Data are presented as mean $\pm$ SD. Statistical significance after two-way ANOVA is indicated as asterisks (*). **(B)** The standard length of WT and KO during growth in the three experiments (**B1**, **B2**, **B3**). Data are presented as mean $\pm$ SD. Statistical significance after multiple *t*-test is indicated as asterisks (*). For all statistics: ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

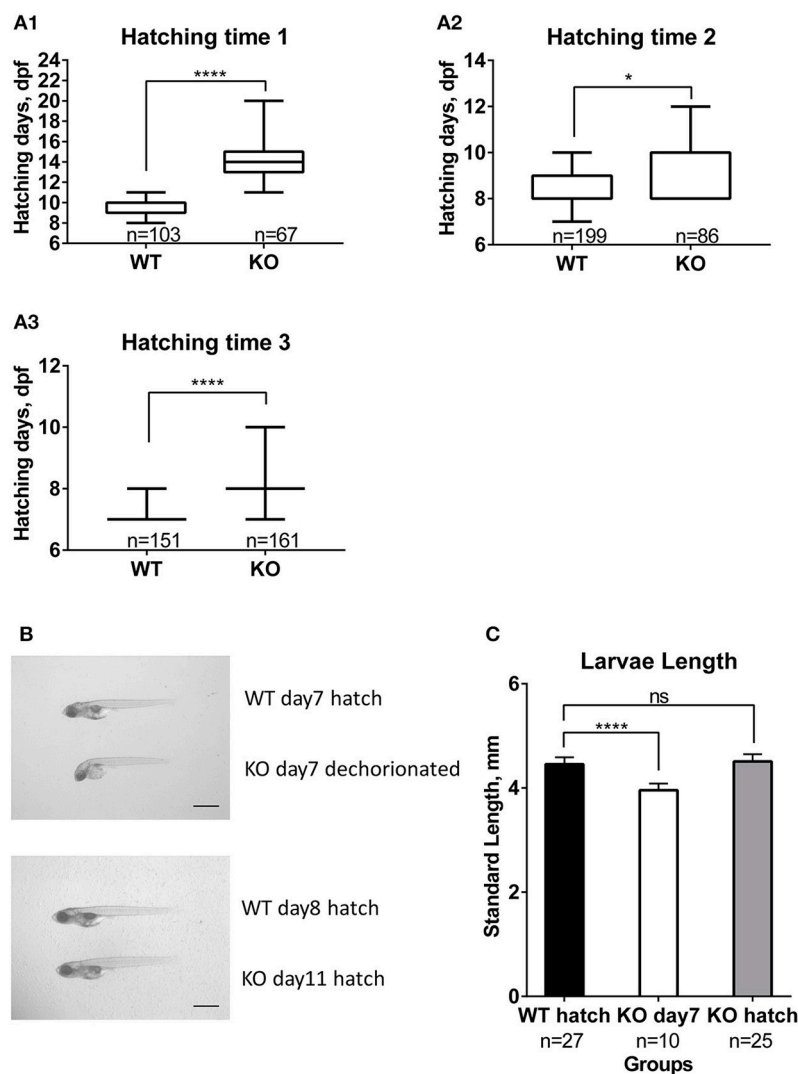


FIGURE 5 | Effect of *mc4r* knockout on medaka development and hatching. **(A)** Differences in hatching time of WT and KO medaka in the three trials (**A1**, **A2**, **A3**). Data are presented in box plots; whiskers are from min to max. Statistical significance after Mann-Whitney test is indicated as asterisks (*). **(B)** Photographs of larvae at 7 dpf and at hatch for WT and KO. Scale bar: 1 mm. **(C)** Graphic showing slower development of KO larvae compared to WT, apparent by a shorter length at 7 dpf. Data are presented as mean±SD. Statistical significance after one-way ANOVA is indicated as asterisks (*). For all statistics: ns: not significant, * $p < 0.05$, **** $p < 0.0001$.

are expressed, indicating that the whole system is in place for full function when the fish start feeding. Intriguingly, *agrp1* showed a dramatic upregulation after hatching, coinciding with first feeding. This may be explained by the important role of *agrp1* in appetite regulation and implies the role of the Mc4r system in energy metabolism regulation from the very first feeding event (45, 46).

Spatial expression analysis revealed that the *mc4r* signaling pathway genes are expressed highly in brains of both sexes consistent with the center of food uptake regulation in the hypothalamus and neighboring regions of the brain, e.g., the preoptic area (46). The expression domains of *mc4r* and *mrp2* were identified in the hypothalamus, and the two genes showed colocalization in certain areas. This indicates interaction of

Mc4r and Mrp2 in those cells. Mc4r and Mrp2 are both membrane proteins, their interaction could tune signal strength and intracellular signal transduction as previously proposed for the zebrafish homologs (47). At some locations where *mc4r* is expressed, *mrp2* is not present, implying that in such regions *mc4r* may be able to transmit signals without its co-factor *mrp2*. Nevertheless, *mrp2* expression was observed only in regions where the *mc4r* signal was present, supporting the specific role of Mrp2 in interacting with Mc4r in the brain (15).

The high transcript levels of *mrp2* in ovary could be explained by the very high maternal RNA contribution. Mature eggs contain high levels of *mrp2* inferred from maternal contribution level, and this is reflected in the high mRNA levels in the ovary. High expression of *mrp2* in the male kidney has

been shown before. In zebrafish, *mrap2* is highly expressed in the head kidney (47). Moreover, *mrap2* is expressed mainly in brain and adrenal gland in human (48) and widely expressed including kidney in mice (49). Fish *mrap2* might be similarly expressed in the interrenal gland on the kidney. A male-specific function of *mrap2* in kidney still needs to be investigated.

Effect of Mc4r on Puberty Timing and Adult Growth

The expression data did not exclude that the Mc4r signaling system in medaka could have the same function in regulating puberty timing as in *Xiphophorus* fish. In *Xiphophorus*, *mc4r* is expressed highest in the brain (5). We find also in adult medaka that the Mc4r signaling system genes are expressed mainly in the preoptic and hypothalamic region, similar as in zebrafish (47). However, our analysis of the Mc4r KO fish revealed that males and females of the WT and Mc4r KO strain reach puberty at a similar time. However, WT females reach puberty significantly later than WT males, while KO females and males do not show a significant difference on puberty timing. In zebrafish, because of the difficulty to monitor puberty onset in this species (50), an effect of *mc4r* on puberty timing was not noted. Our results indicate that Mc4r may not be the critical puberty signal in medaka fish. Alternatively, the lack of a puberty phenotype in our TALEN KO fish might be due to the fact that other genes can compensate for the loss of Mc4r function (51).

Although the timing of puberty onset is not altered in KO fish, female body length at puberty in KO fish is significantly shorter than WT. WT Females are significantly larger than WT males and KO males. KO females and males do not show a difference in body length at puberty. Since puberty is normally related to animal size/weight, earlier puberty and shorter length at puberty in KO females implies an advanced pubertal process in KO females. This suggests that the Mc4r function is relevant for female growth in medaka, rather than male growth. This is unlike the situation in Southern platyfish and other *Xiphophorus* species, where Mc4r is involved in male size but has no noticeable effect on female growth and adult size. In addition, KO fish are slightly shorter than WT, indicating that possibly growth in general is retarded in Mc4r KO fish. In zebrafish, *mc4r* KO led to increased body length at 42 dpf (52). Overexpression of the Mc4r inverse agonists, both *Agrp1* and *Asip1*, increased linear growth in zebrafish (53, 54). *mc4r* KO in medaka surprisingly show retarded growth suggesting that eventually other receptors like Mc3r, which is also related to energy balance and reacts with *Pomc* and *Agrp1*, could compensate for the function of compromised Mc4r by responding to *Pomc* signals and thus reducing growth.

A Role for Mc4r in Medaka Embryonic Development and Larval Growth

Intriguingly, we observed that the hatching time of Mc4r KO medaka was affected. The Mc4r KO fish show delayed

development from day one coinciding with the onset of *mc4r* expression. This effect becomes even more obvious from day three on. It could be speculated that Mc4r has a role in medaka development by influencing growth. Zebrafish Mc4r KO, however, does not show changes in linear growth during embryonic and larval stages. An increased length of Mc4r KO zebrafish was first seen only at 42 dpf (52). The embryogenesis of zebrafish is fast and the fish hatch already at day 2–3, compared to day 7–9 in medaka. Thus, a delay in zebrafish development may have remained undetectable. The overall growth of medaka was clearly slowed down in KO larvae. It should be noted that the knock-out of Mc4r in medaka decreases growth rate, rather than increasing the body length. This is another difference to *Xiphophorus* fish (4), where the non-functional mutant Mc4r leads to increased body size. Also, in zebrafish, the loss of *mc4r* led to increased body length at 42 dpf (52).

In summary, the regulatory network of Mc4r signaling system in medaka appears not to be involved in puberty regulation as in *Xiphophorus*, but rather in growth and development. Our findings suggest that from an evolutionary perspective the role of *mc4r* as the critical *P* locus gene is a specific innovation in the *Xiphophorus* lineage.

AUTHOR CONTRIBUTIONS

RL performed the phylogeny, sampling, RT-qPCR, *in situ* hybridizations, KO adult and embryos functional analysis, and drafted the manuscript. MK established and provided the KO medaka line. MA supervised the experiments and helped to review the manuscript. MS defined and designed the study, coordinated all steps of the research and reviewed all versions of the manuscript.

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Expression Patterns of PACAP and PAC1R Genes and Anorexigenic Action of PACAP1 and PACAP2 in Zebrafish

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with potent suppressive effects on feeding behavior in rodents, chicken, and goldfish. Teleost fish express two PACAPs (PACAP1, encoded by the *adcyap1a* gene, and PACAP2, encoded by the *adcyap1b* gene) and two PACAP receptors (PAC1Rs; PAC1Ra, encoded by the *adcyap1r1a* gene, and PAC1Rb, encoded by the *adcyap1r1b* gene). However, the mRNA expression patterns of the two PACAPs and PAC1Rs, and the influence and relationship of the two PACAPs on feeding behavior in teleost fish remains unclear. Therefore, we first examined mRNA expression patterns of PACAP and PAC1R in tissue and brain. All PACAP and PAC1Rs mRNAs were dominantly expressed in the zebrafish brain. However, *adcyap1a* mRNA was also detected in the gut and testis. In the brain, *adcyap1b* and *adcyap1r1a* mRNA levels were greater than that of *adcyap1a* and *adcyap1r1b*, respectively. Moreover, *adcyap1b* and *adcyap1r1a* mRNA were dominantly expressed in telencephalon and diencephalon. The highest *adcyap1a* mRNA levels were detected in the brain stem and diencephalon, while the highest levels of *adcyap1r1b* were detected in the cerebellum. To clarify the relationship between PACAP and feeding behavior in the zebrafish, the effects of zebrafish (zf) PACAP1 or zfPACAP2 intracerebroventricular (ICV) injection were examined on food intake, and changes in PACAP mRNA levels were assessed against feeding status. Food intake was significantly decreased by ICV injection of zfPACAP1 (2 pmol/g body weight), zfPACAP2 (2 or 20 pmol/g body weight), or mammalian PACAP (2 or 20 pmol/g). Meanwhile, the PACAP injection group did not change locomotor activity. Real-time PCR showed *adcyap1* mRNA levels were significantly increased at 2 and 3 h after feeding compared with the pre-feeding level, but *adcyap1b*, *adcyap1r1a*, and *adcyap1r1b* mRNA levels did not change after feeding. These results suggest that the expression levels and distribution of duplicated PACAP and PAC1R genes are different in zebrafish, but the anorexigenic effects of PACAP are similar to those seen in other vertebrates.

Keywords: feeding behavior, real-time PCR, tissue distribution, intracerebroventricular injection, PACAP receptor, genome duplication

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a bioactive peptide that was originally isolated from the ovine hypothalamus as an activator of cAMP production in pituitary cells (1). It belongs to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon superfamily, and its closest paralog is VIP. PACAP and VIP share three types of G protein-coupled receptors: PACAP-type 1 receptor (PAC1R), VPAC1 receptor, and VPAC2 receptor. The binding affinity of PACAP to PAC1R is ~1,000 times greater than that of VPAC receptors, suggesting that PAC1R is the dominant receptor for PACAP (2). PACAP and PAC1R are widely distributed in central nervous tissues and peripheral tissues in mammals (3, 4). Although PACAP is encoded by the *adcyap1* gene in tetrapods, teleost fish have two PACAPs (PACAP1, encoded by the *adcyap1a* gene and PACAP2, encoded by the *adcyap1b* gene) and two PACAP receptors (PAC1Rs; PAC1Ra, encoded by the *adcyap1r1a* gene, and PAC1Rb, encoded by the *adcyap1r1b* gene) by teleost-specific whole-genome duplication (5, 6). We previously reported the tissue distribution of PACAP2 and *adcyap1b* in zebrafish (7); however, the correlation between expression and distribution of the two PACAPs and PAC1Rs has not yet been clarified.

The amino acid sequence of PACAP is well-conserved in vertebrates, implying that it has important physiological functions for biological activities. Indeed, PACAP contributes a variety of physiological functions, such as neuroprotection (8, 9), retinal protection (3, 10), glial regulation (11–13), neural development (14, 15), immunomodulation (16, 17), exocrine secretion (18, 19), stress response (20–22), and affecting the memory and learning system (23, 24). PACAP also act as a neuromodulator, and its anorectic effects have been widely studied in vertebrates (25, 26). It was reported that intracerebroventricular (ICV) administration of frog PACAP suppressed food intake in goldfish (27). However, the influence of duplicated PACAP/PAC1R genes in fish on feeding behavior has not yet been clarified.

Therefore, the present study used real-time PCR to quantify the distribution and expression levels of the two PACAP and PAC1R genes expressed in adult zebrafish. Furthermore, the influence of administering zebrafish (zf) PACAP1, zfPACAP2, and mammalian PACAP (mPACAP) on food intake was investigated, and the expression patterns of PACAP and PAC1R genes after feeding were evaluated.

MATERIALS AND METHODS

Animals and Housing

Adult short-fin strain zebrafish were purchased from a local commercial supplier in Toyama City. Fish were kept in 35-L housing tanks for at least 2 weeks under standard conditions (28 ± 1°C, 14 h light/10 h dark cycle) prior to experiments. Fish were fed twice per day with brine shrimp (*Artemia salina*) and an artificial diet (Hikari-labo series; Kyorin Co., Ltd., Himeji, Japan). All protocols were conducted in accordance with the University of Toyama guidelines for the care and use of animals.

Reagents

Three types of PACAP were used for ICV injection. mPACAP38 was purchased from Peptide Institute Inc., Japan. zfPACAP1 (HSDGVFTDSYSRYRKQMAVKKYLATVLGKRYRQRYRSK-NH2) and zfPACAP2 (HSDGIFTDIYSRYRKQMAVKKYLA AVL-GRRYRQRVKNK-NH2) were synthesized by Toray Research Center Inc., Japan, with over 95% purity.

Standard DNA Fragment Preparation for Quantitative Real-Time PCR

Standard DNA fragments were prepared for absolute quantification of each gene. Gene-specific primers for standard samples of the two PACAP genes, *adcyap1a* (NM_152885.2) and *adcyap1b* (NM_214715), and the two PAC1R genes, *adcyap1r1a* (NM_001013444.2) and *adcyap1r1b* (XM_677888.7), including the whole open reading frame region were designed using the Primer3Plus website (<https://primer3plus.com/>) (Table 1). Zebrafish were deeply anesthetized in ice-cold water, and the whole brain was removed. Total RNA was isolated from frozen tissues using TriPure isolation reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. cDNAs were synthesized using PerfectScript RT reagent kit using gDNA Eraser (TAKARA Bio, Otsu, Japan). PCR was performed using KOD FX Neo (Toyobo Co. Ltd, Tokyo Japan) with a Takara PCR Thermal Cycler Dice Standard (TAKARA Bio). The PCR step comprised 98°C for the first 2 min, followed by 40 cycles of 98°C for 10 s, 58°C for 30 s, and 68°C for 1 min. After agarose electrophoresis, target bands were cut out and DNA was isolated using NucleoSpin Gel and PCR Clean-up kit (TAKARA Bio). DNA concentration was quantified by Implen NanoPhotometer P-class (Implen GmbH, München, Germany) and the number of molecules of the standard sample for each gene was calculated.

TABLE 1 | Oligonucleotides used as primers of PCR for standard template and quantitative PCR.

Target	Sequence	Amplicon length
FOR STANDARD TEMPLATE DNA		
adcyap 1a-Forward	AGCCTCCATTGGACAGCATC	589 bp
adcyap 1a-Reverse	ATTGATGGTTAGAGAGACGCG	
adcyap 1b-Forward	AAGAGGTGCTGTGAGGAAGA	1,191 bp
adcyap 1b-Reverse	CGTGCCAGCTACTTTTCATC	
adcyap 1r1a-Forward	CACACGAACATACCTCCATCTG	1,621 bp
adcyap 1r1a Reverse	GCAGGTGAAGAGAGTGAGTTGA	
adcyap 1r1b-Forward	TATAATCGCTTCAGTGCTCCA	1,397 bp
adcyap 1r1b-Reverse	GTGTTTGGCCTTCAGTTTGTG	
FOR REALTIME-PCR		
adcyap 1a-Forward	AGCCTCCATTGGACAGCATC	149 bp
adcyap 1a-Reverse	CAGAGGCGAACACACATTGC	
adcyap 1b-Forward	TCTCACACAATCACAGCCGC	158 pb
adcyap 1b-Reverse	AGTCATCCCAATAGCGGTGC	
adcyap 1r1a-Forward	TCTGGTGGGTGATCAAGGGC	95 bp
adcyap 1r1a-Reverse	CTGAAGCTTCTGCACCAGGA	
adcyap 1r1b-Forward	TGATCCCAACAGTGAACCGG	84 bp
adcyap 1r1b-Reverse	ACTTGACCCAGTCTTGACAG	

Quantitative Real-Time PCR

Zebrafish were deeply anesthetized in ice-cold water. For tissue expression analysis, whole brain, eye, gill, heart, gut, kidney, liver, spleen, skin, muscle, and testes were isolated from male zebrafish, and ovaries were isolated from female zebrafish ($n = 5$ in each sample). For brain expression analysis, the whole brain was divided into six regions: telencephalon, optic tectum, diencephalon, cerebellum, brain stem, and spinal cord ($n = 5$ in each brain region). The tissues were immediately stored at -80°C until used. Total RNA extraction and cDNA synthesis were performed using the protocol described above. Gene-specific primers for real-time PCR were designed using the Primer3Plus website (**Table 1**). PCR was performed using TB Green Premix Ex Taq II reagent (TAKARA Bio) with a CFX Connect real-time PCR system (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The PCR reaction was run at 95°C for the first 5 min, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s, and 65°C for 5 s. A dissociation step from 60 to 95°C was performed at the end of the run. PCR was performed using cDNA from tissue and standard samples, and gene expression levels were quantified using a standard curve of Ct values from the standard samples.

ICV Administration and Quantification of Food Intake

Female zebrafish (4 to 5 cm) were used for ICV administration because they are larger; therefore, ICV injection is easier in females compared with male zebrafish. ICV administration was performed as previously reported (28). Zebrafish were maintained under fasting conditions for 24 h in 3-L tanks individually prior to experiments. Zebrafish were anesthetized using 0.01% eugenol (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and placed between a water-soaked sponge. A tiny hole was drilled in the skull on the third ventricle (between telencephalon and optic tectum) using a 25G needle (Terumo Co, Tokyo, Japan; outer diameter = 0.5 mm). Then, a 35G needle (React System, Osaka, Japan; outer diameter = 0.15 mm) attached to a Hamilton syringe was inserted into the third ventricle of the brain and test solution [2 or 20 pmol/g body weight (BW) of mPACAP, zfPACAP1, or zfPACAP2, 7–20 fish in each group] including 0.1% Evans blue dye was injected over a period of 2 min. The volume of the injected-solution is 0.5 μL /g BW. The doses of PACAP was determined with reference to previous paper which showed ICV administration of PACAP in goldfish (29). Saline-injected group was used as control ($n = 26$). The needle was removed, and the hole was filled using cyanoacrylate surgical glue. The zebrafish were then returned to the 3-L tank, and kept for 15 min for recovery from the anesthesia. After the behavior study, zebrafish were anesthetized with ice-cold water, and the brains removed. The brain was dissected in the ICV region, and positive stained by Evans blue in the interior of the cerebral ventricle indicated successful ICV administration. Success rate of ICV injection was ~ 50 –70%. The brain was dissected after behavioral analysis and fish which did not observe Evans blue in the ventricle were excluded from the experimental group.

Quantification of Food Intake

Brine shrimps were divided equally into 20 1.5-mL tubes, and the number of brine shrimps in three tubes were randomly selected and counted. After ICV administration, brine shrimps were placed in the tank and zebrafish were fed the brine shrimp *ad libitum* for 15 min. If most of the brine shrimps in the tank were consumed (approximately <20 brine shrimps), other tubes with brine shrimps were added. After the feeding period had ended, the zebrafish were immediately moved to another tank, and the remaining brine shrimps were filtered and counted. Food intake was calculated using the following formula:

$$(\text{Average no. of brine shrimps in three tubes}) \times (\text{No. of tubes fed}) - (\text{No. of remaining brine shrimps in the tank}).$$

Quantification of Locomotor Activity

After a 15-min recovery interval following ICV administration, zebrafish (10–13 fish in each group) were transferred to the white circle water tank, with a diameter of 26 cm, and 2 L of breeding water at 28°C . Behavior was monitored for 15 min using a 1/3 inch compact and high sensitivity camera (WAT-250D2, Watec Co., Ltd. Tokyo, Japan). The locomotor activity (total distance) was quantified using a video tracking system, ANY-maze (Stoeling Co., Wood Dale, IL).

Changes in PACAP and PAC1R mRNAs Level After Feeding

Male zebrafish were used in this experiment to avoid the influence of the estrus cycle. Twenty zebrafish were transferred to a 40-L water tank, and acclimatized by feeding 2% weight of artificial diet showing above at 10:00 a.m. once a day for 5 days. Five zebrafish were randomly selected from the tank 30 min before, and 1, 2, and 3 h after feeding on day 5, and brains were isolated under ice-cooled anesthesia. The diencephalic brain including hypothalamic region, which is a known feeding center, was used for real-time PCR to quantify PACAP and PAC1R mRNA levels. Ten to fifteen fish were used in each group.

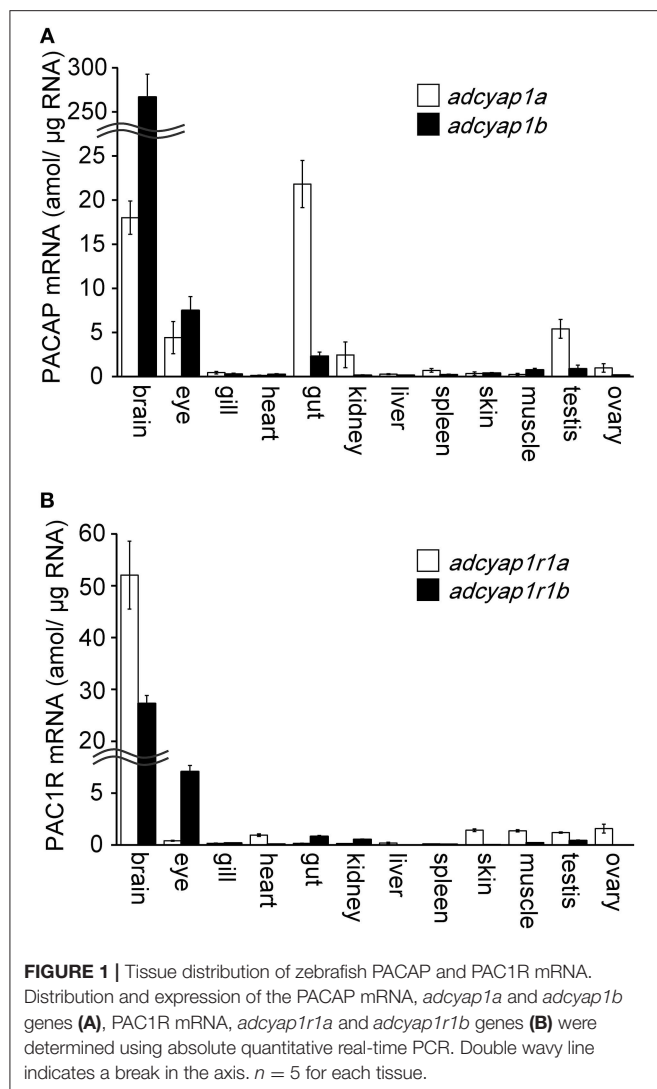
STATISTICS

Experimental data are presented as the mean $\pm$ S.E. (n = sample size). Statistical analysis was performed by one-way ANOVA followed by the Dunnett test. $P < 0.05$ was considered statistically significant.

RESULTS

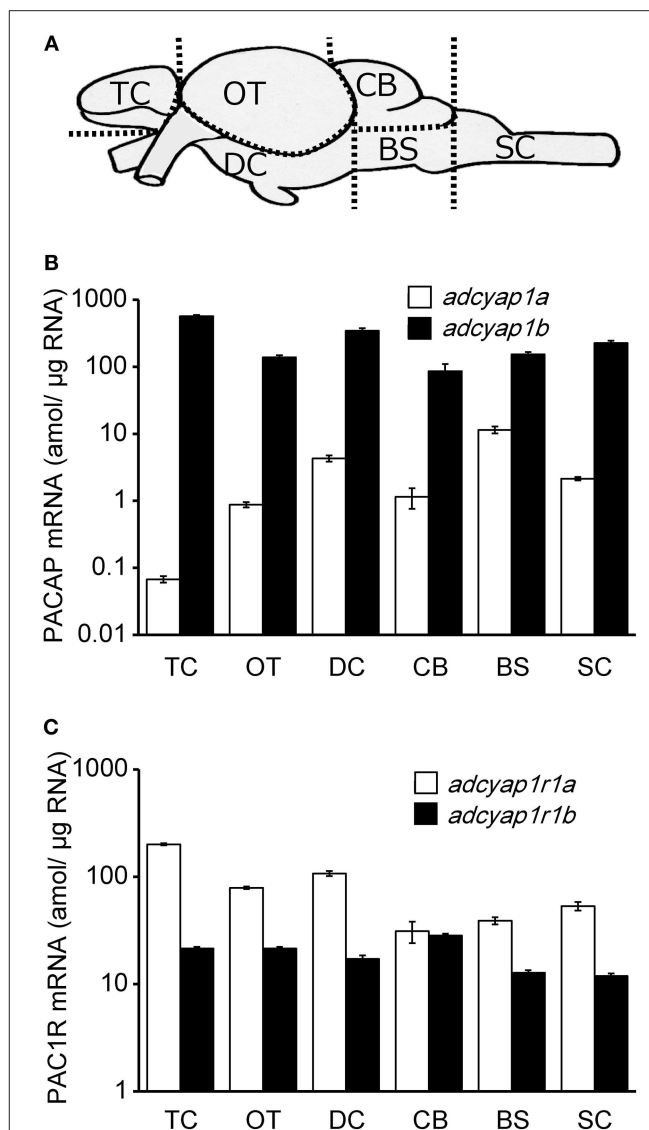
Expression Pattern of zfPACAP and zfPAC1R mRNA in Zebrafish Tissues and Brain Regions

Using real-time PCR, the highest levels of *adcyp1a* and *adcyp1b* mRNA were detected in the brain (**Figure 1A**). Levels of *adcyp1b* mRNA were almost 15 times higher than those of *adcyp1a* mRNA in the brain. *adcyp1b* mRNA was dominantly expressed in the brain and was also expressed at low levels in the eye. Additionally, *adcyp1a* mRNA levels in the gut were slightly similar to those in the brain, and low levels



were detected in the testis and eye. Measurement of PAC1R mRNA revealed the highest levels of *adcyap1r1a* and *adcyap1r1b* mRNA were detected in the brain (Figure 1B). Furthermore, *adcyap1r1a* mRNA levels were almost twice as high than *adcyap1r1b* mRNA levels in the brain. *adcyap1r1b* mRNA was slightly expressed in the eye. However, *adcyap1r1a* and *adcyap1r1b* mRNAs were expressed at very low levels in the peripheral tissues.

Subsequently, the brain was divided into six regions, namely, telencephalic region, optic tectum, diencephalic region, cerebellum, brain stem, and spinal cord (Figure 2A), and PACAPs and PAC1R mRNA was measured in each brain region. In all six brain regions, *adcyap1b* mRNA levels were greater than those of *adcyap1a* (Figure 2B). The highest levels of *adcyap1a* and *adcyap1b* mRNAs were detected in the brain stem and telencephalon, respectively. Both PACAP mRNAs were expressed at relatively higher levels in the diencephalon. Measurement of PAC1R mRNA showed higher levels of *adcyap1r1a* mRNA levels compared with *adcyap1r1b* in all six brain regions



(Figure 2C). High levels of *adcyap1r1a* mRNA were detected in the telencephalon and diencephalon, and the highest levels of *adcyap1r1b* mRNA were detected in the cerebellum.

Effects of PACAP ICV Injection on Feeding Behavior and Locomotor Activity

To determine the effects of zebrafish PACAPs on feeding behavior, feeding behavior and locomotor activity were measured after ICV administration of mPACAP, zfPACAP1, and zfPACAP2. zfPACAP1 injection at 2 pmol/g significantly suppressed food intake for 15 min after injection, but not at 20

pmol/g (**Figure 3A**). zfPACAP2 and mPACAP injection at 2 and 20 pmol/g significantly suppressed food intake (**Figures 3B,C**). At 2 pmol/g, all three PACAP injection groups tended to increase their locomotor activity in a circular tank, but this was not significantly different (**Figure 3D**).

Changes in PACAP and PACAP Receptor mRNA Before and After Feeding

PACAP and PAC1R mRNA levels in the diencephalic region were quantified before and after feeding. *adcyap1a* mRNA levels tended to increase at 60 min after feeding, and peaked significantly at 120 and 180 min (**Figure 4A**). At the peak response at 120 min, *adcyap1a* mRNA levels were 7.5 times higher than the pre-feeding levels. While, mRNA levels of *adcyap1b*, *adcyap1r1a*, and *adcyap1r1b* did not change significantly until 180 min after feeding (**Figures 4B–D**).

DISCUSSION

Tissue Expression and Distribution of Duplicated zfPACAP and zfPAC1R mRNA

The distribution and tissue expression of *adcyap1b* mRNA quantified by relative quantification real-time PCR was described previously (7); however, a comparison of the distribution and expression of the duplicated PACAP or PAC1R mRNA was not studied. Therefore, the present study used absolute quantified real-time PCR for this comparison. A comparison of expression levels in the brain showed levels of *adcyap1b* were around 15 times higher than those of *adcyap1a*, and levels of *adcyap1r1a* were around 2-fold higher than those of *adcyap1r1b*. These data indicate that duplicated zfPACAP and zfPAC1R have very different expression levels in the central nervous system. In the brain expression experiment, *adcyap1b* levels were higher than those of *adcyap1a* in all of the brain regions. While in the telencephalon, *adcyap1b* expression was maximal, but *adcyap1a* expression was lowest. This result is in agreement with previous results showing that *adcyap1b* mRNA and PACAP2 immunoreactivity are highly expressed in the telencephalon (7). Measurements of the PACAP receptor showed that *adcyap1r1a* mRNA was highly expressed in the telencephalon. These results indicate that the brain localization of duplicated PACAP and PAC1R genes is also different, implying that duplicated zfPACAP and zfPAC1R may be responsible for different physiological effects. The telencephalon of fish is thought to closely corresponds to the limbic system of the mammalian hippocampus, amygdala, and cerebral cortex (30). Indeed, PACAP and PAC1R are expressed in the hippocampus and amygdala in rats (4), and PACAP ICV administration to rats improved passive avoidance learning, suggesting that PACAP is involved in the memory and learning system (24). These reports indicate that *adcyap1b* and *adcyap1r1a* may be involved in the memory and learning system in zebrafish.

In the peripheral tissues, *adcyap1a* was expressed at high levels, and was most highly expressed in the intestinal tract. It was reported that PACAP acts on intestinal smooth muscle relaxation in mammals (31). In fish, PACAP-like immunopositive cells and

nerve fibers were observed in the myenteric plexus and smooth muscle in the rectum, and PACAP relaxed smooth muscle in the stargazer (32). PACAP mRNA was detected in the stomach and intestine in largemouth bass (33). Therefore, PACAP may have smooth muscle relaxant actions in the intestinal tract in zebrafish. However, in the present study, despite the high expression of *adcyap1a* in the intestinal tissue, expression of *adcyap1r1a* and *adcyap1r1b* of zfPAC1R genes in the intestinal tract was only detected at very low levels. PACAP has the ability to bind to three receptors: PAC1R (with highest affinity), and two VIP receptors, VPAC1R and VPAC2R (2). PACAP may act in intestinal tissues via VPACR. To verify this, the expression and distribution of VPAC1R and VPAC2R in zebrafish intestinal tissues needs to be clarified.

Various pathological condition models have been developed for zebrafish. It has been reported that administration of PACAP could inhibit adriamycin-induced kidney damage and hydrogen peroxide-induced damage of sensory hair cells in zebrafish (34, 35). The results of the distribution of PACAP and PACAP receptors in the zebrafish obtained in this study are also important knowledge for the pathophysiological study of PACAP in zebrafish.

Effects of zfPACAP1, zfPACAP2, or mPACAP ICV Administration on Food Intake and Locomotor Activity

The anorexigenic effects of PACAP have mainly been studied using rodent models. Local administration of PACAP into nuclei, such as the paraventricular nucleus (36), ventromedial nucleus (37), amygdala central nucleus (38), and the nucleus accumbens (39) reduced food intake, and PACAP exhibited anorexigenic activity via PAC1R (36). In non-mammalian species, ICV administration of human PACAP in chicks (40, 41) and frog PACAP in goldfish (27) reduced food intake. However, these experiments were conducted in PACAP from different species, and there are no reports on whether same species PACAP has truly anorexigenic effect in non-mammals. The present study revealed that ICV administration of both zfPACAPs suppressed food intake without affecting locomotor activity. This result indicates that zfPACAP has potent anorexigenic effects in the brain. PACAP1 and PACAP2 share 82% homology between their amino acid sequences (7). It seems that the 18% of the sequence that differs does not significantly influence the anorexigenic effect.

ICV administration of mPACAP also reduced food intake. This suggests that mPACAP has an affinity to zfPAC1R, and that zfPAC1R has similar ligand selectivity to mPAC1R. In fish, it is known that orexigenic peptides and anorexigenic peptides, which are mainly expressed in the hypothalamus, are involved in appetite regulation, and PACAP is considered to be one of the anorexigenic peptide among them (29, 42). In rats, PACAP increased expression levels of proopiomelanocortin (POMC) by activating the adenylate cyclase/cAMP/protein kinase A pathway via PAC1R, consequently demonstrating its anorexigenic action by secreting the anorexigenic hormone,

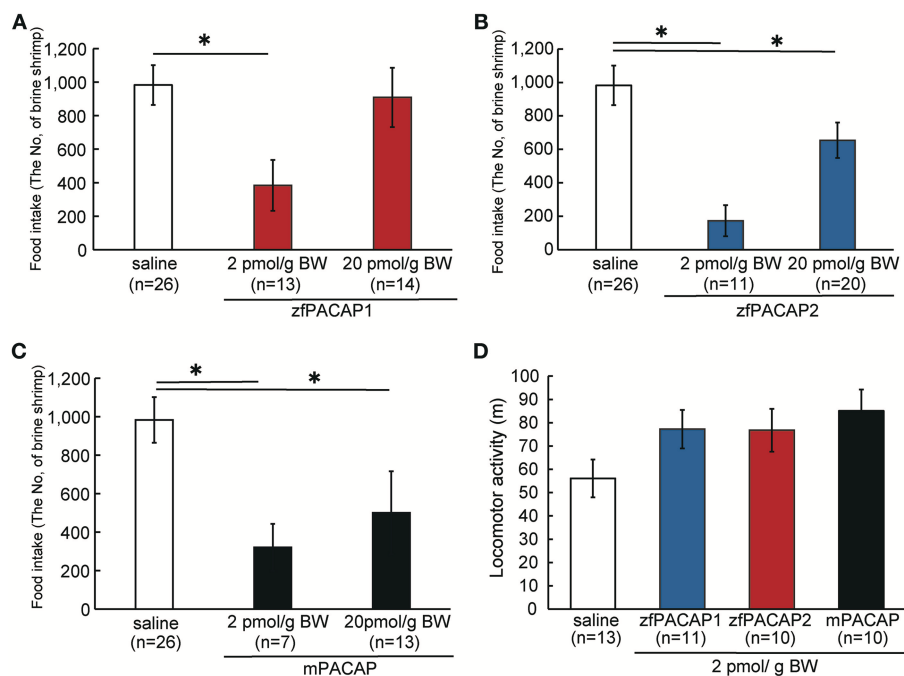


FIGURE 3 | Food consumption and locomotor activity after ICV injection of PACAP. **(A–C)** Food intake was measured for a 15-min feeding period after ICV injection of PACAP. **(D)** Locomotor activity was measured in a white circle water tank for 15 min after ICV injection. * $P < 0.05$.

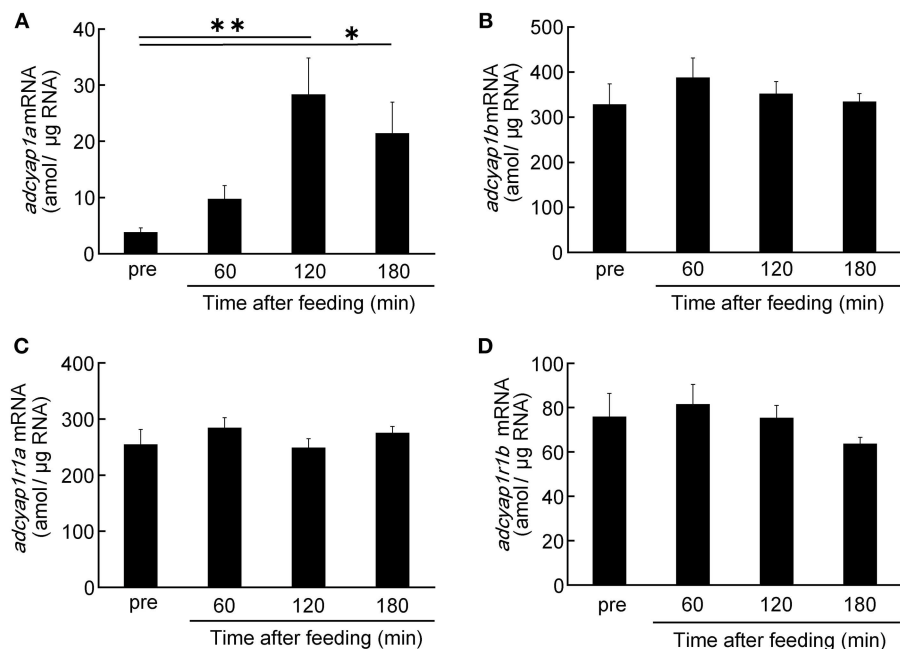


FIGURE 4 | Time-course changes of PACAP and PAC1R mRNAs level after feeding. Expression levels of PACAP mRNA, *adcyap1a* **(A)** and *adcyap1b* **(B)** genes, PAC1R mRNA, *adcyap1r1a* **(C)** and *adcyap1r1b* **(D)** genes were determined using absolute quantitative real-time PCR. The number of samples in the pre, 60, 120, and 180 min groups were 13, 15, 15, and 10, respectively. * $P < 0.05$, ** $P < 0.01$.

alpha melanocyte stimulating hormone (α -MSH), induced by POMC (43, 44). On the other hand, PACAP exerts anorexigenic effects via corticotropin-releasing hormone

(CRH) in goldfish (45). It is possible that the anorexigenic pathway in fish may differ from that of mammals. On the other hand, administration of PACAP to juvenile tilapia

has been reported to enhance growth and feeding (46). This suggests that the effect of PACAP may vary depending on the route of administration, fish species, or stage of development. Further studies are required to determine whether the anorexigenic action of PACAP in zebrafish is via a Gs pathway, and other appetite-regulating hormones, such as α -MSH and CRH.

The PACAP ICV administration experiment showed a significant suppressive effect on food intake only at low concentrations (2 pmol/g BW) of PACAP. The neuroprotective action of PACAP showing rat retinal ganglion cell death (47), mouse retinal injury (48), spinal cord injury (49), and exocrine stimulation in mouse tear secretion (18) demonstrated a bell-shaped dose response, in other words, the action of PACAP peaks and decreases at high concentrations. Although the details remain unknown, there may be a relationship between the response difference of PAC1R with high affinity and VPAC1R and VPAC2R with relatively low affinity. Two VPAC1R genes and one VPAC2R gene were identified in the zebrafish genome on the genome database. Future investigations into the affinity of zfPAC1Rs and zfVPACRs for zfPACAP are required.

Changes in zfPACAP and zfPAC1R mRNAs Levels and Feeding Conditions

There are still few reports on changes in the expression level of PACAP in response to feeding states. Kiss et al. reported that PACAP concentration was elevated in the rat brain at 12 h after food deprivation, and in the chicken brain at 36 h after food deprivation (50). PACAP and PAC1R mRNA expression was increased in overfeeding goldfish (51). Conversely, PACAP mRNA level was decreased in 4 days fasted largemouth bass (33). From these reports, it has been clarified that PACAP shows an expression pattern as an anorexigenic factor in fish, and our results are also consistent with this. However, it is still unclear why the expression change in the feeding states of PACAP differs between tetrapods and fish. This difference seems to have occurred due to evolutionally changes in vertebrate, or difference of the measurement method of PACAP in the peptide level or mRNA level.

In the present study, only *adcyap1a* mRNA was significantly increased at 2 and 3 h after feeding. Although ICV administration of both zfPACAP1 and zfPACAP2 suppressed food intake, it is noteworthy that only *adcyap1a* responded to the feeding state. This result suggests that zfPACAP1 is mainly involved in suppression of food intake after feeding. We previously showed that zfPACAP2 immunoreactivity was localized in the hypothalamus (7); however, the brain localization of zfPACAP1 remains unknown. In future, it will be necessary to prepare a specific antibody against zfPACAP1 and identify the localization of zfPACAP1 in the hypothalamus. Furthermore, the tissue distribution of zfPACAP

mRNA and zfPAC1R mRNA should be analyzed using *in situ* hybridization.

On the other hand, the problem with the protocol in this paper is that the experimental fish was continuously sampled from the same stock tank. Therefore, the remaining fish may have been under the stress. In other words, elevation of *adcyap1a* mRNA after feeding may have been caused by a stress response. Actually, chronic stress increases endogenous PACAP expression in the hypothalamic nucleus, and PACAP can stimulate CRH production and secretion in rodents (21). It is necessary to review and upgrade the protocol, and clarify the relationship between PACAP and food intake under less stressful conditions.

In conclusion, expression levels and the distribution of zfPACAPs and zfPAC1Rs differ in zebrafish. There was almost no difference in the anorexigenic effects of zfPACAP1 and zfPACAP2 by ICV administration. These results suggest that PACAP act as an anorexigenic feeding regulator in the brain, and the anorexigenic effect of PACAP is preserved in vertebrates. Molecular species of the VIP/secretin/glucagon superfamily are thought to have been formed by repeating exon and gene duplication from PACAP-type ancestral genes during evolution (52). It is possible that duplicated zfPACAPs have begun to evolve into another paralogous gene by altering its distribution and responses. Functional analysis of zfPACAP and zfPAC1R, further physiological experiments, and detailed tissue distribution observation will clarify this hypothesis.

ETHICS STATEMENT

All experimental procedures were conducted in accordance with the institutional guidelines of the University of Toyama for the care and use of laboratory animals. However, it was not necessary to receive permission for fish experiment in the University of Toyama. No endangered animal species were involved in the study.

AUTHOR CONTRIBUTIONS

TN and AT designed and performed the experiments. NK, SS, and KM were involved in planning and supervising the work. TN wrote the manuscript with support from NK, SS, and KM.

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Five Neuropeptide Ligands Meet One Receptor: How Does This Tally? A Structure-Activity Relationship Study Using Adipokinetic Bioassays With the Sphingid Moth, *Hippotion eson*

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Adipokinetic hormones (AKHs) play a major role in mobilizing stored energy metabolites during energetic demand in insects. We showed previously (i) the sphingid moth *Hippotion eson* synthesizes the highest number of AKHs ever recorded, viz. five, in its corpus cardiacum: two octa- (Hipes-AKH-I and II), two nona- (Hipes-AKH-III and Manse-AKH), and one decapeptide (Manse-AKH-II), which are all active in lipid mobilization (1). (ii) Lacol-AKH from a noctuid moth showed maximal AKH activity in *H. eson* despite sequence differences and analogs based on Lacol-AKH with modifications at positions 2, 3, 8, or at the termini, as well as C-terminally shortened analogs had reduced or no activity (2). Here we report on N-terminally shortened and modified analogs of the lead peptide, as well as single amino acid substitutions at positions 1, 4, 5, 6, and 7 by an alanine residue. Ala¹ and Glu¹ instead of pGlu are not tolerated well to bind to the *H. eson* AKH receptor, whereas Gln¹ has high activity, suggesting it is endogenously cyclized. Replacing residue 5 or 7 with Ala did not alter activity much, in contrast with changes at position 4 or 6. Similarly, eliminating pGlu¹, Leu², or Thr³ from Lacol-AKH severely interfered with biological activity. This indicates that there is no core peptide sequence that can elicit the adipokinetic effect and that the overall conformation of the active peptide is required for a physiological response. AKHs achieve a biological action through binding to a receptor located on fat body cells. To date, one AKH receptor has been identified in any given insect species; we infer the same for *H. eson*. We aligned lepidopteran AKH receptor sequences and note that these are very similar. The results of our study is, therefore, also applicable to ligand-receptor interaction of other lepidopteran species. This information is important for the consideration of peptide mimetics to combat lepidopteran pest insects.

Keywords: adipokinetic hormone, neuropeptide hormone, AKH receptor, *Hippotion eson*, lipid metabolism, structure-function assay, energy mobilization

INTRODUCTION

Neuropeptide hormones are biologically active peptides that are synthesized in modified neurons and released into the circulatory system to effect an action via a ligand-specific receptor. In insects, neurons of the corpus cardiacum (CC) synthesize and release neuropeptides that belong to the adipokinetic hormone (AKH)/red pigment-concentrating hormone (RPCH) family, so named for their classic action of mobilizing fuel metabolites from storage in the fat body of insects for catabolic purposes, and for their action on integumental pigment cells in crustaceans for camouflage (3). In insects the AKHs may have different chain lengths (octa-, nona-, and/or decapeptides), whereas the RPCHs in crustaceans only occur as octapeptides, to date, and in both animal groups, the ligand operates via a G protein-coupled receptor on the effector cell (4).

The AKH family of peptides has over 60 members, and some of these peptides can be common to different insect orders, for example Peram-CAH-I (one of the so-called cardioacceleratory hormones in *Periplaneta americana*) is synthesized in primitive insects (5), in cockroaches (6, 7), and in beetles (8, 9). On the other hand, certain AKHs are unique to one insect order, as is the case with currently nine order-specific AKH members (see Table 1) of the Lepidoptera, which comprises moths and butterflies. Many lepidopteran species (particularly in the larval stage of the insect lifecycle) are regarded as pest insects that compete for human food resources. One can envisage to produce a “green pesticide” that is harmful to lepidopterans but not to other insects, especially not to beneficial pollinator species, such as certain hymenopteran or dipteran (hoverfly) insects. The rationale of “green pesticides” in food security is to use information about endogenous hormones of pest insects to make peptide mimetics that will act only against the pest insects to alter their behavior or physiology (15). Thus, for such research it is paramount to know how the lepidopteran AKH receptor is activated by its ligands. In the current study we investigate such phenomena for the AKH receptor of a moth species. Here, we review data published previously (1, 2) and complement them with further analog studies emanating from 11 analogs not tested previously. The moth species of choice is the common striped hawk moth, *Hippotion eson*.

Five years ago we demonstrated via electrospray mass spectrometry that *H. eson*, and the silver-striped hawk moth, *H. celerio*, each produce a record number of five different AKHs in their respective corpora cardiaca (1). The five AKHs are identical in these hawk moth species, and biological activity of the neuropeptides was examined with chemically synthesized peptides only in the more-abundant species, *H. eson*. The synthetic peptides were also used to confirm the sequence of the mature hawk moth AKHs [see Table 1; (1)]. All five peptides were active at a low dose to increase the circulating lipid concentration in resting *H. eson* adults, whereas the carbohydrate concentration was not significantly affected. Although not proven yet, it is highly likely that there is only one receptor for all five AKHs

TABLE 1 | Lepidoptera-specific Adipokinetic Hormone (AKH) family peptides.

Name of peptide	Peptide sequence	Species (first elucidated)	References
Hipes-AKH-I#	pELTFTSSWamide	<i>H. eson</i> ; <i>Hippotion celerio</i>	(1)
Manse-AKH#	pELTFTSSWGamide	<i>Manduca sexta</i>	(10)
Lacol-AKH	pELTFTSSWGGamide	<i>Lacanobia oleracea</i>	(11)
Hipes-AKH-II#	pELTFTSTWamide	<i>H. eson</i> ; <i>H. celerio</i>	(1)
Hipes-AKH-III#	pELTFTSTWGamide	<i>H. eson</i> ; <i>H. celerio</i>	(1)
Bommo-AKH	pELTFTPGWGQamide	<i>Bombyx mori</i>	(11)
Manse-AKH-II#	pELTFSSGWGQamide	<i>M. sexta</i>	(12)
Helze-HrTH	pELTFSSGWNamide	<i>Heliothis zea</i>	(13)
Piebr-AKH	pELTFSSGWamide	<i>Pieris brassicae</i>	(14)

Endogenous AKHs in *Hippotion eson*.

in *H. eson*. This has been the case in all other insects where a G protein-coupled receptor has been identified as the AKH receptor (AKHR), regardless of the number of AKH sequences encoded—see, for example, Marchal et al. (16) where AKHR information on *Aedes aegypti* with 1 ligand and *Schistocerca gregaria* with 3 ligands are shown. The AKHR of *H. eson*, is, therefore an interesting candidate to study with respect to ligand interactions, and could provide us with insight into what the structural limits are for adequate receptor-ligand interactions to effect a biological response. With this in mind, a functional study with *H. eson* was initiated when a decapeptide AKH from a water bug, Lacsp-AKH [pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-Gly amide, (17)], with five amino acid substitutions compared with the endogenous decapeptide of the hawk moth, Manse-AKH-II, showed no adipokinetic activity in *H. eson* (1), whereas a decapeptide from another lepidopteran insect (Lacol-AKH: three amino acid substitutions compared with Manse-AKH-II; Table 1) was as active as the endogenous *H. eson* AKHs (2). The present study is a follow-on to elucidate the ligand criteria for the most effective functional response, i.e., an adipokinetic action. There is a tendency to evaluate the activity of neuropeptides only on the basis of their primary sequence and the charge of specific amino acids, whereas it is known that neuropeptides undergo conformational changes to obtain an “active conformation” once docking onto the receptor (18). In the present study we attempt to take such conformational change into account with our analog designs to determine the final activity and functional properties of a modified AKH in the sphingid moth. The AKH analogs are based on the sequence of Lacol-AKH (a Gly-extended version of the endogenous Manse-AKH of *H. eson*, see Table 1), and examine changes to the N-terminus of the peptide, including N-terminally shortened analogs, as well as a series of analogs with the single substitution by Ala in all positions of the peptide, excepting positions 2 and 3—substitutions in these positions were previously shown to have a profound negative effect on lipid-mobilization in *H. eson*, as did C-terminally shortened analogs (2).

MATERIALS AND METHODS

Insects

Eggs and larvae of the common striped hawk moth, *H. eson*, on the leaves of the arum lily, *Zantedeschia aethiopica*, were collected during the austral months of May and June in 2015 and 2016 at the Buitenverwachting vineyard (Constantia, South Africa) and on the property of the University of Cape Town and reared at the University of Cape Town (Rondebosch, S. Africa). The material was placed in a constant temperature room of the Department of Biological Sciences and the insects were reared under the following conditions: 30°C, 60% RH, and a photocycle regime of 17 h light: 7 h dark. The larvae were reared on freshly cut arum lily leaves (in a jar of water) in a wire mesh cage with a wooden floor and dimensions of 45 × 40 × 45 cm (L × H × W). Daily, leaves were replaced and fecal matter was removed from the cage. Pupae were placed into individual small containers in the same rearing room. Under the described controlled holding conditions, adult moths eclosed between 11 and 12 days after pupation (H. G. Marco and G. Gäde, unpublished result) and were used in biological assays on the following day at room temperature.

This study was carried out in accordance with relevant institutional and national guidelines and regulations concerning the use of animal subjects in scientific studies.

Biological Assay

A conspecific bioassay was carried out as described previously (1). Briefly, adult *H. eson* specimens of both sexes that had emerged during the night were used for biological assays on the following day. The cage of emerged moths were taken to the laboratory and left at RT for 1 h before sampling. During this time the moths were not active. Individual moths were removed from the cage one at a time: 0.5 µl hemolymph was sampled from the abdominal dorsal vessel and put into concentrated sulphuric acid; the moth was then injected ventrolaterally into the abdomen with 3 µl of either water, or the synthetic peptides under investigation (reconstituted in water to a concentration of 10 pmol in 3 µl), and the moth was put under a funnel at RT and left there until a second sample of hemolymph was taken 90 min after injection. The hemolymph was thoroughly mixed with the sulphuric acid, and the total vanillin-positive material (= lipids) was measured in the mixture as described previously (19). The number of moths per injectate ranged between 5 and 8 (see details of *n* number in Tables 2–4).

The difference in lipid concentration before and after injection was calculated for each individual animal and a Student's *T*-test was used for calculating statistical significance in Excel.

Synthetic Peptides

Peptides were custom-synthesized by Pepmic Co. Ltd (Suzhou, China). For primary structures see Tables 2–4. The synthetic peptides are based on a decapeptide code-named Lacol-AKH that was previously found as endogenous AKH in *Lacanobia oleracea* and *Mamestra brassicae* (11). Lacol-AKH is 100% active in *H. eson* as shown by *in vivo* lipid-mobilizing assays and

was previously used in structure-activity studies with *H. eson* (2). Lacol-AKH differs in three places from the endogenous decapeptide in *H. eson*, i.e., Manse-AKH-II, which is also present in all sphingid moths investigated to date, and the first eight and nine amino acid residues of Lacol-AKH are, respectively, 100% identical to the endogenous octapeptide (Hipes-AKH-I) and nonapeptide (Manse-AKH) in *H. eson* [Table 1; (14)].

The rationale for the design of the synthetic peptides used in this study is the following: previously (2) we examined the effect of specific amino acid substitutions that cumulatively occur in Lacsp-AKH, a water bug AKH that is not active in *H. eson* (1), and found that the second (Leu) and third (Thr) amino acids are relatively important for biological activity in the sphingid moth, as well as Trp⁸. Here we have designed Ala-substituted analogs of Lacol-AKH to systematically examine the relative importance of charge and side-chains in positions 1 and 4–7. Additional analogs are designed to test the effect of Lacol-AKH without pGlu in position 1, and nonapeptides that differ N-terminally. Previously we looked at the effect of different peptide chain lengths on hyperlipemic activity *in vivo* by using analogs that were C-terminally extended, or truncated, or with a free C-terminus (2). In total, the current study investigates 11 different analogs for the first time (see Tables 2–4 for sequences).

Adipokinetic Hormone Receptor Sequence Alignment

We aligned the amino acid sequences of the AKHR from six lepidopteran genera (moths and butterflies). The AKHR sequences were obtained from BLAST searches. The multiple sequence alignment with hierarchical clustering was performed by using MultAlin version 5.4.1 (20).

RESULTS

The Biological Effect of the N-terminus of Lacol-AKH in *H. eson*

AKH peptides are characterized by a pGlu in position 1. This is believed to be an effective block against exopeptidases in the hemolymph of the insect, resulting in a longer half-life of the peptide (21). Lacol-AKH analogs were designed to specifically explore how lipid mobilization is affected by changes to the N-terminal amino acid of the decapeptide. The results are shown in Table 2. When the N-terminal pGlu is replaced with a free Glu residue, the adipokinetic activity of the ligand was severely reduced from 100% to a mere 23% activity *in vivo*, whereas a free Gln residue in position 1 still had a strong biological effect of over 60% AKH activity relative to the maximal response. In contrast, lipids were not mobilized in *H. eson* when the N-terminal pGlu is replaced with a differently blocked Glu residue (viz. an acetylated Glu), or when a free Ala residue replaced pGlu in position 1.

The Significance of N-terminally Shortened Lacol-AKH Analogs

Functional AKHs are either composed of eight, nine, or 10 amino acids; *H. eson* synthesizes AKHs with all of

TABLE 2 | The biological effect of the N-terminus of Lacol-AKH in the common striped hawk moth, *Hippotion eson*.

Treatment (3 μ l injected) and amino acid sequence ^a	Hemolymph lipids (mg ml ⁻¹)					% Activity relative to maximal increase
	<i>n</i>	0 min	90 min	Difference	<i>P</i> *	
Distilled water	5	33.89 $\pm$ 15.67	34.32 $\pm$ 17.99	0.43 $\pm$ 3.49	NS	NS
Lacol-AKH	7	27.07 $\pm$ 8.78	47.02 $\pm$ 10.13	19.95 $\pm$ 5.90	0.0001	100
pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Lacol-AKH: free N-terminus, Glu ¹	6	32.37 $\pm$ 11.63	36.89 $\pm$ 11.95	4.52 $\pm$ 2.39	0.003	23
Glu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Lacol-AKH: free N-terminus, Gln ¹	5	26.85 $\pm$ 9.30	39.43 $\pm$ 6.31	12.58 $\pm$ 8.75	0.032	63
Gln-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Ala ¹ -Lacol-AKH: free N-terminus, Ala ¹	6	34.74 $\pm$ 13.49	34.16 $\pm$ 14.27	-0.58 $\pm$ 2.47	NS	NS
Ala-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
#[N-Ac]Glu ¹	7	48.73 $\pm$ 10.08	52.55 $\pm$ 11.00	3.95 $\pm$ 6.65	NS	NS [#]
[N-Ac]Glu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						

^aAmino acid substitutions in Lacol-AKH analogs are highlighted.

The data are presented as Mean $\pm$ S.D. [#]Data from Marco and Gäde (2). *Paired *t*-test was used to calculate the significance between pre-and post-injection values. NS, not significant.

TABLE 3 | The biological effect of N-terminally shortened Lacol-AKH analogs in the common striped hawk moth, *Hippotion eson*.

Treatment (3 μ l injected) and amino acid sequence ^a	Hemolymph lipids (mg ml ⁻¹)					% Activity relative to maximal increase
	<i>n</i>	0 min	90 min	Difference	<i>P</i> *	
Lacol-AKH	6	30.88 $\pm$ 12.99	60.96 $\pm$ 22.27	30.08 $\pm$ 11.22	0.001	100
pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Nonapeptide, free N-terminus, Leu ¹	7	33.92 $\pm$ 9.43	44.55 $\pm$ 11.50	10.63 $\pm$ 4.80	0.0001	35
-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Nonapeptide, [N-Ac]Leu ¹	6	45.52 $\pm$ 8.18	49.26 $\pm$ 9.09	3.74 $\pm$ 2.51	0.007	12
[N-Ac]Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Nonapeptide, minus Leu ²	8	28.44 $\pm$ 5.29	39.38 $\pm$ 7.54	10.94 $\pm$ 4.96	0.0002	36
pGlu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						
Nonapeptide, minus Thr ³	8	40.91 $\pm$ 7.31	48.41 $\pm$ 7.70	7.50 $\pm$ 1.60	0.000002	25
pGlu-Leu-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide						

^aAmino acid substitutions or deletions in Lacol-AKH analogs are highlighted.

The data are presented as Mean $\pm$ S.D. *Paired *t*-test was used to calculate the significance between pre-and post-injection values.

these chain lengths and all of these peptides were found to be biologically active in a conspecific bioassay (1). The biological effect of shorter chain lengths (6- and 7-mer) was previously explored with C-terminally truncated, amidated Lacol analogs (2). Here, the relative importance of the first three N-terminal residues are specifically examined in *H. eson* with Lacol-AKH analogs that have one absent residue (either pGlu¹, Leu², or Thr³), effectively producing an amidated nonapeptide.

None of the four nonapeptide Lacol-AKH analogs could achieve a high increase in circulating lipid concentrations and the AKH activity relative to Lacol-AKH injection in *H. eson* was well below 40% (Table 3). Without pGlu¹, the peptide analog now beginning with a free Leu amino acid residue was 35% active, and this activity was further decreased to 12% when the Leu residue was blocked by N-acetylation. The return of pGlu¹ coupled with the absence of Leu² or Thr³ as analogs resulted in 36 and 25% activity, respectively (Table 3).

The Biological Effect of Single Amino Acid Replacements in Lacol-AKH

The relative importance of the middle region (amino acids 4–8) of Lacol-AKH was investigated via a series of analogs in which one amino acid was substituted with an alanine residue (Table 4). In this way we can make inferences about the relative importance of the side chains of the original C-terminal residues in activating the hawk moth AKH receptor. The substitution of an aromatic amino acid residue with Ala (i.e., Phe⁴ or Trp⁸) totally eliminated AKH activity (Table 4). Ala⁶ instead of Ser⁶ resulted in a marked reduction of biological activity (25%), whereas an Ala substitution in position 5 or 7 were more tolerated with 95 and 67% relative activity, respectively (Table 4).

DISCUSSION

Previous studies showed that all five identified AKHs of the common striped hawk moth, *H. eson*, are active in conspecific biological assays to significantly increase lipid concentrations in

TABLE 4 | The biological effect of single amino acid substitutions in Lacol-AKH in the common striped hawk moth, *Hippotion eson*.

Treatment (3 μ l injected) and amino acid sequence ^a	Hemolymph lipids (mg ml ⁻¹)					% Activity relative to maximal increase
	<i>n</i>	0 min	90 min	Difference	<i>P</i> *	
^b Lacol-AKH pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide	6	30.88 $\pm$ 12.99	60.96 $\pm$ 22.27	30.08 $\pm$ 11.22	0.001	100
^c Lacol-AKH pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide	7	27.07 $\pm$ 8.78	47.02 $\pm$ 10.13	19.95 $\pm$ 5.90	0.0001	100
^c Ala ¹ -Lacol-AKH Ala-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-Gly amide	6	34.74 $\pm$ 13.49	34.16 $\pm$ 14.27	-0.58 $\pm$ 2.47	NS	NS
^b Ala ⁴ -Lacol-AKH pGlu-Leu-Thr-Ala-Thr-Ser-Ser-Trp-Gly-Gly amide	6	26.86 $\pm$ 6.30	29.00 $\pm$ 5.26	2.14 $\pm$ 2.97	NS	NS
^c Ala ⁵ -Lacol-AKH pGlu-Leu-Thr-Phe-Ala-Ser-Ser-Trp-Gly-Gly amide	5	27.19 $\pm$ 12.23	46.15 $\pm$ 9.14	18.96 $\pm$ 5.28	0.001	95
^c Ala ⁶ -Lacol-AKH pGlu-Leu-Thr-Phe-Thr-Ala-Ser-Trp-Gly-Gly amide	5	31.49 $\pm$ 10.06	36.46 $\pm$ 8.67	4.97 $\pm$ 2.69	0.007	25
^b Ala ⁷ -Lacol-AKH pGlu-Leu-Thr-Phe-Thr-Ser-Ala-Trp-Gly-Gly amide	7	37.03 $\pm$ 9.56	57.26 $\pm$ 17.18	20.23 $\pm$ 9.48	0.0007	67
[#] Ala ⁸ -Lacol-AKH pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Ala-Gly-Gly amide	8	40.90 $\pm$ 11.43	41.50 $\pm$ 15.65	0.61 $\pm$ 5.62	0.000002	NS [#]

^aAmino acid substitutions in Lacol-AKH analogs are highlighted.

Two data series (^b and ^c) are presented as Mean $\pm$ S.D. The activity of analogs in a particular series was compared with the activity of Lacol-AKH of that series only. *Paired t-test was used to calculate the significance between pre- and post-injection values. NS, not significant. [#]Data from Marco and Gäde (2).

the hemolymph (1). This indicated a degree of tolerance in the hereto unknown *H. eson* AKH receptor for accommodating (i) differences in peptide chain length of octapeptides (Hipes-AKH-I and -II), nonapeptides (Hipes-AKH-III and Manse-AKH), and a decapeptide (Manse-AKH-II; for peptide structures see Table 1), as well as (ii) for particular amino acid substitutions in positions 5 (the polar and neutral Ser or Thr) and 7 (Ser, Thr, or Gly) of the native AKHs. When a decapeptide AKH from a noctuid moth (Lacol-AKH) was 100% active in *H. eson* but Lacsp-AKH, the decapeptide AKH from a water bug was not (1), it presented an intriguing case into unraveling the reasons for the loss of biological activity. Thus, began a study with Lacol-AKH as lead peptide to examine the effect on adipokinetic activity of each of the three Lacsp-AKH amino acids that differed from the *Hippotion* AKHs, viz. Val², Asn³, and Pro⁶ (2): Pro⁶ instead of the conserved *Hippotion* Ser⁶ made very little difference to adipokinetic activity, while Val² or Asn³ in the place of the conserved Leu² and Thr³ drastically curtailed biological activity in *H. eson*. Since Lacol-AKH and Lacsp-AKH both have Gly¹⁰ instead of *Hippotion*'s Gln¹⁰, and since Lacol-AKH is as active as the endogenous *H. eson* decapeptide AKH (Manse-AKH-II), this substitution was not investigated. A different substitution at position 10 in Manse-AKH-II, namely, a bulky and basic Lys residue (K¹⁰-Manse-AKH-II), still had 75% hyperlipemic activity, suggesting that major differences in charge, size and side chain (Gly, Gln, Lys) does not affect activity much, and that the 10th residue is not essential for interaction with the *H. eson* AKH receptor and its activation (2). These earlier studies, thus, also indicated that the N-terminal amino acid residues are quite important for ligand function *in vivo*; however, these alone do not constitute a functional

core peptide, as demonstrated by the much reduced activity of amidated C-terminally truncated Lacol-AKH ligands (6- and 7-mer peptides) (2). The current study evolved from the afore-mentioned work with *H. eson* and Lacol-AKH analogs to study more closely the structure-activity effect of the N-terminus and N-terminal residues in a lepidopteran AKH, and to investigate the role of side-chains and charge of amino acid residues in the mid- and C-terminal part of a lepidopteran AKH in biological activity. Such information is valuable for the consideration of producing biostable peptide mimetics that could bind or block AKH receptors specifically in pest insects, such as lepidopterans.

The *in vivo* adipokinetic assay employed in the current study is very robust and we have used it with other lepidopterans before, such as *Mamestra brassicae* (22) and *Pieris brassicae* (14). Despite a variable starting level of lipids in newly-emerged *H. eson* adult specimens (24 h or less after eclosion), clear increases in circulating lipid levels can be measured within individual moths with a number of Lacol-AKH analogs tested in the present study; the statistically significant adipokinetic responses fall broadly within two categories, relative to a maximum response that is elicited with the same dose of the lead peptide, Lacol-AKH: 20–40%, and above 60%.

The Importance of the N-terminus of Lacol-AKH on Biological Activity in *H. eson*

Among the characteristic features of AKH peptides are their blocked termini: a pyroglutamic acid (pGlu) in position 1, and an amidated C-terminus. Such a blocked ligand is less susceptible to exopeptidases in the hemolymph of the insect, and therefore

results in a longer half-life of the peptide to achieve its hormonal effect. In the current study, Lacol-AKH analogs were designed to specifically explore how lipid mobilization is affected by changes to the N-terminal amino acid. When the N-terminal pGlu was replaced with a free Glu residue, the adipokinetic activity of the ligand dropped to 23% activity *in vivo*; on its own, this result could be interpreted as affirmation that the N-terminally unblocked peptide was enzymatically degraded. What speaks against this interpretation alone, is the result that a free Ala residue in position 1 was not able to mobilize lipids in *H. eson* to the same extent as the free Glu¹ analog, thus suggesting that the analog with the slightly hydrophobic Ala¹ residue has a quite different active conformation than the analog with a polar and negatively charged Glu¹ residue and, hence, ligand-receptor interaction is even further impaired. Alternatively, the data may be interpreted that the Ala¹ analog is degraded much faster by exopeptidases than the Glu¹ analog, and/or that Glu¹ could be converted slowly to pGlu *in vivo* to still achieve the necessary conformation for binding and activating the AKHR. Furthermore, an analog with a differently blocked N-terminus (an acetylated Glu¹) failed to mobilize lipids in *H. eson*, thus could not activate the AKHR significantly despite the peptide being blocked at the N-terminus to delay degradation [current study and (2)]. This result suggests that the acetyl analog does not have the requisite conformation for accessing the receptor binding site, and concurs with an earlier study where Lee et al. (23) found that an unblocked Glu¹-AKH analog was more active than a N-[Acetyl]Glu¹-AKH analog in locusts *in vivo*. Results on other insects also reported lower biological activity when pGlu was replaced with other blocked residues (24, 25). Most surprisingly, however, was the observation that a free Gln¹ Lacol-AKH in the current study attained nearly two-thirds maximal biological activity. This is a strong indicator that Gln was converted to pGlu in the hemolymph either enzymatically by glutamyl cyclases or spontaneously (26) and thus could delay peptide degradation, and activate the AKHR through a suitable conformation. Since Glu¹ and Gln¹ AKH analogs were injected into *H. eson* at the same concentration of 10 pmol, and Gln¹ produced a three times higher biological activity than Glu¹, we may deduce that Gln is the preferred amino acid residue for conversion to pGlu. It has long been known that N-terminal Glu and Gln residues of many biologically active peptides and proteins can form pGlu by intramolecular cyclization, and several years of experimental evidence, reviewed by Abraham and Podell (27), seemed to indicate that either glutamic acid (Glu) or glutamine (Gln) is the direct precursor of pGlu, depending on the experimental system. Cyclization is catalyzed by an enzyme that is called glutamyl cyclase (QC), although this enzyme also catalyzes the conversion of glutamate to pGlu, hence it is an effective glutamyl cyclase (EC) too (28). That Gln seems to be the preferred substrate for QC in insects and crustacean, seems to also be apparent from the amino acid sequence encoded by the mRNA for the AKH precursor sequences known to date [see for example, (4, 29)].

In conclusion, our study has definitively shown that the N-terminal pGlu residue is of crucial importance for the correct

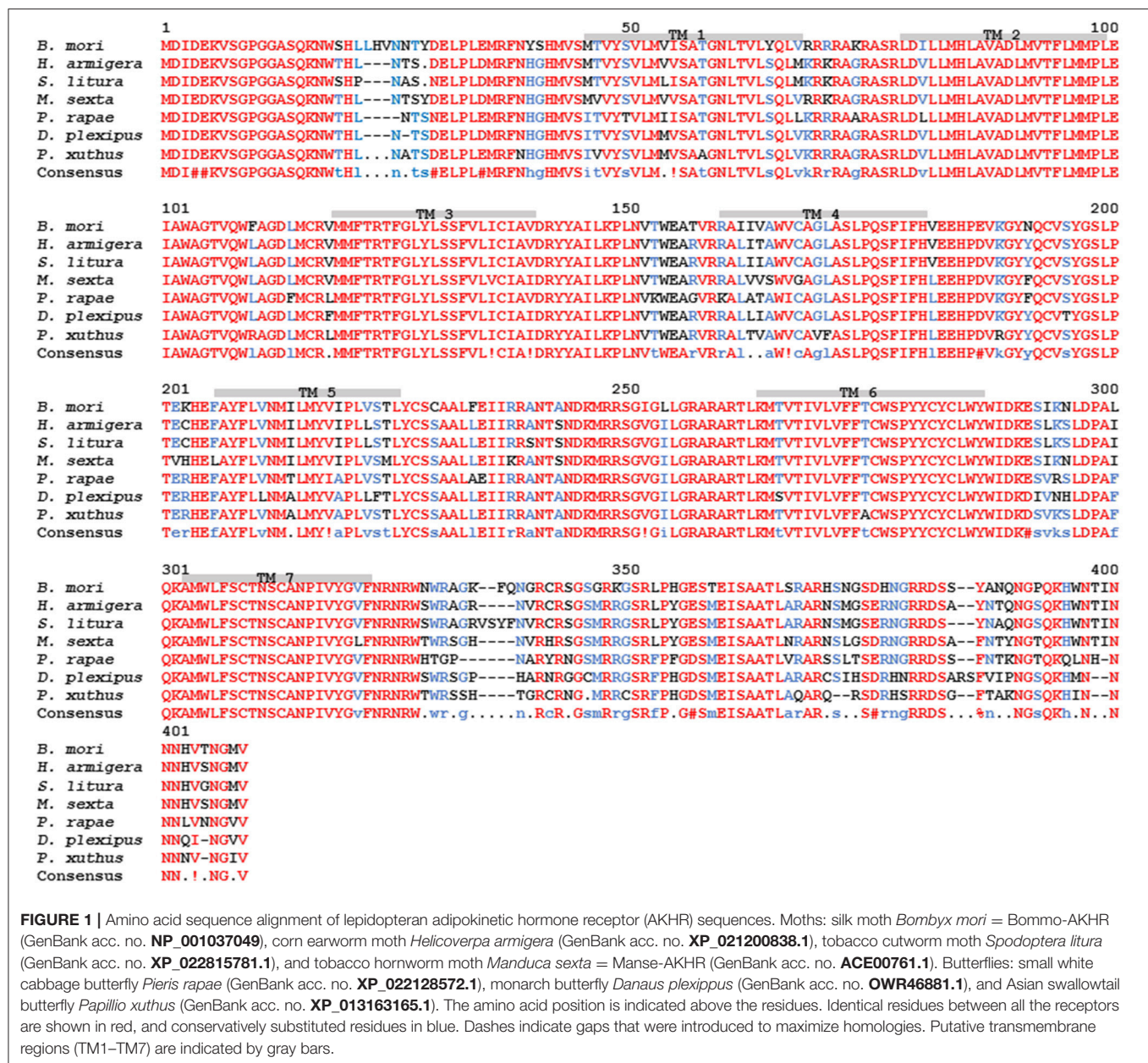
presentation of the AKH ligand to its receptor to achieve and maintain full biological activity.

Importance of N-terminal Amino Acids on Biological Activity Deduced From N-terminal Shortened Analogs

Although nonapeptides bind well to the *Hippotion* AKH receptor and achieve full or 75% of biological activity [Manse-AKH and Hipes-AKH-III, respectively; (1)], distortion of the N-terminus by free or blocked Leu as residue 1, or even peptides with pGlu as N-terminus but missing Leu² or Thr³, failed to result in more than mediocre (>36%) activity in the current study. As argued before (24, 30) it appears to be of great importance to have an alternating amphiphilic orientation of polar (pGlu¹, Thr³, Thr⁵) and hydrophobic (Leu², Phe⁴) amino acids in the 5 positions at the N-terminus to form a beta-strand (31–33). One other reason for the poor adipokinetic response (20–40 %) when interfering with the first three amino acids at the N-terminus, may be the direct consequence of shifting the aromatic amino acid from position 4 to position 3 in all our nonapeptide analogs tested in the present study. Phe⁴ seems to be essential in all AKHs as this is one of the conserved AKH features (see also section Importance of side chains of individual amino acids on AKH biological activity).

Importance of Side Chains of Individual Amino Acids on AKH Biological Activity

It had previously been shown with *H. eson*, that (i) a change from Leu² to another aliphatic polar residue with a shorter side chain, such as Val, negatively influenced the activity of an AKH analog, whereas biological activity was almost restored to full activity when the stereoisomer Ile² was introduced, and (ii) replacement of the neutral Thr³ residue with another neutral amino acid, Asn³ resulted in very little bioactivity (2), thus suggesting the importance of the correct side chains at positions 2 and 3 in the ligand for interaction with the AKH receptor. A similar vital importance of these residues can be deduced from earlier studies on *in vitro* receptor activation in insects as well as in a crustacean AKH/RPCH system (4, 30). The aromatic amino acid residues at position 4 (Phe; this study) and position 8 (Trp; (2)) of AKHs are also essential, as was previously found in all other structure-activity and receptor studies of insects [for example: (24, 25, 30, 34)] and a crustacean (4). It is also not surprising then that all known naturally-occurring AKH ligands have an aromatic amino acid at position 4 (Phe or Tyr) and 8 (Trp) [see (35) review]. The replacement of a polar Thr⁵ with a slightly hydrophobic Ala⁵ in the AKH analog was very well-tolerated in the current study, as was the case in a crustacean system (4). In other insect systems, however, changes at position 5 of AKHs resulted in strong loss of activity probably due to no H-bonding of the missing hydroxylated side chain (24, 30). Residue 5 is supposed to be the cornerstone of a beta-turn commencing residues 5 to 8. Previous structure-activity studies with AKHs also revealed that residue 6 (Pro) is essential for activity in insect systems, whereas residue 7 (Asp, Ala) were shown to be not essential (24, 30). The lead peptide in this study, Lacol-AKH,



as all other lepidoptera-specific AKHs bar one (viz. Bommo-AKH; Table 1) do not possess a Pro⁶ residue; if it is introduced, no reduction in biological activity is measured in *H. eson* (2), suggesting that either a beta-turn is not necessary for the ligand conformation to bind to the receptor or that Ser⁶ is also able to form such a turn although it is generally known that Pro is a preferred residue for this formation [see (34)]. The simple Ala at position 6 cannot provide such conformation and activity is, hence, quite reduced in the current study. Residue 7, as in other systems (4, 24, 30) appears not to be participating in any H-bonding or other interaction with the AKH/RPCH receptor and, hence, an Ala⁷ analog has full activity in *H. eson* in the current study.

Lepidopteran AKHs and Their Cognate Receptors

It is well-known from such model insect species as locusts and tobacco hornworm moth that AKHs are released into the hemolymph upon flight episodes (36, 37) and, from locusts, it is also known that the three endogenous AKHs are co-localized in the same secretory granules in the corpora cardiaca (CC) and, hence, released simultaneously and in the same ratio as found stored (38). By analogy, it is assumed that all five endogenous AKHs of *H. eson* are released together upon flight and in the same ratio as shown to occur in the CC [i.e., Hipes-AKH-I: Hipes-AKH-II: Manse-AKH: Hipes-AKH-III: Manse-AKH-II as 46: 30: 19: 8: 1; see Figure 2 of (1)]. As shown previously (1),

all 5 AKHs are active and cause hyperlipaemia in the moth, thus the peptides must bind to a G-protein-coupled receptor (GPCR) to activate finally a lipase as reviewed previously (39). Do they all bind to the same receptor? Although the AKHR from a number of different insect species has been identified or predicted from genome sequencing projects [see, for example, (40, 41)], only one specific high-affinity AKHR has been found for each species, with the exception of some Diptera where various splice variants have been identified. The latter AKHR splice variants, however, do not have vastly different binding properties when examined experimentally (29, 42–44). Thus, it is postulated that there is only one AKHR for *H. eson* to which all five endogenous ligands bind. Although this receptor has not been cloned or data mined, there are other lepidopteran AKHRs characterized or genome-predicted. **Figure 1** illustrates the alignment of seven butterfly/moth AKHRs. The consensus sequence clearly shows how similar these receptors are, and this is not only true for the transmembrane regions but for the extracellular loop regions as well. The region with the most differences in the lepidopteran AKH receptors spans about 10 amino acids after the seventh transmembrane domain (**Figure 1**).

To date, all AKHs occurring in Lepidoptera are unique to this order and have not been found in any other organism outside of this order. In *M. sexta* two AKHs have been identified, the nonapeptide Manse-AKH (10) and the decapeptide Manse-AKH-II (12). Both of these AKHs are also synthesized in the CC of *H. eson* (1), while the other 3 *H. eson* AKHs are slight modifications of Manse-AKH (see **Table 1**). As the AKHR of *M. sexta* is known (45), it should be possible in future to model the interaction of the 5 endogenous AKHs of *H. eson* with the *M. sexta* receptor, using similar methods as with ligand-receptor interaction studies on the AKH system of a dipteran insect, *Anopheles gambiae* (18), and the crustacean, *Daphnia pulex* (46). Once a model has been produced, the current structure-activity data of this study can be validated, and such a model can hopefully be used to narrow the search for agonists or antagonists, including peptidomimetics in order to utilize the AKH/AKHR system as order-specific “green insecticide.” Of paramount importance to this concept of “green” insecticides is

the notion of specificity and discrimination of such compounds: thus, a pest insect should be harmed, but not a beneficial or mildly harmful insect. Thus, one of the next steps would be to do biological assays (or *in vitro* receptor-binding assays) to test the lepidopteran AKHs for cross-reactivity in non-pest insects. Such information would be critical for assessing whether there is any potential to further investigate the interaction of AKHs with the G protein-coupled receptor in lepidopterans to find a lead to an efficient “green” insecticide. The fact that the lepidopteran AKHs are unique to this order, may be an indication that the ligands are optimized for lepidopteran AKHRs, but this remains to be confirmed.

ETHICS STATEMENT

This study was carried out in accordance with relevant institutional and national guidelines and regulations concerning the use of animal subjects in scientific studies. It is a University of Cape Town and national policy that the use of Insects are exempted from animal ethics applications. Nevertheless, the insects were handled in a proper veterinary manner.

AUTHOR CONTRIBUTIONS

HM and GG designed the research, collected animals (eggs and larvae), and wrote the manuscript. GG reared the moths from egg to adult stage. HM performed the experiments and analyzed the data. Both authors listed have approved the manuscript for publication.

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Sensing Glucose in the Central Melanocortin Circuits of Rainbow Trout: A Morphological Study

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In mammals, glucosensing markers reside in brain areas known to play an important role in the control of food intake. The best characterized glucosensing mechanism is that dependent on glucokinase (GK) whose activation by increased levels of glucose leads in specific hypothalamic neurons to decreased or increased activity, ultimately leading to decreased food intake. In fish, evidence obtained in recent years suggested the presence of GK-like immunoreactive cells in different brain areas related to food intake control. However, it has not been established yet whether or not those neuronal populations having glucosensing capacity are the same that express the neuropeptides involved in the metabolic control of food intake. Therefore, we assessed through dual fluorescent *in situ* hybridization the possible expression of GK in the melanocortinergic neurons expressing proopiomelanocortin (POMC) or agouti-related protein (AGRP). POMC and AGRP expression localized exclusively in the rostral hypothalamus, in the ventral pole of the lateral tuberal nucleus, the homolog of the mammalian arcuate nucleus. Hypothalamic GK expression confined to the ependymal cells coating the ventral pole of the third ventricle but some expression level occurred in the AGRP neurons. GK expression seems to be absent in the hypothalamic POMC neurons. These results suggest that AGRP neurons might sense glucose directly through a mechanism involving GK. In contrast, POMC neurons would not directly respond to glucose through GK and would require presynaptic inputs to sense glucose. Ependymal cells could play a critical role relying glucose metabolic information to the central circuitry regulating food intake in fish, especially in POMC neurons.

Keywords: glucosensing, glucokinase (GK), AGRP, POMC, neuron, brain, fish

INTRODUCTION

Animals must know the energy quantity and essential nutrients available to trigger or block different energy-demanding physiological process. The detection of nutrient levels by the central nervous system (CNS) is essential for the regulation of energy balance and, by extension, for the animal survival. Accordingly, neuronal circuits regulating food intake have developed mechanisms to detect changes in amino acid, fatty acid, and glucose levels that, in turn, have profound effects on their own activity to undertake nutritional demands (1, 2).

In vertebrates, glucose is one of the main nutrients whose changes in levels are sensed through different mechanisms (3). In mammals, the best known glucosensing system involves the phosphorylation of glucose to glucose 6-phosphate (G6P) by glucokinase (GK) that is then metabolized to increase ATP/ADP ratio that subsequently lead to the closure of ATP-dependent inward rectified potassium channels (K_{ATP}^+) or Na^+/K^+_{ATP} (4). Channel closure induces, in turn, membrane depolarization and the successive Ca^{2+} entry into the cell via L-type voltage-dependent calcium channel that results finally into increased neuronal activity [reviewed in (2, 5)]. In addition, alternative glucosensing mechanisms dependent on sodium/glucose cotransporter 1 (SGLT-1), liver X receptor (LXR), or sweet taste receptors has been proposed (2, 5). Neurons sensing glucose are classified in glucose-excited (GE) and glucose-inhibited (GI) based on their response to extracellular glucose levels by increasing or decreasing their firing rates, respectively. Therefore, the activation of glucosensing systems in the CNS regulates the neuronal activity and provokes regulatory responses allowing the animal to control glycemia via control the activity of the autonomic nervous system (6, 7). At central level, one of these responses involves the neural regulation of food intake. Thus, hypo- and hyperglycemia are known to increase and decrease food intake, respectively (7, 8). In fish, available evidence support the presence of comparable glucosensing mechanisms, either dependent or independent on GK [reviewed in (9, 10)]. These mechanisms are probably involved in the regulation of food intake and counter-regulatory mechanisms since hypo- and hyper-glycaemic treatments elicited also comparable changes in food intake (11, 12).

In mammalian species, brain glucose-sensing neurons are located in diverse areas, but especially in hypothalamus and brain stem (13). In the hypothalamus, glucose-sensing neurons are mainly found in the arcuate nucleus and ventromedial and lateral nuclei. In the brain stem, glucosensing cells are located in the area postrema, nucleus of the solitary tract and the dorsal motor nucleus of the vagus (7). In fact, hypoglycemia activates the above hypothalamic and caudal neurons as recorded by c-fos activity (14). There is also evidence supporting that glial cells, particularly astrocytes and tanycytes, located in the lower part of the third ventricle, are also involved in glucose sensing and may control the activity of hypothalamic neurons (5, 15–17). The nature of these glucosensing hypothalamic neurons is controversial (13, 18). Most studies have identified glucosensing neurons in different central areas but the phenotype remains elusive (13, 19). The arcuate nucleus incorporates two key neuronal populations involved in the control of food intake and feeding behavior. The first population co-expresses neuropeptide Y (NPY) and agouti-related peptide (AGRP), and the second population co-expresses pro-opiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART) (1, 20, 21). Glucose regulates the activity of both populations (13) but the molecular mechanisms of glucosensing are still under debate.

Neural circuits and molecular mechanisms regulating feeding behavior are well-evolutionary conserved from fish to mammals (22). In fish, the melanocortin system is key in the regulation of feeding behavior (23–25). Thus, the intracerebroventricular

injection of melanocortin agonists inhibits food intake in fasted goldfish (26). On the contrary, central administration of specific competitive antagonists of the melanocortin 4 receptor (MC4R) promotes feeding in satiated animals (26). Fasting has no effect on hypothalamic POMC expression but sharply stimulates the expression of the MC4R inverse agonist AGRP (27) that depress the activity of the constitutively expressed MC4R (28). In addition, the overexpression of inverse agonist of MC4R in transgenic models promotes feeding and growth in zebrafish (25). Prior experiments in fish using rainbow trout as a model have extensively demonstrated glucosensing capacity of central areas as well as the regulation of hypothalamic expression of neuropeptides by glucose availability through mechanisms comparable in general to those of mammals [reviewed in (10, 22)]. We have also described the presence of GK-immunoreactive cells in brain areas such as hypothalamus (29) and telencephalon (30) putatively involved in the control of food intake. However, there is no direct evidence to date supporting that neurons expressing GK are the same than those expressing the neuropeptides involved in the metabolic control of food intake. Accordingly, in this study, we have assessed, for the first time in fish, the simultaneous expression of marker of glucosensing such as GK in the melanocortinergic neurons expressing AGRP or POMC that actively regulate energy balance.

MATERIALS AND METHODS

Fish

Female juvenile rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) ranging 80–100 g body weight were obtained from a local fish farm (A Estrada, Spain). Fish were maintained for 1 month in 100 liter tanks under laboratory conditions, 12L:12D photoperiod and dechlorinated tap water at 15°C. Fish weight was 98 ± 2 g. Fish were fed once daily (10:00 h) to satiety with commercial dry fish pellets (Dibaq-Diproteg SA, Spain; proximate food analysis was 48% crude protein, 14% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

Synthesis of Riboprobes for ISH

Total RNA was extracted from rainbow trout hypothalamus using Trizol reagent (Life Technologies, Grand Island, NY, USA) and subsequently treated with RQ1-DNAse (Promega, Madison, WI, USA). A 2 µg sample of total RNA was reverse transcribed using Superscript II reverse transcriptase (Promega) and random hexamers (Promega) in approximately 20 µl volume. The cDNA obtained was subsequently used as template for PCR amplification with Taq DNA polymerase (Invitrogen) using specific primers based on AgRP1, GK, and POMCa1. The sequences were retrieved from GenBank sequence database (NCBI) with accession numbers NM001146677, AF053331, and NM001124718, respectively (Table 1). PCR fragments were separated onto 1% agarose gel, purified using EZNA Purification

TABLE 1 | Primer sequences used for generate templates for synthesis of riboprobes for *in situ* hybridization.

	Forward primer	Reverse primer	Size (bp)	GenBank accession number
AGRP1	AGCAGTCCTGTCTGGGTAACACG	ATCCATCTACCCCTATCGGGGAAAA	371	NM_001146677
GK	GCACGGCTGAGATGCTCTTTG	GCCTTGAACCCCTTTGGTCCAG	170	AF053331
POMCa1	TCAGAAACCACTGCTCAGC	GCTAAGATCATCGCTATGTCAGAGGACAG	257	NM_001124718

AgRP, Agouti-related peptide; GK, glucokinase; POMCa1, pro-opio melanocortin a1.

Kit (Omega Bio-tek), and subsequently were cloned into pGEM-T easy vector (Promega). Sense and antisense probes were synthesized using SP6/T7 RNA polymerase (Promega) using digoxigenin (DIG)-labeled UTPs or Fluorescein-labeled UTPs (Roche Diagnostic). The probes were treated with RQ1-DNase-RNase free (Promega) for 15 min at 37°C to remove the DNA template. Finally, the probes were purified using Micro Bio-Spin Chromatography Columns (BioRad) and quantified in a Thermo Scientific Nanodrop 2000c spectrophotometer.

Tissue Preparation

Following 1 month acclimation period, 24-h fasted fish were anesthetized with 2-phenoxyethanol (Sigma, St. Louis, MO, USA; 0.2% v/v), perfused with physiological saline solution (NaCl 0.65%) and subsequently with the same volume of fixative containing paraformaldehyde (PAF, 4%) in phosphate buffer (PB, 0.1 M, pH 7.4). Freshly obtained tissues were removed, post-fixed overnight (O/N) in the same fixative at 4°C, dehydrated and embedded in Paraplast (Sigma). Transverse serial sections were cut at 7 µm using a rotary microtome and sections were mounted on 3-aminopropyltriethoxysilane (TESPA)-treated slides and then air-dried at room temperature (RT) O/N.

In situ Hybridization

Chromogenic ISH was used to detect the location of AgRP1 and POMC1a expression independently, and dual fluorescent *in situ* hybridization (FISH) was used on gene pairs (GK/AgRP1 and GK/POMC1a). Hybridizations with digoxigenin or fluorescein labeled sense or antisense riboprobes were revealed with either colorimetric 4-Nitro Blue Tetrazolium Chloride (NBT) and 5-Bromo-4-chloro-3-indoyl-phosphate (BCIP) staining (Roche Diagnostic), or fluorescent TSA (TSA PLUS Fluorescein kit, PerkinElmer) and Fast Red staining (Roche Diagnostic), respectively. ISH was performed as described by Agulleiro et al. (31), with some modifications as described below. Sections were deparaffinized, re-hydrated, post-fixed in PAF 4% for 20 min, and treated with Proteinase-K solution (20 µg/ml in 50 mM Tris-HCl, 5 mM EDTA at pH 8) for 8 min at RT. Slides were next washed in PB, post-fixed again in PAF for 5 min, subsequently rinsed in sterile water, and acetylated in a triethanolamine (0.1 M, pH 8)/acetic anhydride solution, before incubation with hybridization solution. Anti-sense or sense RNA probes were diluted in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), 10% dextran sulfate (Sigma), and 1X Denhardt's solution (Sigma). Subsequently, 80–100 µl hybridization solution were added to each pretreated slide, which were coverslipped and incubated in

a humidified chamber at 65°C O/N. Coverslips were removed by incubating slides into a solution containing 5X standard saline citrate buffer (SSC, contains 150 mM NaCl, 15 mM sodium citrate at pH 7) for 30 min at 55°C. The slides were then rinsed in 2X SSC and 50% formamide for 15 min at 65°C and three times immersed into NTE buffer (500 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.5) for 5 min at 37°C. After ribonuclease treatment (40 µg/ml ribonuclease in NTE) for 15 min at 37°C, slides were rinsed in NTE buffer for 5 min at 37°C, once in 2X SSC and 50% formamide for 10 min at 65°C, once in 2X SSC for 10 min at RT and once in 0.1X SSC for 10 min at RT. Before incubated with antibody, slides were rinsed twice times in B1 (contains 100 mM Tris, 150 mM NaCl, pH 7.5) for 10 min at RT and washed with 2% blocking buffer (Roche Diagnostic) during 1 h. Slides were incubated at 4°C O/N with primary antibody 1:500 anti-digoxigenin FAB fragments conjugated to alkaline phosphatase (AP) antibody (Roche Diagnostic) in 2% blocking buffer. Redundant antibody was removed by washing twice in B2 (100 mM Tris, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) for 10 min at RT before incubated with chromogen substrates NBT/BCIP (Roche Diagnostic) to develop the staining. Sections were mounted with Mount quick acquoso (Bio-Optica) and visualized with Olympus BX41.

For dual fluorescence detection, slides were incubated O/N at 4°C in darkness with 1:100 anti-fluorescein FAB fragments conjugated to horseradish peroxidase (POD) antibody (Roche Diagnostic) in 2% blocking buffer. The next day, slides were washed twice in B1 with 0.1% triton for 10 min at RT, incubated for 20 min with tyramide-biotine (Fluorescein kit, PerkinElmer) in amplification diluent at RT, washed for 15 min at RT in Solution A with 0.1% triton (contains 100 mM Tris, 100 mM NaCl, pH 9.5), and incubated O/N at RT with Streptavidin (SA)—Alexa 488 (Life Technologies). Finally, slides were washed several times in PBS or PBS with 0.1% triton and subsequently developed with Fast Red staining or tyramide signal amplification (TSA) PLUS Fluorescein Kit. Sections were mounting with Prolong Gold antifade reagent with Dapi (Invitrogen) and visualized with Olympus BX41. Eight to ten sections of the target area (tuberal hypothalamus) from four different brains were used for double ISH.

RESULTS

The *in situ* hybridization with the sense probes never generated specific signals in the trout brain (data not shown). Both POMC1a (**Figure 1C₃**) and AGRP1 (**Figure 1D₃**) localized exclusively in the rostral region of the ventral hypothalamus

(Hv) also called ventral region of the lateral tuberal nucleus (NLTV). GK expression occurred in several brain regions such as hypothalamus, telencephalon, hindbrain, etc. Since neuropeptides localized exclusively in hypothalamus, we only described GK presence in hypothalamus. Thus, hypothalamic expression of GK was mostly associated to the ependymal cells that coat the wall of the third ventricle. GK-expressing ependymal cells localized mainly in the ventral region of the tuberal hypothalamus (**Figures 1C₂,D₂**). These cells imbricated between neurons coating the rostral region of the ventricle where both POMC and AGRP neurons are located. A higher magnification showed that GK did not express, or expressed at undetectable levels, in the hypothalamic POMC1a neurons (**Figure 1E₁**). In contrast, hypothalamic AGRP1 neurons co-expressed GK (**Figure 1E₂**).

DISCUSSION

Central glucosensing has been extensively studied in mammalian species and localized in key hypothalamic and caudal regions involved in the regulation of energy balance (6). However, the phenotypic characterization of central glucosensing neurons remains controversial and elusive (13). In fish, previous studies showed that hypothalamic mRNA abundance of POMC and AGRP respond to peripheral manipulation of systemic glucose (33–36). Furthermore, both types of neurons are involved in the regulation of energy balance in fish (25–27). Accordingly, hyper- or hypoglycaemic treatments inhibit or stimulate food intake in rainbow trout, respectively (11, 12). Our specific question was whether neurons that regulate the energy homeostasis, and particularly the melanocortinergic neurons, are able to sense glucose directly using GK system. To address this question, we evaluate the potential GK expression in both POMC and AGRP central neurons. Thanks to the low affinity for glucose, GK activity changes proportionally with glucose availability and it is thus considered to be a critical marker to predict glucosensing neuronal populations (6, 13) though not all glucosensing neurons express GK (13, 37).

POMC1 and AGRP1 neurons localized exclusively in the ventral pole of the rostral lateral tuberal nucleus of the trout brain. The exclusive expression of POMC and AGRP in the ventral hypothalamus has been also reported in other fish species like goldfish (27), sea bass (38) and zebrafish (39). In the brain of tetrapods, AGRP expressed only in the neurons of the arcuate nucleus but POMC is expressed also in the caudal brain, particularly in the nucleus of the solitary tract (NTS) (40, 41). Based on the coincident phenotype of the neurons, the lateral tuberal nucleus has been proposed to be the homolog of the arcuate nucleus in fish. In mammals, a very high percentage of AGRP neurons co-express NPY (42) and regulate the activity of hypothalamic POMC neurons thanks to the γ -aminobutyric acid (GABA) projections (43, 44). Arcuate POMC neurons also produce CART but hypothalamus is not the only brain area producing this neuropeptide (45). Therefore, these antagonistic melanocortinergic populations, the orexigenic AGRP neurons and the anorexigenic POMC-expressing neurons,

integrate metabolic, and nutrient information to regulate food intake. Recent experiments in fish suggest that the phenotype of AGRP neurons is a derived characteristic of mammalian brain since fish AGRP neurons does not produce NPY, however both zebrafish AGRP and NPY neurons are GABAergic and respond to fasting by increasing neuropeptide gene expression (46).

In mammals, orexigenic AGRP neurons are considered as GI neurons whereas POMC neurons seem to be GE neurons (18). In our hands, POMC1a neurons in the tuberal hypothalamus of rainbow trout do not exhibit GK-mediated glucosensing capacity since GK expression was under detection levels of the highly sensitive TSA technique (47).

Low expression levels of GK in the central nervous system has challenged the assessment of GK role in neuronal glucose sensing in mammalian species (48) and, according to the low level of GK expression detected in the trout hypothalamus, fish do not seem an exception. Therefore, data suggests that trout hypothalamic POMC neurons require presynaptic inputs to sense glucose. Obviously, trout POMC neurons respond to glucose since mRNA abundance of POMC usually increased in hypothalamus in response to a rise in glucose levels (33–36), but probably require presynaptic inputs from upstream neuronal cells. Rainbow trout were fasted for 24 h therefore we cannot rule out that fasting induced a decrease in plasma glucose that subsequently reduced differentially GK expression in POMC but no in AGRP neurons. However, short-term fasting in poikilotherms, including rainbow trout has no severe effects on glycemia (49). Studies using mice hypothalamic POMC dissociated neurons expressing GFP showed that glucose was unable to promote an increase in $[Ca^{2+}]_i$ thus supporting the inefficiency of POMC neurons to directly detect changes in glucose levels (13). The glucose-induced modulation of frequency of excitatory postsynaptic currents onto POMC neurons also supports the requirements of presynaptic inputs of POMC neurons for sensing glucose (50).

How do POMC cells sense glucose then? One possibility would be that these cells respond to glucose through GK-independent mechanisms (2, 5). Another possibility, more likely, is that glucosensing might occur not in neurons alone but in a glucose-sensing unit including POMC neurons and glial cells as demonstrated in mammals (51, 52). Thus, according to our immunological data, we propose that ependymal cells, probably tanycytes, which coat the ventral pole of the third ventricle, send projections contacting with the closely located POMC neurons to convey information about glucose availability in the cerebrospinal fluid (CSF). In fact, tanycytes generate an interphase between CSF and neuronal nuclei that allows the exchange of molecules. Rat tanycytes express GLUT2 and GK and the functionality of the enzyme has been recorded thus suggesting the capability of tanycytes for glucose sensing (53). The adenovirus-mediated suppression of GK expression in the hypothalamic tanycytes after injection in the third ventricle increases feeding levels in rat but also depress hypothalamic POMC expression at the same time that preclude the AGRP neurons response to glucose administration (17). However, the expected response to glucose administration was to stimulate and inhibit POMC and AGRP expression, respectively (17). Our data suggest that trout hypothalamic AGRP1 neurons co-express GK

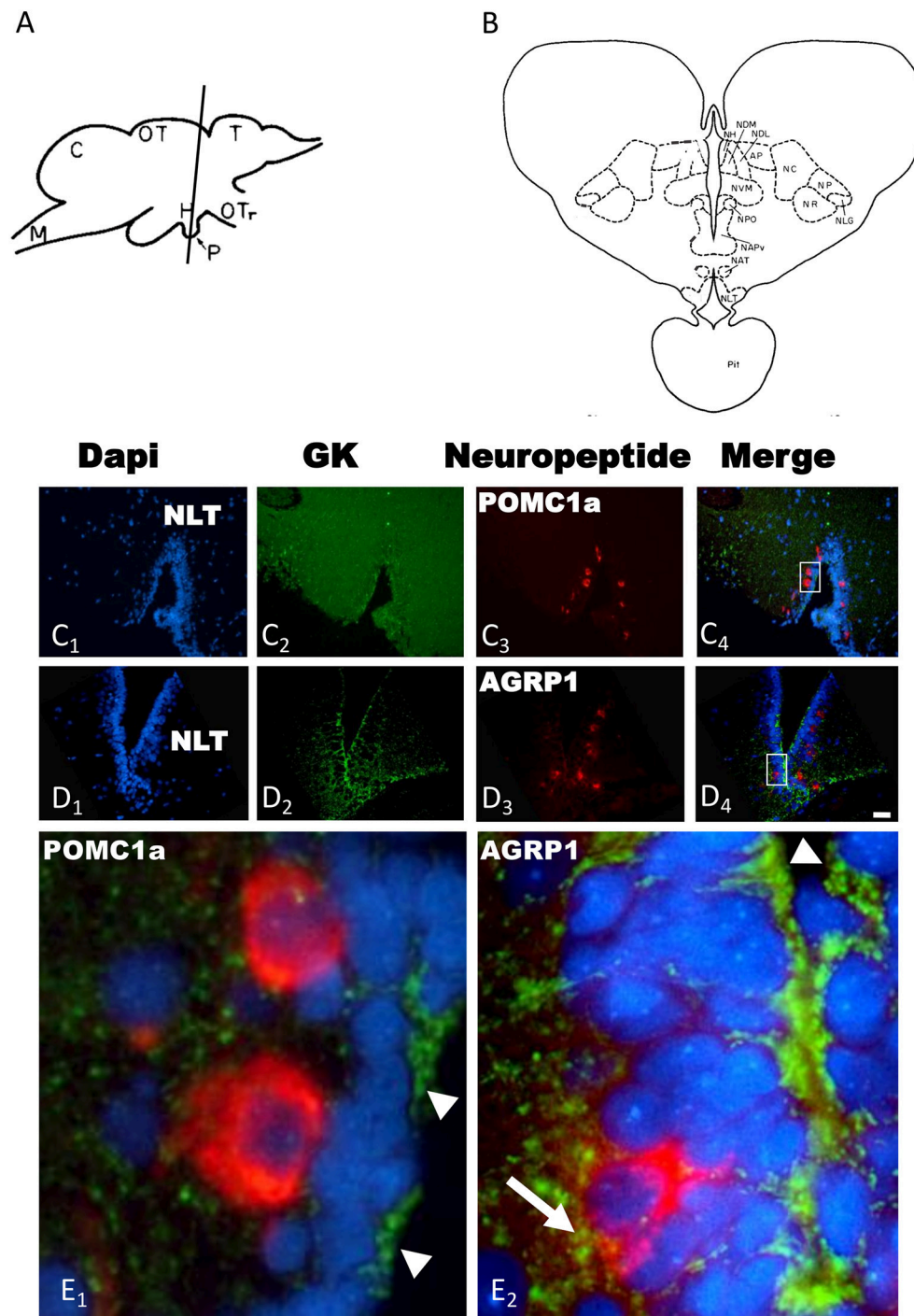


FIGURE 1 | (A) Lateral view of the rainbow trout brain showing the levels of sections in **(C,D)** [adapted from Billard and Peter (32)]. **(B)** Schematic drawing of a transverse section showing the cytoarchitecture of the rostral trout hypothalamus at the level of **(C,D)** [adapted from Billard and Peter (32)]. Transverse sections of rainbow trout rostral hypothalamus showing GK **(C₂,D₂)**, POMC1a **(C₃)** and AGRP1 **(D₃)** mRNA expression by *in situ* hybridization. Slides were incubated with Dapi **(C₁,D₁)** to provide nuclear counterstaining of the same sections. Merge images is provided is **(C₄,D₄)**. Insets in **(C₄,D₄)** show magnification of GK/POMC1a **(E₁)** or GK/AGRP1a **(E₂)** colocalization. Arrowheads indicate GK mRNA expression in the ependymal cells **(E₁,E₂)**, arrow indicate potential coexpression of GK and AGRP1 mRNAs **(E₂)**. AP, pretectal area; C, cerebellum; H, hypothalamus; M, medulla; NAPv, anterior periventricular nucleus; NAT, anterior tuberal nucleus; NC, cortical nucleus; NH, habenular nucleus; NDM, dorsomedial nucleus of the thalamus; NDNL, dorsolateral nucleus of the thalamus; NLG, lateral geniculate nucleus; NLT, lateral tuberal nucleus; NP, preectal nucleus; NPO, preoptic nucleus; NR, nucleus rotundus; NVM, ventromedial nucleus of the thalamus; OT, optic tectum; OTTr, optic tract; Pit, pituitary; T, telencephalon. Scale bar 100 μ m for **(C,D)** and 20 μ m for **(E)**.

indicating that they are potentially true glucosensing neurons based on this mechanism. To our knowledge, there is no data regarding GK expression on AGRP neurons but these orexigenic neurons respond to changes in glucose availability (17). Altered GK activity in the arcuate nucleus regulates the neuronal NPY secretion suggesting that GK modulates the activity of the NPY/AGRP neurons in the arcuate nucleus (54). In addition, GK expression is present in NPY arcuate neurons in rat (55). Since 95% of NPY neurons produce AGRP (42), it is conceivable that AGRP and GK co-expressed in the same neurons. GK co-expression in AGRP neurons and the putative real glucose sensing does not mean that the information coming from tanycytes about glucose was superfluous since suppression of GK in this ependymal cells also abolishes glucose response of AGRP neurons (see above).

In summary, our data support that POMC and AGRP neurons in rainbow trout brain are exclusively located in the ventral region of the lateral tuberal nucleus, i.e., the fish homolog to the mammalian arcuate nucleus. In this area, the main marker of glucosensing (GK) is expressed in the AGRP but not in POMC neurons. Therefore, we suggest that AGRP neurons respond directly to changes in glucose through GK-based glucosensing. In contrast, POMC neurons would not directly respond to glucose through GK and rather would require presynaptic inputs to sense glucose. Ependymal cells, particularly tanycytes coating the ventral region of the third ventricle, could play a key role in sensing CSF glucose and conveying nutrient information to central hypothalamic structures regulating energy

balance, especially POMC neurons. Our results provide original information about specific hypothalamic nuclei in which nutrient sensors may interact with neuropeptides involved in the regulation of food intake.

ETHICS STATEMENT

The experiments described comply with the Guidelines of the European Union Council (2010/63/UE), and of the Spanish Government (RD 53/2013) for the use of animals in research, and were approved by the Ethics Committee of the Universidade de Vigo.

AUTHOR CONTRIBUTIONS

JS and JC-R conceived and designed research. CO-R, RÁ-O, ES, and AR performed experiments. CO-R, RÁ-O, and JC-R analyzed data. CO-R, JS, and JC-R interpreted results. CO-R and JC-R prepared figures. All authors revised and edited the drafted manuscript. All authors approved the final version of manuscript.

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Diurnal Profiles of N-Acylethanolamines in Goldfish Brain and Gastrointestinal Tract: Possible Role of Feeding

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N-acylethanolamines (NAEs) are a family of endogenous lipid signaling molecules that are involved in regulation of energy homeostasis in vertebrates with a putative role on circadian system. The aim of this work was to study the existence of daily fluctuations in components of NAEs system and their possible dependence on food intake. Specifically, we analyzed the content of oleoylethanolamide (OEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA), their precursors (NAPEs), as well as the expression of *nape-pld* (NAEs synthesis enzyme), *faah* (NAEs degradation enzyme), and *pparα* (NAEs receptor) in gastrointestinal and brain tissues of goldfish (*Carassius auratus*) throughout a 24-h cycle. Daily profiles of *bmal1a* and *rev-erbα* expression in gastrointestinal tissues were also quantified because these clock genes are also involved in lipid metabolism, are PPAR-targets in mammals, and could be a link between NAEs and circadian system in fish. Gastrointestinal levels of NAEs exhibited daily fluctuations, with a pronounced and rapid postprandial increase, the increment being likely caused by food intake as it is not present in fasted animals. Such periprandial differences were not found in brain, supporting that NAEs mobilization occurs in a tissue-specific manner and suggesting that these three NAEs could be acting as peripheral satiety signals. The abundance of *pparα* mRNA displayed a daily rhythm in the intestine and the liver, suggesting a possible rhythmicity in the NAEs functionality. The increment of *pparα* expression during the rest phase can be related with its role stimulating lipid catabolism to obtain energy during the fasting state of the animals. In addition, the clock genes *bmal1a* and *rev-erbα* also showed daily rhythms, with a *bmal1a* increment after feeding, supporting its role as a lipogenic factor. In summary, our data show the existence of all components of NAEs system in fish (OEA, PEA, SEA, precursors, synthesis and degradation enzymes, and the receptor PPARα), supporting the involvement of NAEs as peripheral satiety signals.

Keywords: OEA, PEA, SEA, acylethanolamides, PPARα, food intake, rhythms, fish

INTRODUCTION

Acylethanolamides or *N*-acylethanolamines (NAEs) are a family of endogenous bioactive lipid molecules present in animal, plant, as well as in prokaryotic cells (Hansen and Vana, 2018), which play a key role in feeding regulation in vertebrates (Borrelli and Izzo, 2009; Hansen, 2014; Kleberg et al., 2014; Romano et al., 2015). They consist on a fatty acid linked by an amide bound to an ethanolamine and are classified based on the number of carbons and degree of saturation of their acyl chain. Major NAEs in mammalian tissues comprise oleoylethanolamide (*N*-oleoylethanolamine, OEA), palmitoylethanolamide (*N*-palmitoylethanolamine, PEA) and stearoylethanolamide (*N*-stearoylethanolamine, SEA), and several other quantitative minor species including anandamide (*N*-arachidonylethanolamine, AEA; Tsuboi et al., 2013).

Endogenous levels of NAEs are mainly regulated by enzymes responsible for their formation and degradation. Biosynthesis of NAEs is an “on demand” process with two major steps, the formation of *N*-acylphosphatidylethanolamines (NAPEs) from their phospholipid precursors through a Ca^{2+} -dependent *N*-acyltransferase (NAT) activity and the conversion of NAPEs to NAEs via several pathways (Tsuboi et al., 2013; Rahman et al., 2014). In animals, most NAEs result from a NAPE hydrolysis catalyzed in a single enzymatic step by a specific membrane-bound phospholipase D, namely NAPE-PLD, although other multi-step pathways have been described (Borrelli and Izzo, 2009; Tsuboi et al., 2013; Ueda et al., 2013; Inoue et al., 2017). The generated NAEs are rapidly catabolized by fatty acid amide hydrolase (FAAH) to their corresponding free fatty acids and ethanolamine. FAAH is localized in endoplasmic reticulum and functions as a general inactivating enzyme for all NAEs in mammals, with highest activity in liver, small intestine and brain (Ueda et al., 2013; Kleberg et al., 2014; Hansen and Vana, 2018). Moreover, a NAE-hydrolyzing acid amidase (NAAA) localized in lysosomes contributes to NAEs catabolism, and preferentially hydrolyzes PEA over the other NAEs (Ueda et al., 2013).

These NAEs can interact with different receptors which are involved in many physiological processes, playing an important role in the regulation of energy homeostasis (Borrelli and Izzo, 2009; Hansen, 2014; Kleberg et al., 2014; Romano et al., 2015). The most studied NAE in mammals is OEA, which acts as an anorexigenic signal and promotes fat catabolism (Bowen et al., 2017; Sihag and Jones, 2018). In goldfish, OEA also reduces food intake and body weight, and is involved in lipid and glucose metabolism (Tinoco et al., 2014; Gómez-Boronat et al., 2016). A food intake decrease was also observed after exogenous administration of PEA or SEA in the only vertebrates studied to date, rats and mice (Rodríguez de Fonseca et al., 2001; Terrazzino et al., 2004). These functions seem to be mediated via activation of the transcription factor peroxisome proliferator-activated receptor alpha ($\text{PPAR}\alpha$), although OEA and PEA also bind other receptors, such as G protein-coupled receptor GPR119 and a transient receptor potential vanilloid type 1 (TRPV1; Hansen, 2010; Kleberg et al., 2014).

Fed and fasted states modulate NAEs production in vertebrates. Intestinal levels of OEA are decreased by food deprivation and increased upon refeeding in rodents (Rodríguez de Fonseca et al., 2001; Piomelli, 2013; Bowen et al., 2017) and goldfish (Tinoco et al., 2014). A similar feeding-induced PEA mobilization in small intestine, without modifications in SEA, has also been described in rats (Petersen et al., 2006); although another study reported that food deprivation does not change the duodenal or jejunal content of both PEA and SEA in this species (Fu et al., 2007). A postprandial OEA, PEA, and SEA increment has also been described in small intestine of Burmese python (*Python molurus*), the only reptile species so far studied (Astarita et al., 2006). These postprandial variations seem to occur in a tissue-specific manner, since changes in OEA and PEA levels in response to feeding were not observed in other peripheral tissues and brain structures in rats (Fu et al., 2007; Izzo et al., 2010).

Because feeding is usually a rhythmic behavior, the existence of daily fluctuations in some feeding regulators has been reported (Bechtold and Loudon, 2013; Isorna et al., 2017). However, only few studies have investigated the daily rhythmicity in the NAEs system and its possible interaction with the circadian system, with no consistent results. Diurnal fluctuations of endogenous levels of NAEs have been found in brain: while in the cerebrospinal fluid, OEA and PEA concentrations increased during the light-on period; in pons, hippocampus and hypothalamus, these NAEs increased during the dark phase in rat (Murillo-Rodríguez et al., 2006). However, OEA levels in various brain regions of mice did not change between 11:30 a.m. and 11:30 p.m. (Guijarro et al., 2010). In gastrointestinal tissues, OEA levels display diurnal fluctuations in rodents, being higher during the daytime, when animals are satiated, and lower during the night, when they are awaked and actively feeding (Fu et al., 2003; LoVerme et al., 2005; Guijarro et al., 2010). No differences were found in other components of NAEs system, such as PEA, NAPEs and activity of the enzymes NAPE-PLD and FAAH at the midpoint of the light and dark phase in mice jejunum (Guijarro et al., 2010). FAAH activity was also similar in various brain regions, with a decline only in cerebellum (Glaser and Kaczocha, 2009), striatum, and hippocampus (Valenti et al., 2004) at midnight. Although FAAH K_m and V_{max} are affected by time of the day in some brain regions of mice, none of the data support a primary role for FAAH in the circadian regulation of the brain NAEs (Liedhegner et al., 2014). To date, the clearest link between NAEs and the circadian system is the fact that their main receptor, $\text{PPAR}\alpha$, directly regulates the transcription of *BMAL1* and *REV-ERB α* , two core clock genes, which possess a PPAR response element (PPRE) in their promoters (Charoensuksai and Xu, 2010; Chen and Yang, 2014). In addition, $\text{PPAR}\alpha$ is also a direct target gene of the heterodimer *CLOCK/BMAL1*, a key component of the molecular clock which drive rhythms in target genes known as clock-controlled genes. Thus, $\text{PPAR}\alpha$ is considered an output gene and shows daily rhythmic expression in a variety of tissues in mammals (Yang et al., 2006; Chen et al., 2010). This interaction between OEA and the circadian system has also been suggested in fish, since hepatic expression of *bm11a* increases after OEA treatment

in goldfish (Gómez-Boronat et al., 2016) and the expression of *ppara* is rhythmic in gilthead sea bream (*Sparus auratus*; Paredes et al., 2014) and zebrafish (*Danio rerio*; Paredes et al., 2015). Apart from these data, there is no evidence in fish on the possible daily rhythmicity in OEA and other NAEs.

The aim of this work was to study the existence of daily fluctuations in the NAEs system components and their possible regulation by food intake in fish. Specifically, we quantified the content of OEA, PEA, SEA, and their precursors (NAPEs) in central and peripheral tissues of goldfish throughout a 24-h cycle. The daily pattern of expression in gastrointestinal tissues of *nape-pld* (NAEs synthesis enzyme), *faah* (NAEs degradation enzyme), and *ppara* (NAEs receptor) was also measured. Moreover, the enzymatic activity of FAAH was quantified in anterior intestine and hypothalamus. Finally, the daily rhythmic expression of the clock genes *bmal1a* and *rev-erba* was analyzed in gastrointestinal tissues to investigate a possible interaction between NAEs and the circadian system.

MATERIALS AND METHODS

Animals and Housing

Goldfish with a body mass (bm) of 23 ± 6 g were obtained from a local commercial supplier (ICA, Madrid, Spain). Fish were housed in 60 l aquaria with filtered fresh water ($21 \pm 1^\circ\text{C}$) and continuous aeration and maintained under a 12 h light: 12 h darkness (12L:12D) photoperiod (lights on at 8 a.m., considered as *zeitgeber* time 0-ZT0). The aquaria walls were covered with opaque paper to minimize external interferences during the experiment. Fish were fed (1% bm) once daily at 10 a.m. (ZT2) with commercial dry pellets (32.1% crude protein, 5% crude fat, 1.9% crude fiber, 6.8% crude ash, 5.1% water, and the rest nitrogen free extract; Sera Pond, Heinsberg, Germany). Animals were maintained under these conditions for 1 month. The experiments described comply with the Guidelines of the European Union Council (UE63/2010) and the Spanish Government (RD53/2013) for the use of animals in research and were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14).

Experimental Design

Goldfish ($n = 49$) were sampled throughout a 24-h cycle each 4 h ($n = 7$ per sampling point; ZT3, ZT7, ZT11, ZT15, ZT19, ZT23, and ZT3 of next day -ZT3b). Food was offered as scheduled (ZT2) the first day of the experiment, but not the second day before last sampling point (ZT3b). Thus, the possible effect of food intake on the NAEs system was tested by comparing fish sampled at the same time but 1-h postprandial (ZT3) or 25-h fasting (ZT3b). In each sampling point, animals were sacrificed by anesthetic overdose (tricaine methanesulfonate, MS-222, 0.28 g/l; Sigma-Aldrich, Madrid, Spain) followed by spinal cord section. Tissue were quickly dissected: initial and final segments of intestinal bulb, anterior intestine in two sections, liver in three aliquots, and central tissues (hypothalamus and telencephalon)

as a whole. All samples were rapidly frozen in liquid nitrogen and immediately stored at -80°C until analysis.

Determination of Tissue Content of NAEs and NAPEs

A longitudinal half of the final segment of the intestinal bulb, a transversal half of the initial segment of the anterior intestine, and one liver aliquot were weighed (20–30 mg) as well as a longitudinal half of both hypothalamus and telencephalon (5–10 mg). Samples were homogenized in 1 ml of methanol (Thermo Fisher Scientific, Milan, Italy) containing the following deuterated internal standards (IS): OEA- d_4 (100 nM), PEA- d_4 (100 nM), SEA- d_3 (100 nM), and C17:0 NAPE (25 nM) (Cayman Chemical, Ann Arbor, MI, United States). Then, this solution was mixed with 2 ν of chloroform (Thermo Fisher Scientific) and 1 ν of water. Organic phase was collected, dried under nitrogen atmosphere, and fractioned by open-bed silica gel column chromatography, as previously described (Cadas et al., 1997; Tinoco et al., 2014). Briefly, the lipid extracts were reconstituted in chloroform and loaded onto small columns packed with Silica Gel G (60 Å 230–400 Mesh ASTM; Whatman, Clifton, NJ, United States). NAEs and NAPEs were eluted with a methanol:chloroform solution (1:9 and 1:1, respectively). Both eluates were again dried under nitrogen atmosphere and, subsequently, NAEs were reconstituted in 75 μl and NAPEs in 100 μl of methanol:chloroform (9:1). Samples were then analyzed by UPLC-MS/MS on a Xevo-TQ triple quadrupole mass spectrometer coupled with an UPLC (ultra-performance liquid chromatography) system (Waters Inc., Milford, PA, United States). NAEs and its deuterated analogs were loaded on a reversed phase BEH C18 column (50×2.1 mm inner diameter, 1.7 μm particle size, maintained at 45°C ; Waters Inc.) operated at a constant flow rate of 0.5 ml/min. The mobile phase consisted of 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. A step gradient program was developed for the best separation of all metabolites: 0–0.5 min 20% B and 0.5–3.0 min 100% B. The column was then reconditioned to 20% B for 0.5 min. The total run time for analysis was 3.5 min and the injection volume 5 μl . For analysis of NAPEs of PEA and SEA and their deuterated analogs, a reversed phase T3 column (50×2.1 mm inner diameter, 1.8 μm particle size, maintained at 50°C ; Waters Inc.) was used with a constant flow rate of 0.4 ml/min. The mobile phase consisted of 10 mM ammonium formate in acetonitrile:water (60:40) as solvent A and 10 mM ammonium formate in acetonitrile:isopropanol (10:90) as solvent B. A step gradient program was developed for the best separation of all metabolites: 0–0.5 min 50% B, 0.5–3.5 min 50 to 100% B, 3.5–4.5 min 100% B, and 4.5–5.0 min 100 to 50% B. The column was then reconditioned to 50% B for 1 min. The total run time for analysis was 6 min and the injection volume 5 μl . Lastly, conditions for analysis of NAPEs of OEA were the same as above for the other NAPEs with little modifications: constant flow rate of 0.35 ml/min; a step gradient of 0–0.5 min 30% B, 0.5–6.0 min 30 to 100% B, 6.0–7.0 min 100% B, 7.0–7.1 min 100 to 50% B, and reconditioned column to 30% B for 1.9 min; and total run time for analysis of 9 min. For both NAEs and NAPEs,

the mass spectrometer was operated in the positive ESI mode, the capillary voltage was set at 3 kV, the cone voltage was set at 20 V for all transitions, and analytes were quantified by multiple reactions monitoring (MRM). The complete panel of source parameters and MRM transitions are reported in the datasheet of the Supplementary Material (**Supplementary Tables S1, S2**). The source temperature was set to 120°C. Desolvation gas and cone gas (N₂) flows were set to 800 and 50 l/h, respectively. Desolvation temperature was set to 450°C. Data were acquired by MassLynx software and quantified by TargetLynx software. Calibration curves (0.1 to 100 nM range for all compounds) were constructed by plotting the analyte to IS peak areas ratio versus the corresponding analyte concentration using weighted (1/ $\times$) least square regression analysis.

Determination of FAAH Activity

The initial segment of the anterior intestine (the other transversal half) and the other longitudinal half of hypothalamus were weighed, homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) containing 0.32 M sucrose, and centrifuged at 1000 $\times$ g for 10 min at 4°C. Supernatants were collected and protein concentrations determined using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, United States). To measure FAAH activity, 0.5 ml of Tris-HCl buffer (50 mM, pH 7.4) containing fatty acid-free bovine serum albumin (0.05%), tissue homogenates (50 μ g of protein), 10 μ M AEA and AEA-(ethanolamine-³H) (20,000 cpm, specific activity 60 Ci/mmol; American Radiolabeled Chemicals, St Louis, MO, United States) were incubated at 37°C for 30 min. Reactions were stopped with 1 ml methanol:chloroform (1:1), centrifuged at 1400 $\times$ g for 10 min at 4°C, and radioactivity was measured in the aqueous phase by liquid scintillation counting in MicroBeta LumiJET system (Perkin Elmer Inc., Waltham, MA, United States).

Gene Expression Analysis

Total RNA from the initial segment of the intestinal bulb (3 mm), the distal segment of the anterior intestine (5 mm), and the other liver aliquot was isolated using TRI[®] Reagent (Sigma-Aldrich) and treated with RQ1 RNase-Free DNase (Promega, Madison, United States) according to the manufacturer's instructions. Then, an aliquot of total RNA (0.1 μ g of intestinal bulb and anterior intestine, or 0.3 μ g of liver) was reverse transcribed into cDNA in a 25 μ l reaction volume using random primers (Invitrogen, Carlsbad, United States), RNase inhibitor (Promega) and SuperScript II Reverse Transcriptase (Invitrogen). The reverse transcription reaction conditions consisted of an initial step at 25°C for 10 min, an extension at 42°C for 50 min, and a denaturalization step at 70°C for 15 min. Real-Time quantitative PCRs (RT-qPCRs) were carried out by duplicate in a CFX96[™] Real-Time System (Bio-Rad Laboratories, Hercules, United States), using iTaq[™] Universal SYBR[®] Green Supermix (Bio-Rad Laboratories) in a 96-well plate loaded with 1 μ l of cDNA and a final concentration of 0.5 μ M of each forward and reverse primers in a final volume of 10 μ l. Each PCR run also included a 4-point serial standard curve, non-retrotranscribed RNA (as positive control) and water (as negative control). The RT-qPCR cycling conditions consisted of an initial denaturation

TABLE 1 | Accession numbers and primers sequences of the genes employed in the RT-qPCR assays.

Gene	Accession number	Primer sequence 5' $\rightarrow$ 3'	Product (bp)
<i>nape-pld</i>	MH638307	Forward	TGCTCGCTTTGGTTCCAGT
		Reverse	AATGCAGTTTCCCACCCAC
<i>faah</i>	HM231167.1	Forward	TTGGAGGAGGAGGCTCTTTG
		Reverse	CCACTGCAATGAGGATAAGTGC
<i>ppara</i>	AY198322.1	Forward	CCATCCCGACACGAGTTCC
		Reverse	CAGCGACGTGTCTTCTGTCT
<i>bmal1a</i>	KF840401	Forward	AGATTCTGTTCTCGTCCGGAG
		Reverse	ATCGATGAGTCGTCTCCCGTG
<i>rev-erba</i>	KU242427.1	Forward	CGTTCATCTCAGGCACCACT
		Reverse	AACTGACCTGCAGACACCAAG
<i>ef-1a</i>	AB056104	Forward	CCCTGGCCACAGAGATTTC
		Reverse	CAGCCTCGAACTACCAACA

Nape-pld, *N*-acyl phosphatidylethanolamine-specific phospholipase D; *faah*, fatty acid amidohydrolase; *ppara*, peroxisome proliferator-activated receptor α ; *bmal1a*, brain and muscle ARNT-like 1a; *rev-erba*, nuclear receptor subfamily 1 group D member 1 (NR1D1); *ef-1a*, elongation factor-1a.

at 95°C for 30 s and 40 cycles of a two-step amplification program (95°C for 5 s and 60°C for 30 s). A melting curve was systematically monitored (temperature gradient at 0.5°C/5 s from 70 to 90°C) at the end of each run to confirm the specificity of the amplification reaction. The Gene Data Bank reference numbers and the primers (Sigma-Aldrich) sequences employed for target genes (*nape-pld*, *faah*, *ppara*, *bmal1a*, and *rev-erba*) and the reference gene (*ef-1a*) are shown in **Table 1**. The 2^{− $\Delta\Delta C_t$} method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression (fold change). Data obtained were normalized to the group with the lowest expression in each gene.

Data Analysis

All studied parameters were first analyzed by One-way ANOVA followed by the *post hoc* Student–Newman–Keuls (SNK) test (using SigmaPlot 12.0 statistics package). When necessary, data were transformed to logarithmic or square root scale to normalize and to obtain homoscedasticity. In addition, a Student *t*-test was performed to compare data from 1 h postprandial (sampling point ZT3) and 25 h fasting (ZT3b). A probability level of $p < 0.05$ was considered statistically significant in all tests. Furthermore, the existence of daily (24-h) rhythms were determined by Cosinor analysis fitting the data to a sinusoidal function by the least squares method (Duggleby, 1981). The formula used was $f(t) = M + A \cdot \cos(t\pi/12 - \Phi\pi/12)$, where $f(t)$ was the gene expression level at a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours, and Φ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M , A , and Φ , and their standard error (SE), being the SE based on the residual sum of squares in the least-squares fit (Duggleby, 1981; Delgado et al., 1993). Significance of Cosinor analysis was defined by the noise/signal of amplitude calculated from the ratio $SE(A)/A$ (Nisembaum et al., 2012). Data were considered to display a daily rhythm if it had both $p < 0.05$ by ANOVA and $SE(A)/A < 0.3$ by Cosinor analysis.

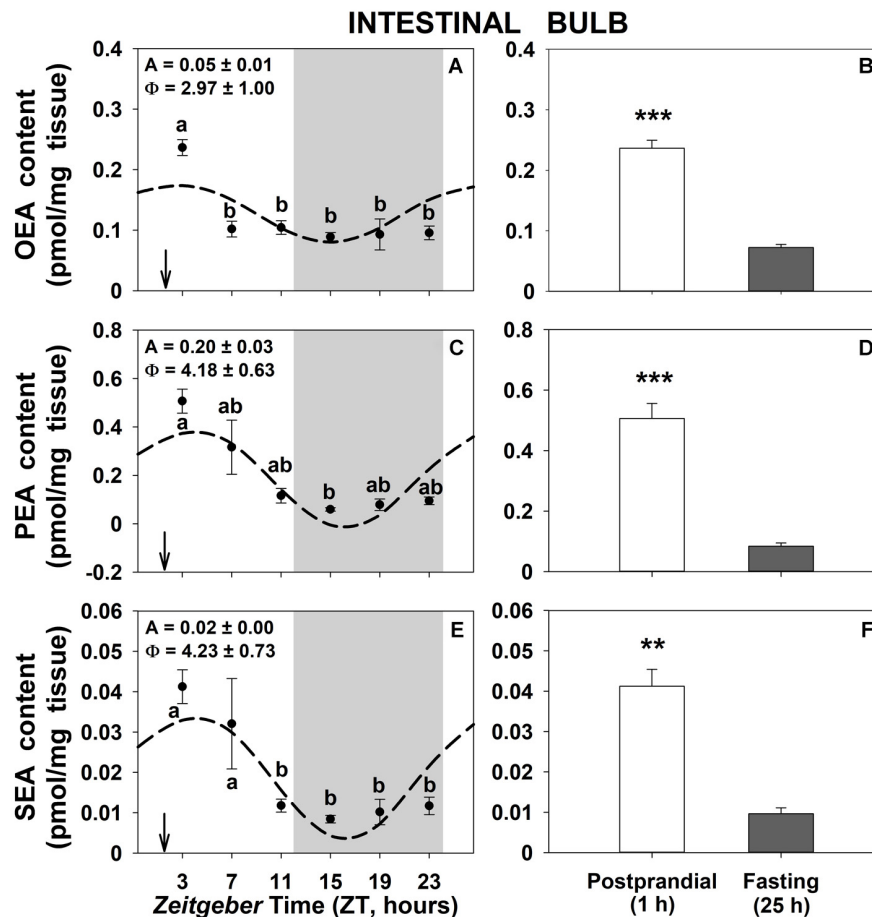


FIGURE 1 | Daily variations of NAEs (OEA, PEA, and SEA) content in intestinal bulb of goldfish. **(A,C,E)**, profiles throughout a 24-h cycle. Gray area indicates the dark phase and arrows the feeding time (ZT2). Different letters indicate significant differences among groups (ANOVA, $p < 0.05$ and SNK *post hoc* test). Dashed lines represent periodic sinusoidal functions when rhythms are significant [Cosinor, $SE(A)/A < 0.3$; and ANOVA, $p < 0.05$]. Amplitude (A, fold change) and acrophase (Φ , h) values of the rhythms are shown in the top-left of the graphs. **(B,D,F)**, comparison between 1 h postprandial and 25 h fasting. Asterisks indicate significant differences by Student *t*-test analysis (** $p < 0.01$, *** $p < 0.001$). Data are shown as mean $\pm$ SEM ($n = 6-7$).

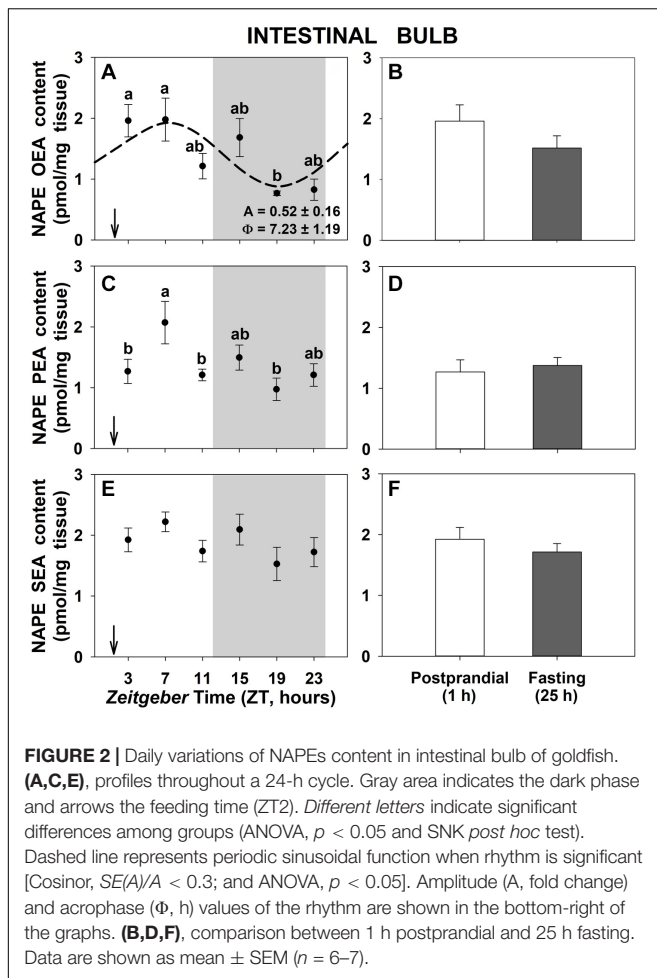
RESULTS

Daily patterns of OEA, PEA, and SEA levels in intestinal bulb of goldfish are shown in **Figure 1**. All three NAEs displayed significant rhythms with low amplitudes and the acrophase (time of the day with maximum levels) 3–4 h after mealtime (**Figures 1A,C,E**). The content of the three NAEs was 3–4 fold higher in the 1-h postprandial fishes than in 25-h fasting ones (**Figures 1B,D,F**). Similar results were obtained in the other two gastrointestinal tissues (**Supplementary Figures S1, S3**), being rhythmic PEA and SEA in the anterior intestine and OEA in the liver. As for intestinal bulb, the content of NAEs was higher in the 1-h postprandial than in 25-h fasting fish in both peripheral tissues, although the trend not was statistically significant in the case of SEA in the liver (**Supplementary Figure S3F**).

Regarding the precursors of NAEs, the daily profiles of NAEs corresponding to each NAE in intestinal bulb of goldfish are shown in **Figure 2**. We can observe daily variations in the

content of NAE of OEA and PEA in intestinal bulb with the maximum levels occurring during the day time and the lowest in the middle of the night (**Figures 2A,C,E**). Only the NAE of OEA displayed a significant rhythm (**Figure 2A**). On the other hand, 25-h fasting did not modify the total amount of NAEs respect to 1-h postprandial (**Figures 2B,D,F**). In the other two gastrointestinal tissues, the anterior intestine and the liver (**Supplementary Figures S2, S4**, respectively), no significant daily oscillations of NAEs were found and only the PEA-NAE showed a significant higher content in the 1-h postprandial compared to 25-h fasting animals.

Daily patterns of NAEs levels in the hypothalamus are shown in **Figure 3**. Contrary to gastrointestinal tissues, no significant differences were found neither throughout the 24-h cycle (**Figures 3A,C,E**) nor when 1-h postprandial and 25-h fasting animals were compared (**Figures 3B,D,F**). Similar results were obtained in the other studied brain tissue, the telencephalon (**Supplementary Figure S5**), except for SEA that showed daily oscillations with the lowest content at the beginning of the



dark phase, although rhythmicity did not reach the threshold of significance (Supplementary Figure S5E).

Figure 4 shows the daily profiles of the NAEs corresponding to each NAE in the hypothalamus of goldfish. No significant oscillations were obtained throughout the 24-h cycle (Figures 4A,C,E). However, significant differences between 1-h postprandial and 25-h fasting were noticed for all NAEs in this encephalic tissue (Figures 4B,D,F). While hypothalamic levels of NAE of OEA was increased by fasting, both NAEs of PEA and SEA were decreased. Obtained results in the other central tissue, the telencephalon (Supplementary Figure S6), showed daily oscillations in all NAEs with only a significant 24-h rhythm in the NAE of OEA (Supplementary Figures S6A,C,E), but did not exhibit feeding-induced changes in the levels of any studied NAEs (Supplementary Figures S6B,D,F).

The enzymatic activity of FAAH, the degradation enzyme of NAEs, in anterior intestine and hypothalamus of goldfish is shown in Figure 5. There are no daily changes in none of the studied tissues (Figures 5A,C). Instead, a significant threefold increase were noted in 1-h postprandial fish respect to 25-h fasting in the anterior intestine, while FAAH activity in hypothalamus remained unchanged with feeding (Figures 5B,D, respectively).

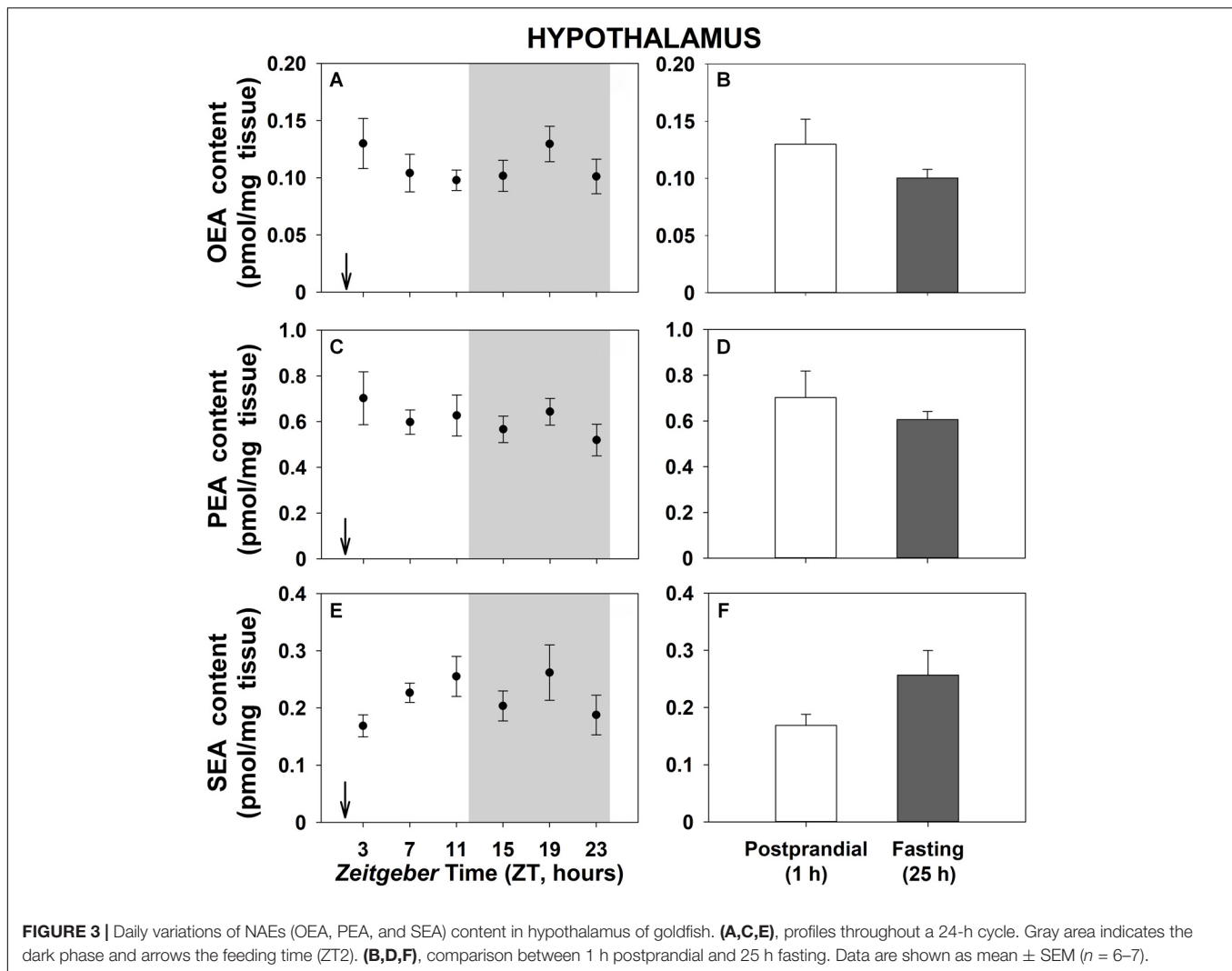
The mRNA abundance of *nape-pld* and *faah* genes (which codify for the NAEs synthesis and degradation enzymes, respectively) in intestinal bulb presented significant daily rhythms (Figure 6), with low amplitudes, and no differences were found between 1 h-postprandial and 25 h fasting (data not shown). The acrophase of the *nape-pld* gene took place in the interphase dark-light, 2 h before the mealtime, while the acrophase of *faah* gene took place around ZT4, 2 h after the mealtime. A similar pattern was observed for *nape-pld* in liver but not in the anterior intestine (Supplementary Figures S7A,C) and for *faah* in both anterior intestine and liver (Supplementary Figures S7B,D), although such daily differences were not associated with significant rhythms.

Daily significant expression rhythms of the NAEs receptor (*ppara*) and of the two studied clock genes (*bm11a* and *rev-erba*) were found in the intestinal bulb of goldfish (Figure 7). The acrophase of *ppara* (Figure 7A) took place at ZT1, 1 h before mealtime and 1 h after the onset of the light, while the acrophase of *bm11a* (Figure 7B) took place around ZT8, 6 h after mealtime. The acrophase of *rev-erba* (Figure 7C) occurs at ZT17, almost in the middle of the scotophase. With regards to the other two peripheral tissues, the anterior intestine and the liver (Supplementary Figure S8), the circadian rhythms were maintained as for the intestinal bulb, with comparable acrophases and amplitudes.

DISCUSSION

Our results show for the first time in a fish species the existence of all components of NAEs system: OEA, PEA, SEA, their precursors, enzymes of synthesis and degradation, and the receptor PPAR α . Endogenous levels of NAEs and NAEs found in gastrointestinal and brain tissues in goldfish are similar to that previously reported in other vertebrates, although very few species have been studied (Astarita et al., 2006; Murillo-Rodriguez et al., 2006; Fu et al., 2007; Guijarro et al., 2010; Liedhegner et al., 2014). These data suggest that these bioactive lipids may be widespread across vertebrate groups.

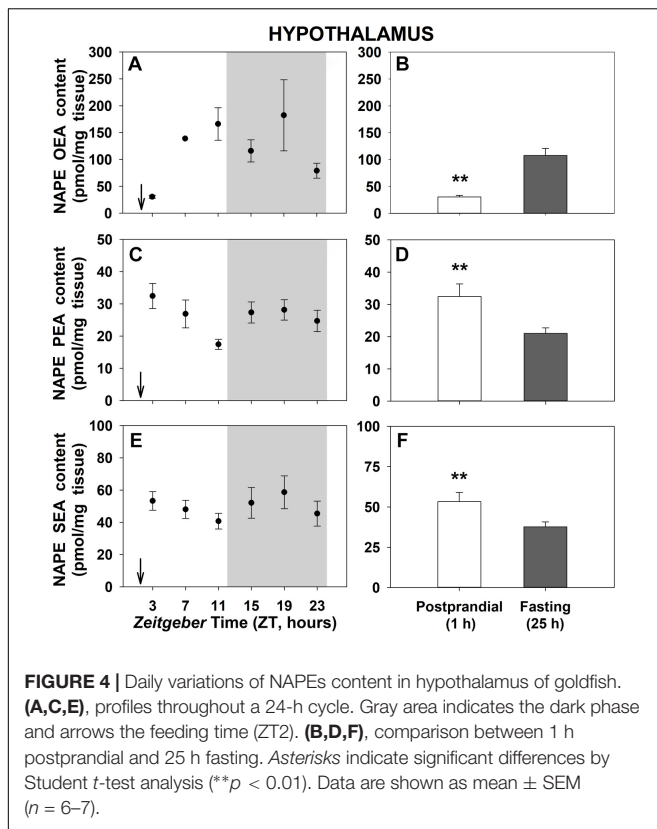
NAEs (OEA, PEA, and SEA) exhibit daily variations in the goldfish gastrointestinal tissues, which seemed to follow daily rhythmic patterns, being mainly driven by food intake. In fact, the most interesting result of this study is a pronounced and rapid postprandial increase in the content of the three NAEs analyzed in intestinal bulb, anterior intestine, and liver of goldfish, compared to levels found in 25-h fasting fish. These results agree with the OEA formation promoted by feeding in the small intestine previously found in mammals, reptiles, and goldfish (Astarita et al., 2006; Piomelli, 2013; Tinoco et al., 2014; Bowen et al., 2017). Both rat and Burmese python also exhibit these fasting/refeeding-induced changes in the intestinal content of PEA and SEA (Astarita et al., 2006; Petersen et al., 2006; Diep et al., 2011). These periprandial fluctuations in the gastrointestinal content of NAEs suggest that this family of bioactive lipids may contribute to the regulation of feeding behavior in vertebrates, possibly acting as satiety signals, since intestinal levels are elevated in the post-ingestive



state. Pharmacological acute studies in rodents support this idea, demonstrating anorectic effects of OEA, PEA and SEA in rats and mice, which are peripherally mediated (Rodríguez de Fonseca et al., 2001; Terrazzino et al., 2004; Piomelli, 2013). Similar results have also been found in goldfish, where a reduction in food intake was produced after intraperitoneal administration of OEA (Tinoco et al., 2014). Thus, all results support that NAEs are involved in feeding regulation, acting as short-term anorectic signals.

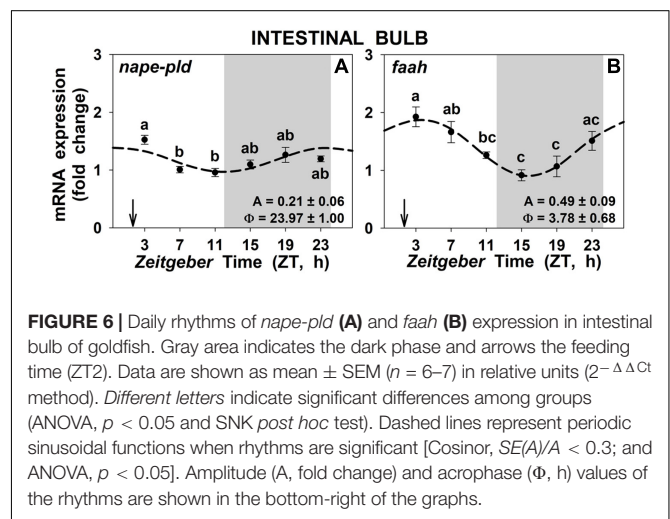
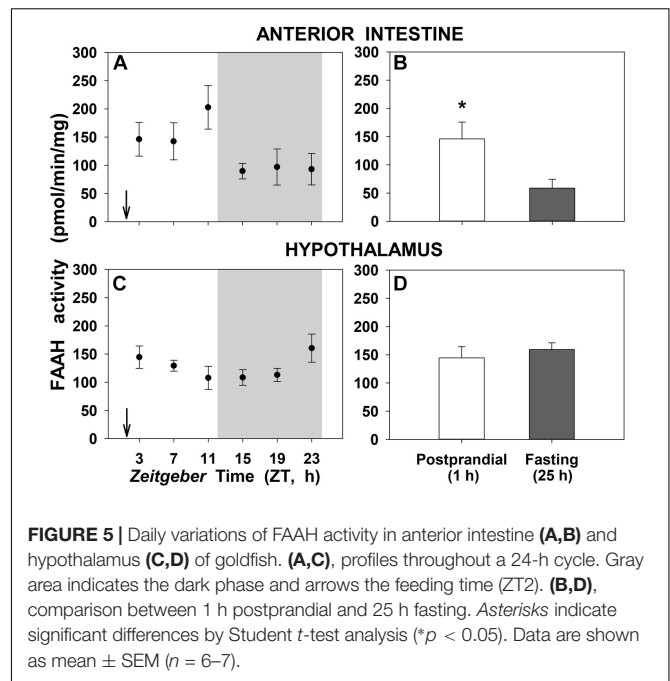
The postprandial increase of NAEs in intestinal tissues may be due to an increase of their precursors and/or changes in the activity of enzymes involved in their synthesis and degradation (Fu et al., 2007; Bowen et al., 2017). Some studies in mammals have suggested that NAEs levels are regulated in intestinal tissue in parallel with the formation of their precursor molecules, the NAEs (Petersen et al., 2006; Fu et al., 2007; Gillum et al., 2008). In addition, Fu et al. (2007) found that feeding stimulates OEA mobilization in duodenum and jejunum by increasing activity and expression of NAPE-PLD. However, most data in the present study indicate that there are no feeding-related differences in the

gastrointestinal content of NAEs nor relative mRNA expression of *nape-pld*, even though significant changes in NAEs levels were observed during fasting/feeding cycles. Some hypotheses could explain this lack of differences in precursors and synthesis enzyme in fish. On one hand, it should also be taken into account that, in addition to the direct hydrolysis from NAEs to NAEs by NAPE-PLD, other multistep pathways of NAEs formation exist (Hussain et al., 2017; Inoue et al., 2017). These alternative pathways involve intermediate molecules, such as glycerophospho-NAE, lyso-N-acyl-phosphatidylethanolamine, or phospho-N-acylethanolamine, and a possible decrease of these intermediates could explain the increase of NAEs levels observed in gastrointestinal tissues in goldfish, without feeding-related modifications in levels of NAEs and the NAPE-PLD enzyme. In addition, Lin et al. (2018) demonstrated that dietary fatty acids can modulate tissue NAEs levels in the absence of NAPE-PLD, which suggest that NAPE-PLD is not necessary for NAEs synthesis, thereby highlighting the important role of alternative pathways in maintaining NAEs levels. Other possibility is that the NAEs regulation by feeding occurs at level of the degradation

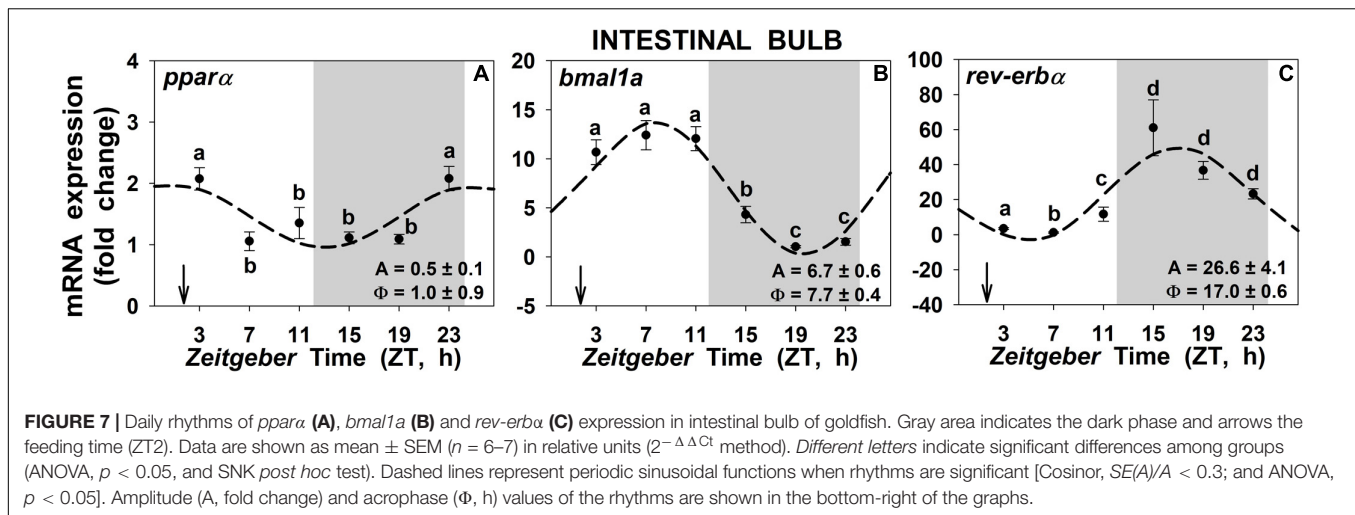


enzyme, since a decrease in FAAH activity and expression in rodents' intestine was found after feeding (Fu et al., 2007), being responsible, at least in part, of feeding-induced OEA increase. However, this expected negative correlation between NAEs concentration and FAAH was not found in goldfish tissues. Other studies in mammals have indicated that FAAH activity has a small contribution in NAEs levels, suggesting the existence of other amidases, such as NAAA, also responsible for NAEs metabolism (Borrelli and Izzo, 2009; Liedhegner et al., 2014; Bowen et al., 2017). Thus, other forms of amidase not yet characterized in fish could also contribute to the postprandially NAEs-increased levels in goldfish. The postfeeding increase in the expression and activity of FAAH in some gastrointestinal tissues of goldfish could be a physiological response to the rise in NAEs levels due to the upregulation of any enzyme involved in the formation of NAEs, as it has been previously suggested in rats (Diep et al., 2011).

Less clear is the role of NAEs at central level and very few studies have examined daily changes in the brain content of NAEs. In the present study, the NAEs content in the goldfish hypothalamus and telencephalon did not display significant rhythms. Similar results were found in mice, where daily oscillations were not detected in hypothalamic content of OEA and PEA, although these NAEs exhibited diverse daily rhythms in other brain regions, such as cerebellum, amygdala, and hippocampus, suggesting that these daily changes in NAEs are brain region-specific (Liedhegner et al., 2014). Controversial results have been reported in rats. While no



effect of daytime (photophase versus scotophase) was found in OEA content in various encephalic tissues in rats (cerebellum, hippocampus, hypothalamus, thalamus, cortex, striatum, and brainstem; Guijarro et al., 2010), diurnal variations of OEA and PEA were detected in pons, hippocampus, and hypothalamus in the same species (Murillo-Rodríguez et al., 2006). Independently of existence or not of daily modifications in the NAEs content in different encephalic tissues, it has been suggested that the brain content of these compounds seem to be feeding independent. Thus, OEA did not respond to food deprivation in different rat brain tissues, including structures involved in the control of feeding, such as hypothalamus, thalamus, cortex, striatum, and brainstem (Fu et al., 2007; Izzo et al., 2010). Similarly, no differences in NAEs content in hypothalamus and telencephalon were observed between fasting and feeding states



in goldfish. This feeding-independent regulation of NAEs in the brain suggests that nutritional status could be regulating NAEs mobilization in a tissue-specific manner only at gastrointestinal level. In addition, it would support the above discussed idea that NAEs play a role in the feeding regulation at peripheral level. Nevertheless, it cannot be ruled out that NAEs play other physiological roles at the brain level, although they have not been investigated in fish yet.

Although NAEs have been considered for a long time as simply phospholipid precursors of the NAEs, the increasing body of evidence in mammals has suggested that NAEs also seem to be bioactive molecules that are involved in several physiological functions, without the involvement of NAEs (Coulon et al., 2012; Romano et al., 2015). Particularly, it has been demonstrated that hypothalamic administration of C16:0 NAPE (N-palmitoyl-phosphatidylethanolamine, the most abundant plasmatic NAPE) decreases food intake in rats, and its effect does not seem to be mediated by a NAPE metabolite (NAE) (Gillum et al., 2008; Wellner et al., 2011). In the present study, we have found postprandial changes in NAEs at hypothalamic level in goldfish, which could suggest a possible involvement of these NAEs in the central regulation of feeding. Although, because OEA-NAPE content decreases and PEA- and SEA-NAPE content increases after feeding, the interpretation of these results is difficult. Further studies must to be performed in order to clarify the exact role of NAEs in fish brain and if they are signaling lipids able to control important biological functions on their own.

The expression of the NAEs receptor, *ppara*, displayed a clear daily rhythm in all the studied gastrointestinal tissues, suggesting a possible rhythmicity in the functions of NAEs. In nocturnal rodents, *ppara* expression in liver increases during the daytime, having its maximum at the beginning of the night (Yang et al., 2006; Chen et al., 2010; Wang et al., 2014). Our data indicate that in the diurnal-species goldfish, *ppara* expression rises during the nighttime peaking in the early morning (1 h before feeding) in intestinal tissues and liver, similar to that reported in the liver of sea bream, another diurnal fish (Paredes et al., 2014). In both nocturnal and diurnal animals, the PPAR α is upregulated

during the rest phase, which coincides with the fasting state of the animals (Liu et al., 2014). During this fasting state, animals obtain energy from increasing the hepatic fatty acid oxidation with the synthesis of ketone bodies (Ribas-Latre and Eckel-Mahan, 2016). In fact, it has been demonstrated in mammals that PPAR α stimulates fatty acid oxidation and lipid catabolism (Charoensuksai and Xu, 2010; Chen and Yang, 2014; Liu et al., 2014). In addition, *bmal1a* is also rhythmic increasing after feeding, supporting its role as a lipogenic factor in mammals (Shimba et al., 2005, 2011; Zhang et al., 2014), and also in goldfish (Gómez-Boronat et al., 2016). Moreover, PPAR α has been largely proposed as a link between lipid metabolism and circadian system in mammals (Yang et al., 2006; Chen and Yang, 2014; Ribas-Latre and Eckel-Mahan, 2016), in which circadian rhythms of PPAR α are essential for the temporal coordination of genes involved in energy and metabolic process (Charoensuksai and Xu, 2010; Chen and Yang, 2014; Ribas-Latre and Eckel-Mahan, 2016). Thus, it is widely known that PPAR α directly regulates the transcription of *bmal1* and *rev-erbα* via binding to the peroxisome proliferator response element (PPRE) sites in their respective promoter regions. In addition, BMAL1 induces *ppara* and *rev-erbα* by binding to an E-box rich region in their respective promoters (Canaple et al., 2006; Charoensuksai and Xu, 2010; Chen and Yang, 2014; Lecarpentier et al., 2014). In accordance with this regulatory loop, in the three gastrointestinal tissues of goldfish here analyzed (intestinal bulb, anterior intestine, and liver), the acrophases of *ppara* and *bmal1a* are ~ 8 -h shifted, as previously reported in mammals (8–14-h shift; Canaple et al., 2006; Yang et al., 2006; Chen et al., 2010). The existence of the expected daily rhythms in all studied clock genes supports the idea that clocks in gastrointestinal tissues are functional.

In summary, the identification in goldfish of the NAEs system, including precursors, enzymes of synthesis and degradation and receptor, suggests that this endogenous system can be an important pathway for physiological functions as regulation of energy homeostasis in fish, as it is mammals. The gastrointestinal regulation of NAEs levels by the fed and fasted metabolic states supports that NAEs are involved in the feeding regulation, acting

as a peripheral satiety signal. In addition, the present results are in agreement with a putative role of PPAR α as a functional link between the circadian clock and lipid metabolism in fish.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Guidelines of the European Union Council (UE63/2010) and the Spanish Government (RD53/2013). The protocol was approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014) and the Community of Madrid (PROEX 107/14).

AUTHOR CONTRIBUTIONS

MG-B, EI, MD, and NdP conceived and designed the experiments, carried out sampling, and interpreted findings. MG-B, EI,

NdP, AA, and DP analyzed the samples. All authors drafted and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnins.2019.00450/full#supplementary-material>

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Regulation of Gastrointestinal Motility by Motilin and Ghrelin in Vertebrates

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The energy balance of vertebrates is regulated by the difference in energy input and energy expenditure. Generally, most vertebrates obtain their energy from nutrients of foods through the gastrointestinal (GI) tract. Therefore, food intake and following food digestion, including motility of the GI tract, secretion and absorption, are crucial physiological events for energy homeostasis. GI motility changes depending on feeding, and GI motility is divided into fasting (interdigestive) and postprandial (digestive) contraction patterns. GI motility is controlled by contractility of smooth muscles of the GI tract, extrinsic and intrinsic neurons (motor and sensory) and some hormones. In mammals, ghrelin (GHRL) and motilin (MLN) stimulate appetite and GI motility and contribute to the regulation of energy homeostasis. GHRL and MLN are produced in the mucosal layer of the stomach and upper small intestine, respectively. GHRL is a multifunctional peptide and is involved in glucose metabolism, endocrine/exocrine functions and cardiovascular and reproductive functions, in addition to feeding and GI motility in mammals. On the other hand, the action of MLN is restricted and species such as rodentia, including mice and rats, lack MLN peptide and its receptor. From a phylogenetic point of view, GHRL and its receptor GHS-R1a have been identified in various vertebrates, and their structural features and various physiological functions have been revealed. On the other hand, MLN or MLN-like peptide (MLN-LP) and its receptors have been found only in some fish, birds and mammals. Here, we review the actions of GHRL and MLN with a focus on contractility of the GI tract of species from fish to mammals.

Keywords: energy homeostasis, ghrelin, motilin, gastrointestinal motility, vertebrates, evolution

INTRODUCTION

Food intake, digestion of foods, and absorption of nutrients through the gastrointestinal (GI) tract wall are fundamental physiological events for living vertebrates. The GI system is the gateway for food entry, and it is well known that GI motility positively influences feeding behavior and contributes to the regulation of energy homeostasis. In general, GI motility of vertebrates is regulated by contractility of smooth muscles controlled by extrinsic parasympathetic and sympathetic neurons, intrinsic enteric sensory and motor neurons, and some GI hormones (1–3). Hormones are signal transduction molecules carried through the bloodstream to transmit biological information from one cell to another by activation of specific receptors on the target

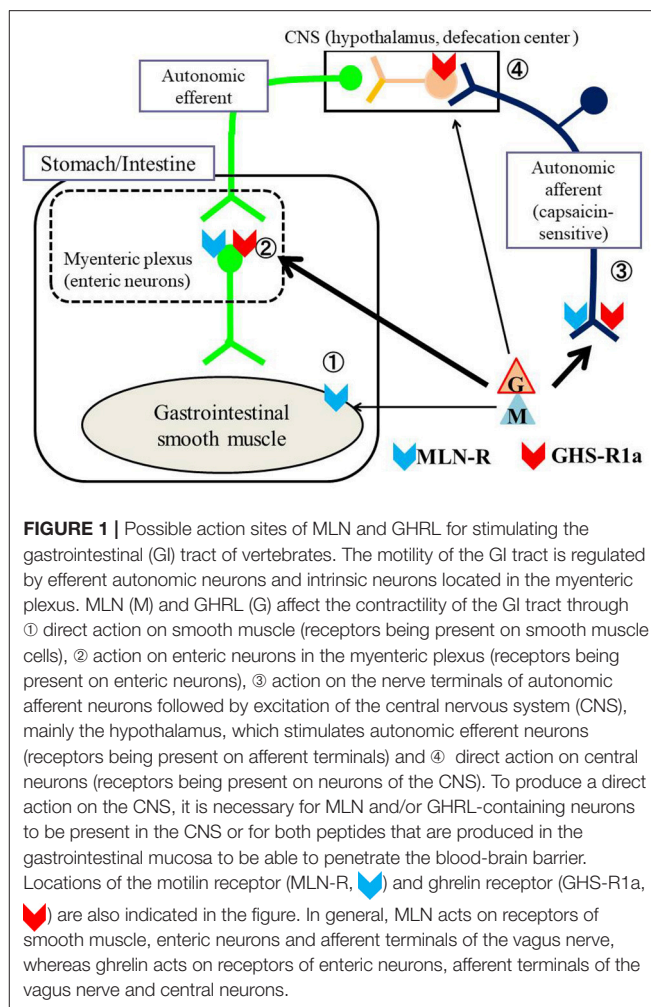
cells. Many GI hormones, including secretin, peptide YY, neurotensin, gastrin, gastrin-releasing peptide (GRP), cholecystokinin (CCK), somatostatin, ghrelin (GHRL), and motilin (MLN) have been identified. GI hormones are produced in specialized gut endocrine or enteroendocrine cells of the GI epithelium, and they act on other digestive organs, associated cells and vagal nerve afferent terminals. The GI tract has functions in controlling energy homeostasis through nutrient absorption and excretion, and several gut hormones have functional significance in the regulation of food intake, secretion of other hormones and control of GI motility (4–8).

MLN was identified in the 1970s (9, 10) and GHRL was identified in the 1990s (11). From their similarity in amino acid sequences of both ligands and receptors, it is thought that these two peptides originate from the same ancestral gene and form a peptide family (5, 12, 13). GHRL and MLN are mainly produced in the gastric and upper small intestinal (duodenum) mucosa, respectively, and they have some common functional characteristics such as regulation of GI motility and appetite, which have crucial roles for regulation of digestion and absorption of nutrients. Therefore, MLN and GHRL must be important GI peptides for energy homeostasis of living vertebrates (4, 5, 14, 15).

In this review, we present results of recent studies regarding the regulation of GI motility by MLN and GHRL, with comparison to different vertebrates including mammals and non-mammals. **Figure 1** shows the possible action sites of MLN and GHRL for stimulating GI motility in vertebrates: (i) smooth muscle cells, (ii) enteric neurons in the myenteric plexus, (iii) terminals of autonomic afferent nerves to evoke an afferent-efferent reflex such as a vago-vagal reflex, and (iv) direct action on the central nervous system (CNS). Generally, although it is possible in *in vivo* experiments on GI motility to detect the actions of peptides in all of the action sites indicated in **Figure 1**, in *in vitro* studies using isolated GI strips, only the actions on enteric neurons and smooth muscle cells can be detected.

REGULATION OF GASTROINTESTINAL MOTILITY BY MLN

MLN, a 22-amino-acid peptide hormone that was firstly discovered from the upper intestinal mucosa of a pig (9, 10) (**Figure 2**), is produced in enteroendocrine cells called M or Mo cells located in the mucosal epithelium in the upper small intestine, the duodenum. The name MLN originates from its stimulatory role in gut motility. Actually, MLN is known to contract the GI tract in several mammals through activation of smooth muscle cells, local enteric neurons and afferent terminals of vagus nerves (**Figure 1**). The mechanisms that have been identified depend on the experimental conditions (*in vitro* or *in vivo*) and animal species, such as dogs (*Canis lupus familiaris*), rabbits (*Leporinae Trouessart*) and Asian house musk shrews (*Suncus murinus*) (4, 16–20). The MLN receptor (MLN-R) was identified in the human (*Homo sapiens*) stomach as an orphan G protein-coupled receptor (GPR38) for which the ligand is



unknown, and it was deorphanized in 1999 (21). The MLN-R couples with $G_{q/11}$ protein, stimulates phospholipase C that synthesizes inositol-trisphosphate, and increases intracellular Ca^{2+} and diacylglycerol to excite smooth muscles or neurons (22). *In vivo* and *in vitro* contraction studies for MLN have been extensively performed using GI tracts of various vertebrates in experiments.

Mammals Rat and Mouse

Mice (*Mus musculus*) and rats (*Rattus norvegicus*) are widely used as experimental animals in physiological and pharmacological studies. It has been known for a long time that MLN does not affect GI contractility *in vitro* (23, 24) and gastric emptying *in vivo* (25). Regarding *in vivo* recording of GI motility, interdigestive migrating contraction-like motility, with short intervals (about 15 min), was observed in the mouse and rat GI tracts in fasting periods (26, 27), but this motility pattern is actually caused by a family peptide, GHRL (see below). Recent genome-wide analysis revealed that these rodentia lack genes for MLN and its receptor (28, 29).

Human	FVPIFTYGELQRM--QEKERNK-GQ	(22)
Rhesus monkey	FVPIFTYGELQRM--QEKERSK-GQ	(22)
Cattle	FVPIFTYGEVRRM--QEKERYK-GQ	(22)
Horse	FVPIFTYSELQRM--QEKERNR-GQ	(22)
Rabbit	FVPIFTYSELQRM--QERERNR-GH	(22)
Cat	FVPIFTHSELQRI--REKERNK-GQ	(22)
Dog	FVPIFTHSELQKI--REKERNK-GQ	(22)
House shrew	FMPIFTYGELQKM--QEKEQNK-GQ	(22)
Chicken	FVPFFTQSDIQKM--QEKERNK-GQ	(22)
Turkey	FVPFFTQSDIQKM--QEKERIK-GQ	(22)
Japanese quail	FVPFFTQSDFQKM--QEKERNK-GQ	(22)
Zebra finch	FMPFFTQSDFQKM--QEKERNKAGQ	(23)
North Island brown kiwi	FLPFFTQSDFRKM--QEKERNK-GQ	(22)
Band-tailed pigeon	FVPFFTQSDFRKMQLQEKERNKAGQ	(25)
Chinese alligator	FLPIFTHSDMQRM--QDRERNK-GQ	(22)
Three-toed box turtle	YLAFTRSDIERMQLQEKQRNK-AQ	(24)
Common garter snake	YLAFYSREDFRRM--QEKEKNQ-AQ	(22)
Western painted turtle	YLAFTRSDIERM--QEKQRNK-AQ	(22)
Central bearded dragon	YTALYSWEDFRM--QERERNQ-AQ	(22)
Green anole	YTAFFTREDFRKM--QENEKNK-AQ	(22)
Spotted green pufferfish	HITFFSPKEMMVLK-QEQEG-----	(19)
Three-spined stickleback	HITFFSPKEMMLMK--EREG-----	(18)
Turbot	HITFFSPKEMMLMK--EREG-----	(18)
Japanese medaka	HITFFSPKELLHMLRLEQQE-----	(20)
Zebrafish	HIAFFSPKEMRELK--EKEG-----	(18)
Coelacanth	FISFFSPSDMRRM--MEKEKSKAL-	(22)
Spotted gar	FLSFISPSDMRRM--MEQEKVKAG-	(22)
		

FIGURE 2 | Comparison of amino acid sequences of mature motilin in vertebrates. Asterisks and dots indicate amino acids that are identical in all species or identical in more than half of the species. The number of amino acids is shown in parenthesis. Amino acid sequences were obtained from the DDBJ/EMBL/GenBankTM databases (acc#: NP_002409.1 for human, NP_001027979.1 for rhesus monkey, NP_776363.1 for cattle, NC_009163 for horse, X63860.1 for rabbit, NC_018727 for cat, XP_005627282.1 for dog, AB325968 for house shrew, NP_001292058.1 for chicken, XP_010722636.1 for turkey, BAU80773.1 for Japanese quail, XP_004175023.1 for zebra finch, XP_013812966.1 for North Island brown kiwi, OPJ88883.1 for band-tailed pigeon, XP_006025154.1 for Chinese alligator, XP_024054930.1 for three-toed box turtle, XP_013912065.1 for common garter snake, XP_005309417.1 for Western painted turtle, XP_020650577.1 for central bearded dragon, XP_008107992.1 for green anole, ALD51563 for spotted green pufferfish, ALD51564 for three-spined stickleback, AWP03197 for turbot, XP_023810781 for Japanese medaka, XP_002665930.1 for zebrafish, XP_005995529.1 for coelacanth, and NC_023181 for spotted gar).

Guinea-Pig

The guinea-pig (*Cavia porcellus*) belongs to rodentia, as do rats and mice, but several findings indicate the possible presence of an MLN system. In a previous molecular biological study, an MLN precursor was identified in the duodenal mucosa (GenBank accession number AF323752) and the structure of guinea-pig MLN is estimated to be FVPIFTYSELRRRTQEREQNKRL (30). The results of an immunohistological study, using a human MLN antibody and a human MLN-R antibody, suggested the presence of MLN and MLN-R protein in the GI tract (31). However, human MLN did not cause any contractions of the intestine either in a non-stimulated or electrically stimulated condition *in vitro* (23, 32). The guinea-pig MLN was also ineffective at contracting GI strips or at modifying neural responses in the

guinea-pig (33) (Table 1). There has been no *in vivo* study in which the GI motility of the guinea-pig was recorded. In the guinea-pig MLN system, the MLN gene might be expressed but the MLN-R gene is degenerated, as in other rodent species such as the kangaroo rat (genus *Dipodomys*) (29, 33).

Dog

Canine MLN is a 22-amino-acid peptide that has differences in five amino acids at positions of 7, 8, 12, 13, and 14 when compared with human MLN (Figure 2). Dogs have been used for a long time to examine the regulation of GI motility by autonomic nerves and gut hormones. The GI motility (circular muscle direction) of conscious dogs is measured by force transducers sutured on the serosal surface of the stomach and the intestine.

TABLE 1 | Comparison of gastrointestinal motility-stimulating actions of motilin in several vertebrates.

Species	In vitro study		In vivo study	Action site	References
	Unstimulated	Stimulated			
Fish					
Rainbow trout		Potentialiation of EFS			(34)
Zebrafish*	No effect				(35)
Amphibians					
Bullfrog	Contraction (human MLN)				(36)
Japanese fire belly newt	No effect (human MLN)				(36)
Reptiles					
Research not available					
Birds					
Chicken*			Contraction (rhythmic oscillatory complex)		(37)
	Contraction	Potentialiation of EFS		Enteric neurons (proventriculus)	(38)
				Smooth muscle (proventriculus and ileum)	(39, 40)
Quail*	Contraction			Enteric neurons (proventriculus)	(41)
				Smooth muscle (proventriculus and ileum)	(41)
Mammals					
Human*	Contraction	Potentialiation of EFS		Smooth muscle and enteric neurons	(42)
	Contraction			Smooth muscle	(43)
			Contraction (phase-III)		(44, 45)
Dog*	Contraction			Smooth muscle	(46)
			Contraction (phase-III)	Enteric neurons and vago-vagal reflex	(4, 17, 47–49)
Pig*	No effect	No effect (EFS)			(50)
Rat	No effect				(23, 25)
			No effect (gastric emptying)		(25)
Mouse	No effect				(24, 35)
Guinea-pig*	No effect	No effect (ganglion stimulant)			(23, 33)
		No effect (EFS)			(32)
Rabbit*	Contraction			Enteric neurons and smooth muscle	(16, 51–53)
			Contraction	Enteric neurons	(16)
Asian house musk shrew*	Contraction			Enteric neurons	(18, 19)
			Contraction (phase-III)	Enteric neurons	(20, 54)

*Motilin structure has been identified in the marked animal species.

EFS, Electrical field stimulation for excitation of enteric neurons.

The GI motility in dogs can be divided into two patterns, interdigestive and digestive (postprandial) contractions. GI contraction in the interdigestive state is called the interdigestive migrating motor complex (IMC), which is characterized by three different phases: phase-I (motor quiescent period), phase-II (irregular and low-amplitude contraction period) and phase-III (regular and high-amplitude contraction period) (4, 47, 49, 55). The IMC pattern in fasted periods has also been reported in the GI tracts of humans and house musk shrews (44, 54, 56). The physiological significance of the IMC is thought to be for flushing out and cleaning up the GI lumen, mechanically and chemically,

to make it ready to receive the next food, and for preventing bacterial overgrowth in the intestinal lumen (4, 56, 57). MLN has been thought to be an endogenous regulator of phase-III activity of the IMC evoked in the stomach in a fasting state (47–49, 58). The following findings support the involvement of MLN in gastric phase-III: (i) the peaks of endogenous MLN change cyclically and the peaks of plasma MLN concentration are highly associated with gastric phase-III contractions, (ii) exogenously applied MLN causes phase-III-like gastric contraction, and (iii) phase-III contraction is disrupted by administration of anti-MLN serum or MLN-R antagonists such as MA-2029. MLN-induced

gastric contractions in dogs were shown to be sensitive to a muscarinic receptor antagonist, atropine, and to a ganglion blocker, hexametonium, and it was shown that a neural pathway including acetylcholine (ACh) was stimulated by MLN and that vagus nerves have an important role in GI contraction induced by MLN *in vivo* (4, 17).

In an *in vivo* study using conscious dogs, erythromycin caused a phase-III-like contraction that is similar to the response of exogenous MLN in a fasted state (17, 55). This macrolide compound was shown to bind to the MLN-R of mammals (21, 51). From the words “motilin” and “macrolide,” macrolide antibiotics (including erythromycin) are called “motilides.” Motilides, which are erythromycin derivatives, have been known to induce GI contractions in humans (59, 60) and rabbits (61) as observed by MLN.

In comparison with many *in vivo* studies, little is known about the *in vitro* studies using isolated canine GI strips. Poitras et al. (46) showed that bath-applied canine MLN, but not human MLN, causes a contraction of canine duodenum muscle strips by direct action of MLN on muscle cells, which is different from results obtained in the *in vivo* studies.

Human

Human MLN is a 22-amino-acid peptide with the same structure as that of pig MLN (**Figure 2**). Similar to the GI motility pattern in dogs, GI motility in humans can be divided into interdigestive and digestive contractions. IMC-like motility is observed in the interdigestive state and its physiological significance is the same as that described in the section for dogs (44, 56). MLN is thought to be the mediator of the IMC elicited in the stomach because exogenous MLN causes an active front of the IMC and because plasma MLN concentration fluctuated in a cyclic manner with phase-III of the IMC being consistent with the peak of MLN concentration (44, 56, 62). Recently, it was proposed that the IMC in the GI tract signals hunger sensation from the periphery to the brain in humans. Therefore, MLN mediating the IMC is a hunger hormone in humans. Erythromycin derivatives (motilide) and MLN-R agonists also caused phase-III contraction and hunger sensation in humans (59, 60). Due to their GI motility stimulation action and their resistance to degradation in a stomach, clinical use of erythromycin derivatives as gastroprokinetic agents is being investigated (63).

In vitro mechanical studies show that MLN causes contraction of human GI muscle strips. Ludtke et al. (43) reported the region-dependent direct actions of MLN on GI smooth muscle strips. Broad et al. (42) demonstrated both indirect action through activation of enteric neurons and direct action on smooth muscles with electrically stimulated GI strips. Low concentrations of MLN act on neural MLN-R, whereas high concentrations of MLN act on smooth muscle MLN-R (42). Therefore, it is thought that neural MLN-R might be function for regulation of GI contractility.

Rabbit

Rabbit MLN is also a 22-amino-acid peptide hormone with the structure of FVPIFTYSELQRMQERERNRGH, and with 5 amino acids at positions 8, 16, 19, 20, and 21 being different from those

of human MLN (**Figure 2**). An immunohistochemical study indicated that MLN-producing cells were localized preferentially in the mucosa of the upper small intestine (duodenum), as has been reported in other species (64).

An *in vivo* study, in which myoelectric activity of the GI tract was recorded in conscious rabbits, indicates that the migrating myoelectric complex (MMC) originates from the proximal jejunum, not the stomach, and that the MMC appears both in feeding and fasted rabbits at almost the same intervals (65). Therefore, the character of the rabbit MMC is different from that of the IMC observed in a fasted state of dogs and humans. The effects of MLN on rabbit GI motility have been examined in an *in vivo* experiment under an anesthetized condition, and it was found that MLN caused contractions of the stomach and colon but not ileum. Atropine significantly decreased the contraction in the stomach but not in the colon, indicating that different contractile mechanisms are present between stomach and colon (16). The GI region-dependent motility stimulation action of MLN has also been demonstrated in an *in vitro* study using different parts of isolated muscle strips. Pharmacological analysis using atropine and tetrodotoxin (a neuronal blocker) indicated that MLN acted on smooth muscle MLN-R and in addition to the neural MLN-R located on cholinergic nerves (52, 53). The magnitude of MLN-induced contraction differed depending on the GI region (duodenum = jejunum > colon > stomach > ileum), probably due to different expression levels of the MLN-R, although the expression pattern has not been examined (16). High sensitivity of the colon to MLN is a characteristic of the rabbit GI tract. Rabbits belong to lagomorpha, not rodentia, and are coprophagous grass-eating animals with a property of hindgut fermentation. Therefore, regulation of colonic motility must be important, and in this situation, MLN might play a physiological role in the regulation. Regarding regulation of the rabbit MMC described previously, although the jejunum, the starting region of the MMC, is highly sensitive to MLN, the physiological role of MLN in regulation of the MMC has not yet been clarified.

House Musk Shrew

The effects of MLN on GI motility have been mainly investigated using dogs (*in vivo*) and rabbits (*in vivo*, *in vitro*) as mentioned earlier. However, since the body sizes of these animals are relatively large. In this point, house musk shrew (*Suncus murinus*) belongs to insectivora, different from mice, rats and guinea-pigs. The body size is similar to that of rats, and is easy to handle. Interestingly, because house musk shrew has both MLN and GHRL (66, 67), it is a good animal model to investigate their actions and interactions.

A molecular study revealed the primary amino acid sequence of the house musk shrew MLN (FMPIFTYSELQKMQEKEQNKGGQ), and showed that three amino acids at positions 2, 12 and 18 were different from those of human MLN [(67), **Figure 2**]. MLN-producing cells localizes in the mucosa of the upper small intestine (duodenum) as in other mammals (67).

An *in vivo* study in which GI motility was recorded using conscious house musk shrews indicated that the GI motility

pattern differs depending on the feeding conditions, and both interdigestive (IMC) and digestive motor patterns, that are similar with humans and dogs, were observed (54). Although changes in the plasma MLN concentration during the three phases of the IMC have not been examined, MLN caused phase-III activity of the IMC, and the phase-III activity was inhibited by a MLN-R antagonist (19, 54). These results suggest that MLN is involved in the induction of phase-III of the IMC as observed in humans and dogs. A functional role of the vagus nerves for regulation of MLN-induced contraction was demonstrated in a digestive state but not in the interdigestive state. MLN does not cause contraction in the digestive state in the vagus nerve-intact animals but causes contraction in vagotomized animals, suggesting that the vagus nerve suppresses the action of MLN in the digestive state (20). MLN also causes contraction of gastric strips in an *in vitro* study, and the contraction was completely abolished by atropine and tetrodotoxin, indicating that MLN-induced response is a pure neural origin (18). Namely, the MLN-R is located only in enteric neurons in the house musk shrew and mediates the MLN-induced contraction (18). In this animal species, interaction of MLN and GHRL for the regulation of GI motility was examined in both *in vivo* and *in vitro* studies [(19, 20, 68), see below].

Pig

Pigs (*Sus scrofa domestica*) are the species in which MLN was first identified (9, 10), and the amino acid sequence of MLN is the same as that of human (Figure 2). An immunohistochemical study indicated that MLN is co-localized and secreted together with GHRL from the endocrine cells of the pig small intestine (69). However, the effect of MLN on regulation of GI motility has not yet been examined *in vivo*. Only an *in vitro* study has indicated that MLN does not cause contraction of GI strips and does not modify neural responses in the stomach and the intestine (50).

Birds

Chicken

The morphology and function of the GI tract in birds are different from those in mammals in the following three aspects: (i) the crop in the middle of the esophagus stocks food, (ii) there are two distinct stomach structures, the proventriculus that secretes digestive enzymes for chemical digestion and the gizzard for mechanical digestion, and (iii) a pair of a long cecum and short colon/rectum. Electromyogram measurements showed that the MMC, consisting of three phases like the mammalian IMC, is also present in the GI tract of avian species including chickens (*Gallus gallus domestica*) (70, 71). However, the MMC originates from the duodenum, not the stomach, and it appears in both fed and fasted states, being different from the mammalian IMC. The detailed mechanisms of regulation of the MMC in chickens have not been reported yet, but it is known that the appearance of the MMC is modulated by some gut hormones such as CCK and gastrin. The difference in the motility of the GI tract in fed and fasted states is unclear at present in the case of the bird MMC (70, 71).

In birds, MLN was first identified as a 22-amino-acid peptide in the extract of chicken duodenal mucosa (38). Six amino acids are different from those of human MLN at positions 4, 7–10, and 12 (Figure 2). The primary structure of MLN has been identified in several birds including the quail (*Coturnix japonica*), turkey (*Meleagris gallopavo*) and pigeon (*Columbidae*), and the structural difference in MLN among birds is small compared with that in mammals (Figure 2). Chicken MLN is produced in the mucosa of the duodenum but not in the proventriculus and gizzard, as in mammals (38). The chicken MLN-R has also been identified, and the homology to the human MLN-R was shown to be 59.1% (72). This value is lower than the homology to the MLN-R of other mammals such as the rabbit (84%), house musk shrew (76%), and dog (71%) (73–75), suggesting a different structure of the chicken MLN-R from that of mammals. The insensitivity to a mammalian MLN-R agonist, erythromycin, and low sensitivity of human MLN in the chicken intestine (38, 40) support the possibility of a structural difference of the chicken MLN-R from the mammalian one.

The GI-stimulating action of MLN has been mainly investigated using isolated smooth muscle strips *in vitro*, and it has been shown that MLN causes contraction of the proventriculus and small intestine but that the crop and colon are insensitive. The underlying mechanisms of MLN-induced contraction in the proventriculus are myogenic and neural mechanisms, being different from those in the small intestine only by myogenic action (40) (Table 1). The small intestine is highly sensitive to MLN, independent of aging due to a high expression level of MLN-R mRNA (76), but the physiological roles of MLN for regulation of GI motility in avian species have not been fully elucidated. Jimenez et al. (70) found rhythmic oscillating contraction in the chicken ileum, and Rodriguez-Sinovas et al. (37) reported that the plasma MLN concentration was high during spontaneous rhythmic oscillating contraction and that exogenous MLN triggered the contraction. These results suggested the involvement of MLN in regulation of the MMC in the small intestine. The proventriculus also shows an appropriate sensitiveness to MLN, but MLN-R mRNA expression significantly decreases with aging, and MLN-induced contraction also decreases markedly with aging (72, 76). This suggests the age-dependent regulation of proventriculus motility by MLN in adult chickens.

Japanese Quail

MLN has also been identified in the Japanese quail. Quail MLN consists of 22-amino-acids and only one amino acid at position 10 is different from that in chickens [(41), Figure 2]. The distribution of MLN-immunopositive cells in the quail is almost the same as that in the chicken (38). The effect of MLN on the quail GI tract is only examined in *in vitro* using isolated muscle strips. Chicken MLN is capable of causing contraction of the quail GI tract. The region-related contractile responsiveness (small intestine > proventriculus > crop = colon) and region-related different contraction mechanisms reported by Apu et al. (41) are similar to those in chickens (39, 40, 77) (Table 1). Although it is necessary to examine the responses of MLN in other avian species, the characteristics of the MLN-induced GI

contraction in the two species are almost the same, and the high sensitivity of MLN in the small intestine suggests that MLN might regulate motility of the small intestine by spontaneous oscillating contraction as seen in chickens (37).

Reptiles

Little is known about the MLN system in reptiles. An immunohistochemical study using an anti-human MLN antibody failed to show the presence of MLN in the digestive tract of three reptiles (*Testudo graeca*, *Mauremys caspica*, and *Lacerta lepida*), though the presence of other gut peptides, including neurotensin, gastrin, glucagon, and somatostatin, was demonstrated (78). On the other hand, MLN immunoreactivity was detected in the alimentary tract of the King's skink (*Egernia kingii*) (79). The presence of an MLN peptide possessing a mammalian MLN structure is dependent on the species of reptiles, and reptiles might therefore be the boundary for the presence of a mammalian-like MLN structure. Concerning the MLN structure, Liu et al. (80) reported that the deduced amino acid sequence of the lizard MLN was YTAFFTRDFRKMQENENKAQ, which is quite different from the N-terminal structure of human MLN (FVPIFTYGEL) and chicken MLN (FVPFFTQSDI). **Figure 2** also indicates the different N-terminal structures of reptile MLNs from those of birds and mammals. However, the N-terminal structure of alligator MLN (FLPIFTHSDM) is close to that of the chicken.

Concerning the contraction study, the effects of human MLN have never been examined despite the fact that GI motility of isolated strips has been investigated in reptiles (81). Identification of MLN peptide and examination of its effects on reptile GI motility should be carried out in the future.

Amphibians

No evidence has been obtained for the immunohistochemical localization and the structural difference of MLN in amphibians.

Previous studies using the isolated spinal cord of a toad (*Bufo marinus*) indicated that human MLN depolarized the motor-neurons of hemisected spinal cord *in vitro* (82). Our previous study *in vitro* using the isolated GI tract of the bullfrog (*Lithobates catesbeiana*) showed that human MLN induced small contractions of longitudinal muscle strips of the upper intestine at a relatively high concentration (1 μ M) (36), while longitudinal muscle strips from the middle and lower intestine and gastric longitudinal and circular muscle strips were quite insensitive (**Table 1**). These results indicate that the upper intestinal strip, corresponding to the duodenum, was sensitive to human MLN. In mammalian and avian species, the GI region-related contraction of MLN, probably due to the heterogeneous expression of MLN-R, is one of the characteristics of the MLN-induced contraction in the GI tract (16, 39, 40, 76). The characteristic of region-dependent sensitivity might be preserved in the bullfrog GI tract. The above described results suggest the presence of MLN-R in the bullfrog GI tract. However, preliminary data indicated that erythromycin did not cause any contraction of the upper intestine of the bullfrog, suggesting the possibility that the structure of MLN-R is different from that in mammals. In contrast to the bullfrog, human MLN did not

induce any contraction of the upper small intestine from another amphibian, the Japanese fire belly newt (*Cynops pyrrhogaster*) (36) (**Table 1**). Amphibians are divided into three groups, anuran, urodelal, and gymnophional species. The results suggest species differences in response to MLN among evolutionary states of amphibians, similar to that for rodentia, and even in mammals that do not have a MLN system.

Teleost Fish

The presence of a MLN peptide in the GI tract of a teleost was first investigated immunohistochemically by Pan and Fang (83), who reported that no MLN-immunoreactive cells were detected in the gut of a grass carp (*Ctenopharyngodon idellus*) (stomach-less fish) by using an anti-human MLN antibody. However, a recent molecular cloning study for MLN and MLN-R genes indicated the presence of MLN-like peptide (MLN-LP) and MLN-R genes in the zebrafish (*Danio rerio*) (80). The genomic structure was similar to that of the human MLN and GHRL genes (84, 85): The MLN-LP genes contain five exons and four introns, and the mature peptide was encoded by the second and third exons, although the amino acid sequence of the mature peptide is quite different from that of the known MLN, suggesting that the MLN-LP is unique in teleost (80) (**Figure 2**). The well-known MLN is composed of a 22-amino-acid sequence in both mammalian and avian species, but the structure of zebrafish MLN-LP is unique (80) (**Figure 2**). The N-terminal structure (FVPIFT) is essential for biological activity of MLN (86, 87), but the N-terminal structure (HIAFFS) of the zebrafish MLN-LP is quite different. The MLN-LP gene has also been identified in other fish, and the deduced amino acid sequences were HITFFSPKEMMVLKEQE (17 amino acids) in Takifugu (*Takifugu rubripes*) and HITFFSPKELLHMLRLEQQE (20 amino acids) in Medaka (*Oryzias latipes*) and these MLN-LPs are considered to be orthologs of the known MLN (80) (**Figure 2**).

In zebrafish, the MLN-LP gene was highly expressed in the intestine, moderately expressed in the liver, and expressed at low levels in the brain, heart and kidney (80). Interestingly, high expression of the MLN-LP gene in the liver has not been reported in mammals and birds. The ortholog of the MLN-R gene has been identified in zebrafish (34), and mammalian cells transfected with this receptor mRNA responded to the newly identified zebrafish MLN-LP (35, 80). These results indicate that an MLN system related to MLN-LP is present in zebrafish.

GI motility stimulation actions of MLN is mainly investigated *in vitro*. In isolated intestinal bulb and mid/distal intestine preparations of zebrafish, human MLN caused contraction of the intestinal smooth muscle. Erythromycin also induced contraction (34), though this compound was insensitive in the chicken GI tract (40). Interestingly, zebrafish MLN-LP caused contraction of the rabbit duodenum, but the affinity and efficacy were smaller than those of human MLN, indicating that zebrafish MLN-LP is a partial MLN-R agonist for mammalian MLN-R, although there is a substantial sequence difference (35). In the zebrafish intestinal bulb, zebrafish MLN-LP and human MLN induced only very small contractions at high concentrations (1–10 μ M), although ACh caused marked contraction at a concentration of 1 nM–1 μ M (**Table 1**). Liu et al. (80) examined

the expression of MLN-R mRNA in zebrafish and reported that the expression level was highest in the brain (especially in the hypothalamus) and low in the intestine. This might explain the low responsiveness of MLN-LP in zebrafish and suggests that the MLN system is not positively involved in the regulation of intestinal motility in zebrafish (35).

On the other hand, high expression of the MLN-R gene in the hypothalamus and hindbrain suggests functions of MLN in the CNS other than GI motility in zebrafish, such as food intake and energy homeostasis (80). In mammals, similarly, MLN-binding sites (MLN-R) in rabbit cerebellum cells (88) and human cerebellum cells (89) have been reported. However, our data indicated that the expression level of MLN-R genes was low in the zebrafish brain, similar with that in GI tracts (35). Detailed experiments are needed to clarify the presence of MLN-R in the CNS of zebrafish. Taken together, the results indicate that MLN-LP is expressed in the GI tract of fish but that its physiological role for regulating GI motility is skeptical due to low expression of the MLN-R. Expression of the MLN-R in the CNS suggests a novel function of MLN in fish.

REGULATION OF GASTROINTESTINAL MOTILITY BY GHRL

GHRL, a gut peptide consisting of 28 amino acids, was identified from rat stomach extracts as an endogenous ligand of a G protein coupled receptor (GPCR), growth hormone secretagogue receptor 1a (GHS-R1a), and it was shown to primarily stimulate the release of growth hormone (GH) from a pituitary (11, 14, 90). GHRL has a unique structural feature with fatty acid modification at the third serine residue (Ser-3), and acylation is essential for binding of GHRL to GHS-R1a and for eliciting its biological actions (11, 14). GHRL is mainly produced in cells called X/A-like endocrine cells in the mucosa of the stomach as an unacylated-type, and Ser-3 is modified by a middle-chain fatty acid such as *n*-octanoic acid or *n*-decanoic acid through GHRL-O-acetyltransferase (GOAT) that co-localizes in the same X/A-like cells (91). A relatively high concentration of unacylated GHRL is present in the stomach and plasma, but its physiological roles have not been fully clarified (14, 92). Regarding the sites of action (receptors), two types of GHS-R, designated type 1a (GHS-R1a) and type 1b (GHS-R1b), have been found, and GHS-R1a with seven transmembrane domains is activated by GHRL, whereas GHS-R1b with five transmembrane domains is not (14, 90, 93, 94).

GHRL was first discovered as an endogenous GH secretagogue, but subsequent studies indicated that GHRL is also produced in the hypothalamic arcuate nucleus and is involved in the regulation of food intake through GHS-R1a expressed on neuropeptide Y and orexin neurons (95–97). Consistent with its effect on feeding, plasma GHRL concentration increases after fasting, and the increased concentration of GHRL stimulates food intake (12, 14, 98). On the other hand, it has been shown that GHS-R1a mRNA and protein is expressed in regions of the CNS other than the hypothalamus, such as the hippocampus, substantia nigra, ventral tegmental area and dorsal and medial raphe, as well as in various peripheral organs including the

stomach, intestine, pancreas, thyroid, adrenal gland, kidney, heart and blood vessels (99–102). This ubiquitous expression of GHS-R1a mRNA and its protein suggests that GHRL has multiple functions, and accumulating evidence indicates its involvement in glucose metabolism, lipid metabolism, endocrine/exocrine functions, GI motility, cardiovascular functions, and reproduction (14, 84, 90).

GHRL has been identified not only in mammals but also in non-mammalian vertebrates from elasmobranch fish to birds (84, 103) (**Figure 3**). Evidence indicates that GHRL is predominantly produced in the stomach of all species with stomachs and that it is mainly produced in the intestine in stomach-less animals such as goldfish. The fundamental structure of GHRL, such as a common sequence at the N-terminal seven amino acids with an acyl modification at Ser-3 (Thr-3 in the bullfrog), has been conserved during vertebrate evolution, with some exceptions in fish and amphibians (**Figure 3**). Phylogenetic tree analysis indicated that GHRL falls into two lineages, mammalian-type and cartilaginous fish-type (103), and it was very recently proved that the relationship between the two is not orthologous but paralogous (13). GHRL receptors have also been identified in non-mammalian vertebrates and have been roughly divided into two groups, GHS-R1a and GHRL receptor-like receptor (GHS-R1a-LR) (84, 103–105). Results of studies on the effects of GHRL in vertebrates have suggested that the GH-releasing action is a common action of GHRL (106–110).

From the genetic structural features with strong similarity in peptide precursor and receptor levels, GHRL and MLN originate from a common ancestral gene and form a family (13): GHS-R1a and MLN-R belong to a family including G protein-coupled receptor (GPR) 39, neurotensin and neuromedin-U receptors (111) and show 52% overall amino acid identity and 86% identity in the seven-transmembrane regions (5, 12). As mentioned earlier, MLN regulates GI motility in several mammals, and GHRL is known to regulate GI function in mammals and non-mammalian vertebrates (**Table 2**).

Mammals

Effects of GHRL on GI motility were investigated using *in vivo* and *in vitro* experimental conditions in several mammals.

Rat and Mouse

Rats and mice are naturally deficient in the MLN system, as mentioned earlier (29), but they have the GHRL system instead [(11, 14), **Figure 3**]. GHRL bears GI motility regulating actions both *in vivo* and *in vitro* and increases gastric emptying (118, 119, 122, 127). The motility stimulation action of GHRL is caused by direct stimulation of the enteric neural pathway and capsaicin-sensitive vagal afferent neurons, which in turn stimulates parasympathetic efferent cholinergic neurons (118, 119, 122) (**Figure 1**, **Table 2**). Since GHS-R1a is not expressed in smooth muscles (99) and non-stimulated isolated gastric strips do not respond to GHRL (118, 122), direct action on smooth muscle cells is excluded as a stimulatory mechanism, different from the case of MLN. Interdigestive migrating complex (IMC)-like activity with short intervals (15–20 min) was also observed in the stomach of mice and rats (26, 27, 119), and GHRL enhances the appearance of the gastric IMC. Furthermore, a GHS-R1a

Human	GSSFLSP-EHQ-RVQQRKES-KK-PPAKLQPR---	(28)
Pig	GSSFLSP-EHQ-KVQQRKES-KK-PAAKLKPR---	(28)
Dog	GSSFLSP-EHQ-KLQQRKES-KK-PPAKLQPR---	(28)
Golden hamster	GSSFLSP-EHQ-KAQQRKES-KK-PQAKLQPR---	(28)
Rat/Mouse	GSSFLSP-EHQ-KAQQRKES-KK-PPAKLQPR---	(28)
Guinea pig	GASFRSP-EHH-SAQQRKES-RK-LPAKIQPR---	(28)
Cattle	GSSFLSP-EHQ-KLQ-RKEA-KK-PSGRLKPR---	(27)
Cat	GSSFLSP-EHQ-KVQ-RKES-KK-PPAKLQPR---	(27)
Rabbit	GSSFLSP-EHQ-KAQ-RKDA-KK-PPARLQPR---	(27)
House shrew	GSSFLSP-EHQ-KGP-KKDP-RK-PP-KLQPR---	(26)
Opposum	GSSFLSP-EHP-KTQ-RKET-KK-PSVKLQPR---	(27)
Chicken	GSSFLSP-TYK-NIQQQKDT-RK-PTARLH-----	(26)
Turkey	GSSFLSP-AYK-NIQQQKDT-RK-PTARLHPR---	(28)
Goose	GSSFLSP-EFK-KIQQQNDP-AK-ATAKIH-----	(26)
Duck	GSSFLSP-EFK-KIQQQNDP-TK-TTAKIH-----	(26)
Emu	GSSFLSP-DYK-KIQQRKDP-RK-PTTKLH-----	(26)
Green anole	GSSFLSP-EQP-KMQQRKVS-QK-SVTKFH-----	(26)
Red-eared slider turtle	GSSFLSP-EYQ-NTQQRKDP-KK-HT-KLN-----	(25)
Bullfrog	GLTFLSPADMQ-KIAER-QS--Q---NKL RHGNMN	(28)
Tropical clawed frog	GTSFLSP-A-D--LQ--KSSVKR-PPRKLQH-NEH	(27)
Zebrafish	GTSFLSP-T-Q-KPQGR-----R--PPRVG-----	(20)
Goldfish	GTSFLSP-A-Q-KPQGR-----R--PPRMG-----	(20)
Japanese eel	GSSFLSP-S-Q-RPQGR-D---K-KPPRVG-----	(22)
Channel catfish	GSSFLSP-T-Q-KPQNRGD---R-KPPRVG-----	(23)
Rainbow trout	GSSFLSP-S-Q-KPQVRQG---K-GKPPRVG-----	(24)
European seabass	GSSFLSP-S-Q-KPQSR-G---K--SSRVG-----	(21)
Mozambique tilapia	GSSFLSP-S-Q-KPQNKV----K--SSRIG-----	(21)
Hammerhead shark	GVSFH-P-RLKEKDDNSSGNSRK---SK-NP----	(25)
Stingray	GVSFH-P---QPRSTSKPSA-----	(16)
	*	

FIGURE 3 | Comparison of amino acid sequences of mature ghrelin in vertebrates. Asterisks and dots indicate amino acids identical in all species or identical in more than half of the species. The number of amino acids is indicated in parenthesis. Amino acid sequences were obtained from the DDBJ/EMBL/GenBank™ databases (acc#: AB029434 for human, AY028942 for pig, AB060700 for dog, NM_012488 for mouse, NM_021669 for rat, NC_037349 for cattle, AB089201 for cat, XM_002722463 for rabbit, AB364508 for house shrew, XM_001375640 for opossum, AB075215 for chicken, XM_003210209 for turkey, AY338465 for goose, EF613551 for duck, AY338467 for emu, NW_003338820 for green anole, AB161457 for red-eared slider turtle, AB058510 for bullfrog, NC_030680 for tropical clawed frog, NM_001083872 for zebrafish, AF454389 for goldfish, AB062427 for Japanese eel, AB196449 for channel catfish, AB096919 for rainbow trout, DQ665912 for European seabass, AB077764 for Mozambique tilapia, AB254128 for hammerhead shark, and AB4800033 for the red stingray).

antagonist, [D-Lys³]-GHRP-6, attenuates the frequency of IMC (26, 27), suggesting that GHRL serves as an alternative to MLN with regard to regulation of the gastric IMC in rats and mice.

In addition to the regulation of gastric motility, GHRL and its agonist applied into the lumbo-sacral spinal cord (region of the defecation control center) stimulated defecation of rats through activation of pelvic nerves and the connected enteric neurons (120). Intravenously injected GHRL failed to stimulate defecation (120), but a centrally acting GHRL receptor agonist, GSK894281, which is able to pass through the blood-brain barrier, was effective for stimulating defecation (128). GHRL-sensitive neurons are located in the lumbosacral defecation center and regulate defecation in rats (121) (**Figure 1**, **Table 2**). Therefore, in rodentia, GHRL is a GI motility regulator in both the stomach (digestion) and colon (defecation).

Guinea-Pig

Guinea-pig GHRL has recently been identified and demonstrated to locate in the mucosa of the stomach (125). As shown in

Figure 3, GHRL in most mammals has a common ten-amino-acid sequence at the N-terminus (GSSFLSPEHQ), but in the guinea-pig, three amino acids are different at positions 2, 5, and 10 within the sequence (GASFRSPEHH). In spite of the unique amino acid sequence, guinea-pig GHRL is able to activate both guinea-pig and rat GHS-R1a with almost the same affinity (125). Guinea-pig GHRL increases food intake when injected intraperitoneally (125).

The GI motility-stimulating action of GHRL in the guinea-pig was investigated using both rat and guinea-pig GHRL under an anesthetized condition *in vivo* and isolated muscle strips *in vitro*. Rat GHRL increased gastric motility *in vivo* and its stimulatory action was decreased by atropine, hexamethonium, and capsaicin. Unacylated GHRL was not effective at inducing gastric contraction (123). In addition, non-stimulated and stimulated isolated GI strips were insensitive to rat GHRL and guinea-pig GHRL *in vitro*. ACh release from enteric neurons was not enhanced by rat GHRL (123–125). These results suggest that only the vago-vagal reflex pathway was involved in

TABLE 2 | Comparison of gastrointestinal motility-stimulating actions of ghrelin in several vertebrates.

Species	In vitro study		In vivo study	Action site	Reference
	Unstimulated	Stimulated			
Fish					
Rainbow trout	No effect				(112)
Gold fish	No effect	No effect (EFS)			(112)
Zebrafish	No effect				(35)
Amphibians					
Bullfrog	No effect				(36)
Japanese fire belly newt	No effect				(36)
Reptiles					
Research not available					
Birds					
Chicken	Contraction			Smooth muscle (crop and proventriculus)	(77)
		Potentialiation of EFS		Enteric neurons (proventriculus)	(77)
Quail	No effect				(113)
					(41)
Mammals					
Human	No effect				(99)
			Contraction (Premature phase-III)		(114)
			Increase in gastric emptying		(115)
			Increase in gastrointestinal transit		(116)
Dog			No effect (motility and gastric emptying)		(117)
			Inhibition of phase-III	Decrease in motilin release	(49)
Rat	No effect	Potentialiation of EFS		Enteric neurons	(99, 118)
			Contraction	Enteric neurons and vago-vagal reflex	(118, 119)
			Increase in phase-III		(26)
			Contraction (colon)	Defecation center	(120, 121)
Mouse	No effect	Potentialiation of EFS		Enteric neurons	(24, 122)
			Increase in gastric emptying	Enteric neurons	(122)
			Increase in phase-III		(27)
Guinea-pig	No effect				(123–125)
		No effect (ganglion stimulant)			(124)
			Contraction (stomach)	Vago-vagal reflex	(123)
Rabbit	No effect				(126)
Asian house musk shrew	Contraction			Enteric neurons (presence of motilin required)	(19, 68)
			Contraction (phase-II), potentiation of phase-III	Enteric neurons and vago-vagal reflex	(20)

EFS, Electrical field stimulation for excitation of enteric neurons.

GHRL-induced gastric contractions and that GHRL mainly acted on the terminals of primary afferent neurons (123) (**Figure 1**, **Table 2**). Although the guinea-pig is a kind of rodentia, the actions of GHRL on enteric neurons reported in rats and mice have not been included in the mechanisms of GHRL-induced GI-stimulating actions. This may be due to very scant expression of GHS-R1a mRNA in the guinea-pig GI tract (125). Although the guinea-pig belongs to rodentia, IMC-like gastric motility observed in conscious mice and rats (26, 27) has never been reported. Therefore, the functional role of GHRL in regulation of the IMC has not been clarified.

Dog

Since dog indicates two patterns of GI motility depending on the feeding, the effect of GHRL has been investigated in the interdigestive state. GHRL intravenously applied at phase-I of the interdigestive state did not cause any mechanical changes and did not enhance gastric emptying in postprandial dogs, though GH-releasing activity was observed (117). However, interestingly, GHRL applied during phase-II and phase-III of the gastric IMC inhibited the appearance of phase-III and decreased plasma MLN level through activation of GHS-R1a (49). Regarding plasma GHRL concentrations, endogenous GHRL level changes in a

cyclic pattern, i.e., the peak of plasma GHRL concentration is observed in the phase-I period, and the lowest GHRL concentration appears in the phase-III period. The cyclic changes of plasma GHRL are opposite to those of plasma MLN (49, 129). Consistent with this fact, exogenous GHRL decreases plasma MLN level and, conversely, exogenous MLN decreases plasma GHRL level. This indicates mutual regulation of MLN release and GHRL release for maintaining a regular interval of the IMC in dogs (49). There have been no *in vitro* studies regarding the action of GHRL in a dog GI tract. Gathering these results, GHRL might regulate dog GI motility indirectly through control of MLN release. The underlying detailed mechanisms of the cyclic changes of GHRL release in the dog remain to be determined.

Human

Different from the action of GHRL in the dog GI tract, it has been shown that GHRL stimulates gastric motility and accelerates gastric emptying in healthy human volunteers (114–116, 130, 131). Therefore, GHRL has been proposed as a target for therapeutics of GI motility disorders, such as delayed gastric emptying and postoperative ileus (132). Regarding regulation of the IMC in fasting periods, intravenously applied GHRL induces a premature gastric phase-III in phase-I quiescent periods without stimulating MLN release. This premature IMC was accompanied by a prolonged increase in gastric tonus (114). However, plasma GHRL concentrations did not change in a cyclic manner, different from the change in MLN concentrations during IMC cycles, and the magnitude of change in plasma GHRL levels was small compared with that of MLN (45). In addition, the concentration of GHRL that caused the premature IMC was considerably high compared with the normal endogenous GHRL level, suggesting that GHRL causes phase-III-like contraction at pharmacological doses, but it is not a physiological regulator of the IMC in humans, actually MLN acts the role for regulation of the IMC (45).

Rabbit

Isolated muscle strips from the rabbit stomach did not respond to GHRL even at a relatively high concentration (10 μ M) (126). The results of an *in vivo* study have not been reported, but this kind of study is needed to examine the actions of GHRL on afferent vagus nerve terminals or the central nervous system.

House Musk Shrew

GHRL has been identified in the Asian house musk shrew (66) (**Figure 3**) and has been reported to stimulate gastric contraction in the latter half of phase-I and to enhance phase-II contractions. On the other hand, a GHS-R1a antagonist ([D-Lys³]-GHRP-6) suppressed the occurrence of spontaneous phase-II contractions and prolonged the time of occurrence of the peak of phase-III contraction. The pathway through the vagus nerve is essential for the GHRL-stimulating phase-II contraction (19, 20) (**Figure 1**, **Table 2**). Different contractile patterns in the interdigestive and digestive periods have been reported in the house musk shrew and interestingly, MLN has been shown to cooperate with GHRL in the initiation of phase-III-like gastric contractility (20, 54): in an *in vitro* study, treatment with GHRL alone did not cause

any gastric contraction, but GHRL showed contractile activity in the presence of a very low concentration of MLN. On the other hand, pretreatment with GHRL enhanced MLN-induced gastric contraction, and a positive correlation between GHRL and MLN was also found in anesthetized animals *in vivo*. Therefore, GHRL is essential for the phase-II contraction, and coordination of MLN and GHRL is necessary to initiate the phase-III contraction of the stomach (19, 20). Intrinsic primary afferent sensory neurons that are located in the mucosa are necessary for the synergistic responses between GHRL and MLN, and GHRL enhances MLN-induced contraction by disinhibition of the GABA neuron-mediated tonic inhibition (68). Coordination between GHRL and MLN has so far been shown only in the house musk shrew, and this animal has been proposed as a suitable small laboratory animal for neuro-gastroenterological study of the gastric IMC and for study on the coordination of MLN and GHRL.

Birds

Chicken

GHRL has been identified in chickens (**Figure 3**) and it has been shown that homologous GHRL stimulates GH release (108), but food intake is decreased by central and peripheral applications, different from the action observed in mammals (110, 133, 134). On the other hand, fasting increases plasma GHRL concentration as in mammals (135).

Since the opposite inhibitory effect on feeding regulation is observed in chickens, it would be interesting to examine the effect of GHRL on GI motility. Actually, GHRL acts on the chicken GI tract. In isolated GI muscle strips, chicken GHRL, but not rat GHRL, causes contraction of the upper intestine (such as the crop) and proventriculus and the lower intestine (such as the colon) but not of the middle intestine (such as the duodenum, jejunum and ileum). Interestingly, pharmacological analysis indicated different contractile mechanisms depending on the region: in the crop, GHRL acts on smooth muscle cells directly, and in the proventriculus, GHRL stimulates both smooth muscle cells and enteric neurons (77). Direct action of GHRL on smooth muscle cells is a characteristic of the chicken GI tract that has never been reported in mammals (**Table 2**, (136)). The region-dependent contractile responses correlate to the expression level of GHS-R1a mRNAs, indicating that the different degrees of responsiveness of the GI tract to GHRL are due to the heterogeneous expression of GHS-R1a (76). The region-dependency of the GHRL-induced contraction is in contrast to that of the MLN-induced contraction (77): in the crop, proventriculus and colon, which are sensitive to GHRL, MLN showed a weak effect, and in the middle intestine, MLN caused strong contraction, whereas GHRL caused only a small contraction. This has never been observed in mammals.

Ontogenic and developmental changes in morphology and function of the GI tract in the chicken have been reported. Since GHRL stimulates GH release and regulates energy homeostasis, we hypothesized that the degree of GHRL-induced contraction may change in growing chickens (76). In fact, GHRL-induced responses in the proventriculus decreased depending on age from day 0 to day 100 after hatching, and the decreased contraction

was correlated with reduction of GHS-R1a mRNA expression. However, age-dependent decreases in contraction and GHS-R1a mRNA expression did not occur in the crop. Therefore, the crop is a physiological target for GHRL in the chicken. A negative significant correlation between plasma GHRL level and GHS-R1a mRNA expression level in the proventriculus suggests down-regulation of GHS-R1a mRNA expression by increased plasma GHRL concentration to maintain the GHRL-induced response within a certain level (76, 137). The detailed unique mechanism of down-regulation of GHS-R1a mRNA in the proventriculus is unknown. However, GHS-R1a is mainly expressed on smooth muscle cells in the crop, whereas it is expressed on both smooth muscle cells and neural components in the proventriculus (77, 137). These different distributions of neural and muscular GHS-R1a may be responsible for the different aging-dependent changes: neural GHS-R1a may be easily down-regulated by increased endogenous GHRL. Similarly, contraction of the proventriculus induced by MLN, which involves both myogenic and neural mechanisms, decreases depending on age, but such a decrease does not occur in the ileum, where MLN acts on muscle receptors (76). In addition, contractions induced by carbachol and serotonin, which act on myogenic muscarinic and serotonin receptors, do not decrease with aging (76). Therefore, the age-dependent decrease in the responses to GHRL is due to the decreased neural GHS-R1a in enteric neurons of the proventriculus. In contrast to those *in vitro* studies, no *in vivo* study focusing on the regulation of GI motility by GHRL has been performed. The physiological significance of GHRL has not been fully clarified.

Quail

Quail GHRL and GHS-R1a have been identified (103, 113, 138). Intraperitoneal (ip) injection of a small dose of GHRL, but not intracerebroventricular (icv) injection of small doses, stimulates food intake, whereas large doses of GHRL, injected as both ip and icv, inhibit feeding as seen in chickens (139). Despite the different actions of GHRL on food intake, the pattern and degree of GHS-R1a mRNA expression in the GI tract are similar to those in chickens: i.e., the expression level of GHS-R1a mRNA is high both in the upper regions (esophagus, crop, and proventriculus) and lower regions (colon) of the GI tract and relatively low in regions of the middle intestine (duodenum, jejunum, and ileum) (113). In this condition, chicken GHRL-induced contraction

was very weak in all GI regions of the quail despite the fact that similar levels of GHS-R1a mRNA were expressed (113). Ineffectiveness of chicken GHRL in the quail proventriculus and duodenum has also been reported (41). Such a discrepancy between GHS-R mRNA expression level and contractile response has been reported in humans: substantial amounts of GHS-R mRNA and its protein are expressed in the stomach and colon (140), but GHRL did not cause mechanical responses or modify neural contractions (99). The underlying mechanisms are still not known, but there are some possibilities: (i) GHS-R1a mRNA has not been translated in GHS-R1a protein and (ii) most of the GHS-R1a is not involved in intestinal contraction and is linked to other intestinal functions as has been suggested in fish (141, 142). Since GHRL actions that we observed in the chicken and quail are quite different, it is necessary to examine the effects in other bird species in order to clarify the common action of GHRL in the avian GI tract. In addition, *in vivo* experiments are needed because GHRL regulates the GI tract by activation of the vago-vagal reflex and/or by activation of the CNS.

Reptiles

A GHRL peptide has been identified in the red-eared slider turtle (*Trachemys scripta elegans*) [Figure 3, (143)] and the partial sequence of squamata has been deposited in the NCBI database. However, nothing is known about its physiological roles such as GH release, food intake and GI motor functions in reptiles.

Amphibians

A GHRL peptide has been identified and its amino acid sequence has been determined in several species of amphibians [Figure 3, (84, 103, 107, 144, 145)]. GH-releasing activity has been demonstrated (107) and it has been shown that the endogenous GHRL level is increased by fasting (144) and GHRL stimulated food intake in the bullfrog larvae (146), indicating involvement in energy homeostasis. However, neither bullfrog GHRL nor rat GHRL caused contraction of the stomach and intestinal muscle strips, despite the fact that other stimulants, such as substance P and a muscarinic agonist, caused marked contraction (36). Similar to the bullfrog, GI strips from Japanese fire belly newts were also insensitive to newt GHRL and rat GHRL *in vitro* (36). The expression level of GHS-R1a in the bullfrog is comparable to that in chickens (36, 113), and this is the same as the case of the quail GI tract. GHS-R1a mRNA was expressed

TABLE 3 | Summary of the ghrelin and motilin systems in various vertebrates and their actions on gastrointestinal motility.

	Motilin	Motilin receptor	GI motility-stimulating action	Ghrelin	GHS-R1a	GI motility-stimulating action
Fish	○	○	×	○	○	×
Amphibians	ND	○	Δ (only bullfrog)	○	○	×
Reptiles	○	○	NE	○	○	NE
Birds	○	○	○	○	○	○ (only chicken)
Mammals	○ (except rodenita)	○ (except rodentia)	○ (except rodentia)	○	○	○

○, presence or determined; Δ, very weak and doubtful; ×, No effect.
ND, Not determined; NE, Not examined.

more predominantly in the mucosa than in the smooth muscle in the bullfrog intestine (36), indicating the possibility that GHRL in the bullfrog does not regulate GI motility but regulates mucosal functions, such as absorption of nutrients and secretion. On the other hand, the expression level of GHS-R1a mRNA in the GI tract of the Japanese fire belly newt was only about 10% of that in the GI tract of the bullfrog, and GHRL did not affect GI motility in the newts. A functional approach using isolated strips failed to demonstrate GHRL function in GI motility in an *in vitro* study, and it is necessary to investigate functions such as gastric emptying, intestinal transient and GI motility in an *in vivo* study.

Teleost Fish

The gene and peptide structures of GHRL have been identified in various fish species [Figure 3, reviewed by Kaiya et al. (84)]. As in mammals, GHRL mRNA was mainly expressed in the stomach of fish and in the intestine in stomach-less fish, such as goldfish and zebrafish. GHRL regulates energy balance through its actions on release of pituitary hormones, food intake, and lipid metabolism in fish (103, 110, 147).

In vitro experiments on the GI motility stimulation actions of GHRL have been performed in several fish. In the isolated rainbow trout (*Oncorhynchus mykiss*), stomach and intestinal strips, two types of rainbow GHRL [20 and 23 amino acids, (148)] did not cause any mechanical changes (112). The goldfish (*Carassius auratus*) is a stomach-less fish and has a thick intestine called the intestinal bulb instead of the stomach. Two types of goldfish GHRL, with different fatty acid modifications of Ser-3 (octanoyl and decanoyl forms) (149), and rat GHRL did not cause any mechanical changes in a preparation from the intestinal bulb. Stimulation of enteric nerves caused contraction by excitation of both cholinergic and non-cholinergic nerves, but both the octanoyl and decanoyl forms of goldfish GHRL applied during the stimulation did not modify the neural responses (112) (Table 2). These results indicate that GHRL does not play a crucial role for GI motor function in the rainbow trout and goldfish, being different from the results for chickens, mice and rats.

It is thought that GI motor functions might be markedly different in land-dwelling creatures and underwater creatures because of different kinds of food, living temperature and surrounding osmotic environment. Therefore, the lack of GI motility regulatory actions of GHRL and MLN observed in the rainbow trout, zebrafish, and goldfish might reflect these differences. However, little has been reported about the difference in the motor functions of GI between fish and mammals.

CONCLUSION

This review focused on control of GI motility by MLN and GHRL in vertebrates. Both peptides are thought to originate from the same ancestral gene, and they are predominantly produced in and released from the mucosa of the GI tract and act on MLN-R

and GHS-R1a. The two peptides have various functions, and their target is not only the GI tract. The GI motility-stimulating actions are different from animal species and seem to be reflected during the vertebrate evolution process (Table 3).

GHRL and MLN are present in fish, and the structure (amino acid sequence) of the identified MLN (called MLN-LP) is slightly different from that of avian and mammalian MLN. Their functional receptors are expressed in the GI tract, but neither of the peptides is involved in regulation of GI motility. In amphibians, neither MLN nor GHRL shows a remarkable effect on GI motility despite the considerable presence of receptors in the GI tract. Depending on species, MLN may have begun to control GI motility partially as observed in the bullfrog, but not by GHRL. Nothing is known about GI motility stimulating actions of the peptides in reptiles, but the structure of the MLN-like peptide changes into an avian and mammalian type in some species. In avian species, although expression levels of receptors are sufficient to function, species where GHRL and MLN stimulate GI contraction or not are more apparent, and a region-dependent control of GI motility by GHRL (upper and lower intestines) and MLN (middle intestine) has begun to observe. Only after this evolutionary process, the ligand-receptor-GI motility regulation system begins to link to causing contraction, and the mechanism varies depending on the part of the GI tract. In chickens, it has been demonstrated that GHS-R1a is expressed on both smooth muscle cells and enteric neurons and that age-dependent down-regulation of neural GHS-R1a occurs. Furthermore, when the evolutionary stage is reached in mammals, the diversity of stimulatory mechanisms is further increasing. In rats and mice, the MLN system is lost and GI contraction is controlled only by the GHRL system. The guinea-pig is losing the MLN system at the molecular level and is also losing regulation of GI motility by local actions of GHRL. Moreover, in the house musk shrew, GHRL and MLN show a cooperative effect in regulation of GI motility, and the action was specialized in the regulation of phase-III contraction. GHRL is a physiological regulator of the IMC in rodentia and the house musk shrew, and MLN regulates the gastric IMC in a fasting state in humans and dogs and translates the hunger signal from the periphery to the brain (Table 3).

In this way, while MLN and GHRL are hormones produced in the GI tract, they are not initially involved in the regulation of GI motility. However, it seems to act on the regulation of GI motility more when nearer the region of producing cells (GI tract), with animal evolution (Table 3). If that is so, what is the action of MLN or GHRL to begin with? Why did they not act in the GI tract despite being produced there? Why did they come to affect GI motility? It is interesting to anticipate it from the change of action when considering vertebrate evolution.

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Dynamics of Insulin Signaling in the Black-Legged Tick, *Ixodes scapularis*

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Insulin-like peptides (ILPs) have been identified in several invertebrates, particularly insects, and work on these ILPs has revealed many roles including regulation of energy homeostasis, growth, development, and lifespan to name a few. However, information on arthropod ILPs outside of insects is sparse. Studies of Ixodid tick ILPs are particularly scarce, despite their importance as vectors of infectious agents, most notably Lyme disease. The recent publication of the genome of the black-legged tick, *Ixodes scapularis*, has advanced opportunities to study this organism from a molecular standpoint, a resource sorely needed for an organism with challenging life history requirements for study in the laboratory, such as a long life cycle and obligate, prolonged, blood-feeding at each life stage. Through bioinformatics searches of the tick genome and other available *I. scapularis* databases, we identified four putative ILP sequences. Full-length sequences of these ILP transcripts were confirmed, and quantitative RT-PCR was used to examine expression levels of these ILPs in different life stages, feeding states, and adult tissues. This work serves as an initial characterization of ILP expression in ticks and provides the foundation for further exploration of the roles of ILPs in these important arthropod vectors.

Keywords: insulin, tick, ILPs, peptide, blood-feeding

INTRODUCTION

Insulin-like peptides (ILPs) are recognized to have several important functions in arthropods. This has been most extensively characterized in the dipterans *Drosophila melanogaster* and *Aedes aegypti* (1–4). Insects have multiple ILPs, ranging from as few as four in planthoppers (5), to eight in *Ae. aegypti* and *D. melanogaster* (2, 6, 7), to over 30 in *Bombyx mori* (8). The functions of these ILPs in most arthropods are largely unexplored, but, where investigated, ILPs have demonstrated pleiotropic roles, regulating metabolism, growth, reproduction, longevity, and larval molt, among other functions (9–11). Tissue expression of ILPs is also variable, with many produced in nervous tissue for neurohemal release. Others are produced in the midgut, fat body, or imaginal discs (7, 12, 13).

ILPs are variable in amino acid sequence, but are united in sharing conserved cysteine residues consistently spaced throughout the molecule. These cysteines form disulfide bridges, folding the peptide into a domain structure required for insulin receptor (IR) activation. The synthesis and post-translational processing of ILPs also is conserved. A prepropeptide is translated with B, C, and A chains (14). After disulfide bonds form, the C-chain is processed at dibasic cleavage sites, leaving

behind the joined B and A chains. Some speculate that the C-chain is left intact in certain arthropod ILPs as is the case with human Insulin-like Growth Factor I (IGF-I) (2). However, to date, few native arthropod ILPs have been isolated and none of these examples retain the C-peptide (15).

Rapid genome and transcriptome sequencing, and associated bioinformatics processing of such data, have opened up previously unavailable resources for the study of neuropeptides and peptide hormones in arthropods. Our initial understanding of insect ILP structure, function, and signaling dynamics is the result of the isolation of bombyxin from *B. mori* (16) and *Locusta* Insulin-Related Peptide (LIRP) from *Locusta migratoria* (17). Characterization of individual ILPs through painstaking genetic methods such as PCR amplification with degenerate primers eventually gave way to available genomic and transcriptome databases. The availability of these sequences has permitted the use of genetic techniques such as RNAi and quantitative PCR to advance our knowledge of specific ILP functions. However, extraction, quantification, and synthesis of the ILP molecules themselves for study has remained a challenge for many arthropods.

The above mentioned research on ILPs is dominated by more easily studied model insects, not to mention the exhaustive vertebrate literature on insulin, IGFs, and relaxin, and little information on neuropeptide signaling in arachnids exists. In ticks, immunocytochemical evidence for the presence of ILPs has been reported in *Ornithodoros parkerii* (18) and *Rhipicephalus appendiculatus* (19) using an antibody to bombyxin. Immunostaining in *Dermacentor variabilis* using an anti-insulin antibody demonstrated a decrease in immunoreactivity in the synganglion after blood-feeding and mating (20). A partial fragment of an apparent A-chain of a *D. variabilis* ILP was detected by 454 pyrosequencing of the transcriptome (21), and a subsequent study showed a decrease in expression of this ILP in partially fed and replete ticks in comparison to unfed ticks (22). In *Amblyomma americanum*, upregulation of an ILP was detected in pathogen-challenged ticks (23), and knockdown of Insulin-like Growth Factor Binding Protein (IGFBP) in this tick prevented blood-feeding females from feeding to repletion (24).

In the United States, ticks are responsible for 95% of vector borne diseases (25). The publication of the genome sequence of *Ixodes scapularis*, the black-legged tick, has provided a wealth of information on this important vector (26). This was a much needed resource in our efforts to prevent the debilitating diseases transmitted by these ticks and others, including Lyme disease, babesiosis, anaplasmosis, ehrlichiosis, Rocky Mountain Spotted Fever, Southern Tick-Associated Rash Illness, Tick-Borne Relapsing Fever, tularemia, Colorado tick fever, Powassan encephalitis, and Q fever (25). In addition to its utility as a way to study vector-pathogen interactions, the *I. scapularis* genome and transcriptomes provide a unique opportunity to examine neuropeptides in a non-model arthropod. Therefore, in this study, we characterized the sequence and expression dynamics of ILPs in *I. scapularis*, to provide a basis for further explorations of these signaling molecules in ticks.

METHODS

Bioinformatics Identification of *Ixodes scapularis* ILPs

Amino acid sequences from previously identified ILPs from *Ae. aegypti*, *Anopheles stephensi*, *An. gambiae*, *D. melanogaster*, and vertebrate ILPs [*Mus musculus* insulin and insulin-like growth factor I (IGF-I)] were used as queries in tBLASTn searches of the *I. scapularis* genome and expressed sequence tag (EST) databases, and *I. ricinus* transcriptome (TSA) databases through the National Center for Biotechnology Information (NCBI) website. To ensure detection of ILPs that might deviate from canonical insect ILP sequences, these queries were further searched against all available arthropods, from whole genome sequencing reads, transcriptomes, and EST databases. From these results, putative ILP amino acid sequences from representative arthropods outside of Hexapods were selected with an emphasis on arachnids (e.g., Aranae, Acari, Scorpiones, Opiliones, Amblypygi, Pseudoscorpiones, Solfugae), but also including the outgroup Merostomata (Atlantic Horseshoe Crab, *Limulus polyphemus*) (**Supplementary Table 1**). These were then leveraged as queries in searches against the *I. scapularis* genome and EST database to identify ILP signatures potentially unique to non-Hexapod arthropods.

Analyses of signal peptide sequences within putative prepropeptides were conducted using both the Neural networks and the Hidden Markov models contained in the SignalP program [<http://www.cbs.dtu.dk/services/SignalP/> (27)].

I. scapularis Colony

A colony of *I. scapularis* was maintained at 20°C under 16:8 light:dark conditions at 95% humidity. Larvae and nymphs were fed on mice as described previously (28). Adults were fed on rabbit (29). All animals were treated humanely according to the guidelines of the Institutional Animal Care and Use Committee (IACUC protocol # 00682).

Sample Collection and RNA Extraction

RNA was extracted from three separate biological cohorts of *I. scapularis* eggs (~500), unfed larvae (10 individuals), nymphs (10 individuals), adult female (2 individuals), and adult male (5 individuals) ticks by placing them separately into 1.7 ml tubes, and extracting with TRIzol™ reagent (Invitrogen), following a slightly modified protocol from the manufacturer's instructions. The modified protocol is as follows: *I. scapularis* were collected into 1.7 ml tubes and were ground in liquid nitrogen with autoclaved plastic pestles. These samples were further homogenized in 100 µl of TRIzol™ with a tissumizer, then brought to 800 µl with additional TRIzol™. Solid tissue debris was pelleted by centrifugation at 12,000 x g at 4°C for 10 min, and the supernatant was transferred to a fresh tube. Thereafter, the manufacturer's protocol was followed for extraction of total RNA.

The resulting RNA pellet was resuspended in 20 µl of RNase/DNase free water incubated at 55–60°C for 10 min. RNA was treated with DNase I from the Direct-zol™ RNA MiniPrep Kit (Zymo Research) at RT for 30 min. Columns from this kit

were pre-wet with RNA Wash Buffer, briefly centrifuged, then the DNase-treated RNA was added directly to the columns. Manufacturer protocols were followed for subsequent RNA recovery. Total RNA quantity was measured with Nanodrop spectrophotometer. Purity was determined by 260/280 and 260/230 ratios, with acceptable values in the ~1.8 and 2.0–2.2 range, respectively (all samples collected met purity standards). RNA samples were immediately stored at -80°C .

Sequencing Confirmation of *I. scapularis* ILPs

To confirm sequences, primers were designed to the identified *I. scapularis* ILP sequences using Primer3 (30, 31) and estimated optimal annealing temperatures were determined using Integrated DNA Technologies' online Oligoanalyzer tool (IDT) (Table 1). Synthesis of *I. scapularis* cDNA was accomplished using the SMARTer cDNA synthesis kit according to manufacturer's instructions (Takara Bio USA, Mountain View, CA) and 1 μg total RNA obtained from nymphs. These cDNA template and primers were used in polymerase chain reactions (PCR) to amplify sections of *I. scapularis* cDNA using Biotool Taq polymerase (Biotool, Ely, UK) with the following program: 40 cycles at 94°C , 30 s; 60°C , 30 s; 72°C , 1 min. This included an initial 5 min denaturing step at 94°C , and a final 10 min, 72°C extension step. These reactions were separated with gel electrophoresis and bands of the expected size were extracted from the gel and sent for Sanger sequencing (Genewiz, South Plainfield, NJ). Resulting sequences were aligned with the predicted sequences.

To confirm 5' and 3' ends of ILP transcripts not accessible to primer design, 5' and 3' rapid amplification of cDNA ends (RACE) was performed. For 3' RACE, the anchor primer NotI d(T)2 (Table 1) was used to initiate cDNA synthesis using SMARTer cDNA synthesis kit and 1 μg total RNA from blood-fed nymphs. This template was used in nested PCR reactions as above, using gene-specific reverse primers and the NotI anchor primer. For 5' RACE, specific ILP reverse primers were used with the SMARTer cDNA synthesis kit for generation of cDNA. These cDNA strands were purified and the 3' end was polyadenylated (A) using Terminal Deoxynucleotidyl Transferase (TdT) and dATP (Thermo Fisher Scientific Inc.). Poly-A tailed cDNA was again isolated and used as template in nested PCR reactions employing NotI d(T)2, NotI and gene specific reverse primers. Resulting products were separated by gel electrophoresis and subcloned using the TOPOTM TA CloningTM Kit for Subcloning (Invitrogen, Waltham, MA, USA). Plasmids were isolated from successful transformants and were Sanger sequenced as above.

Expression of *I. scapularis* ILPs

ILP expression was characterized using quantitative RT-PCR (qRT-PCR). The iScriptTM cDNA Synthesis Kit was used to convert 500 ng–1 μg *I. scapularis* total RNA samples from eggs, larvae, nymphs, and male and female adults (see above) into cDNA following manufacturer's protocols (BioRad, Hercules, CA, USA). Briefly, cDNA of ticks from different life stages was diluted 5x with deionized H₂O before using it as a template in qRT-PCR experiments. One microliter cDNA was used in each 10 μl qRT-PCR reaction. Four different housekeeping genes

TABLE 1 | Primer sequences used to amplify IsILP sequences for confirmation by Sanger sequencing or for relative quantification by qRT-PCR.

Primer	Nucleotide sequence
SEQUENCING PRIMERS	
NotI d(T)2	AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTT TTTTTTTTTTTTTTTTTTVN
NotI-d(T)	AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTT TTTTTT
ILP1F2	GATCGGTCAGCGACTGTTC
ILP1Fwd5	TTTCTGGGCACTCAACAC
ILP1R210	GGACACCAGAGGAAACAGTC
ILP3F3	TGCTCGACTTCCTCTGTGAAG
ILP3R3	CAGTACGCGAGAAGCTCCAG
ILP4F2	CTCTAGCCTGGACGCCCTTC
ILP4Fwd87	AACCACCGAAGAACAACAGC
ILP4R1	GAGGGCCTGTAGATCACGAA
ILP5F1	CGGAGATGACGGACCTGTTT
ILP5Fwd343	AGCTCGTCTCACACATGAAC
RT-QPCR PRIMERS	
ILP1Fwd131	AGGCAGAGTTCTACGATCCG
ILP1Rev210	GGACACCAGAGGAAACAGTC
ILP3Fwd108	CTTTTGAGGCTCTGCCAAGA
ILP3Rev201	CCCGTGCGCTTGTGTATC
ILP4Fwd87	AACCACCGAAGAACAACAGC
ILP4Rev189	GCCTCCACCAGGTCTACGAA
ILP5Fwd343	AGCTCGTCTCACACATGAAC
ILP5Rev452	CAGGAACAGGTCGTCATCT
β -tubulinFwd	TGAATGACCTGGTGTCCGAG
β -tubulinRev	GACAAGCTGTTCAAGCCTCT

were tested: β -tubulin, ubiquitin, ribosomal protein 4 (rps4), and I13A. β -tubulin was selected for final experiments because of its consistent expression in all life stages and tissues. Sequences of specific primers for IsILPs and the housekeeping gene, β -tubulin, are listed in Table 1. Each sample was run in triplicate wells of 96-well plate. qRT-PCR was performed on CFX touch Real-Time PCR Detection system using SYBR green master mix (BioRad, Hercules, CA, USA). All reactions were performed with initial 5 min at 95°C , followed by 40 cycles of 10 s at 95°C , 15 s at 64°C , and 15 s at 72°C , and a melt curve was analyzed at 70– 95°C . Relative expression was calculated using $2^{-\Delta\Delta\text{Ct}}$ method. Experiments were replicated two to four times with different tick cohorts.

Statistical Analysis

All data were analyzed using GraphPad Prism 7 software (La Jolla, CA, USA). Relative expression values of IsILP expression in different developmental stages and tissues were compared with one-way ANOVA, followed by Dunnett's multiple comparison test.

RESULTS

I. scapularis ILP Identification

Four sequences with ILP characteristics were identified through bioinformatics searches of *I. scapularis* genome, transcriptome,

and EST databases. The use of mosquito ILPs as queries in tBLASTn searches was sufficient to detect these sequences and no additional sequences were found with subsequent searches employing other arthropod ILPs. Sequences containing complete features of ILPs (start and stop codons, B and A chains, and signal peptide) were found for three *I. scapularis* ILPs. For IsILP4, only a partial sequence was obtained for *I. scapularis* which contained no stop codon. However, a highly similar transcriptome sequence from *I. ricinus* (accession: GANP01013821) was found that did contain a stop codon and untranslated regions further downstream. Reverse primers designed to these regions in the *I. ricinus* sequence were capable of amplifying PCR products in combination with forward primers from *I. scapularis*.

Complete cDNA sequences of four ILPs were confirmed by Sanger sequencing of PCR products and 3' and 5' RACE products. These sequences contain features consistent with ILPs in other animals, including start and stop codons, signal peptides, conserved cysteine residues, and putative dibasic cleavage sites (Figures 1, 2). IsILP1 was named as such considering it was the first *I. scapularis* ILP noted in the literature (21). The remaining ILPs (3, 4, and 5) were named according to their amino acid similarity to *An. gambiae* ILPs, particularly ILP5 which contains additional amino acids between key Cys residues of the A-chain in comparison to other ILPs (Figure 2 and see section Discussion).

We encountered but were unable to amplify a putative additional *I. scapularis* ILP sequence that contained spacing of cysteines characteristic of an ILP A-chain: ILVFFSVKPDKKPAFSSKCCDGDCTKSVWKGSKISFHVFLPAPFVRVRE (accession: ABJB010579748). This may represent a pseudogene or an unrelated sequence that was picked up in our bioinformatics search by chance, or genome assembly in this region is of insufficient quality to design functional primers.

***I. scapularis* ILP Developmental Expression**

ILP expression varied markedly between the developmental stages sampled. For fold difference calculations, eggs samples were used as a baseline (control). IsILP1 was significantly more expressed in the unfed larvae and adult females compared to eggs, nymphs, and adult males. In contrast, IsILP3 and IsILP5 expression was significantly higher in unfed larvae as compared to other life stages. IsILP4 was significantly more expressed in adult females, followed by larvae and nymphs having significantly more expression than adult males and eggs. For most IsILPs, there was little to no expression in adult males and eggs (Figures 3A–D).

IsILP1 and IsILP5 expression was significantly higher in unfed stages (larvae, nymphs, and adults) compared to the levels at 2 and 7 days after detachment from the host (Figures 3A,D). For IsILP3, expression was highest in unfed larvae compared to detachment from the host (Figure 3B); whereas in nymphs and adult females expression increased after detachment from the host and was higher at 2-day post detachment in adults and 7 day post detachment in the nymphs. For IsILP4, expression was significantly higher in unfed larvae and adults, whereas there was no difference in expression before and after a blood meal in the nymphal stage (Figure 3C).

***I. scapularis* ILP Adult Female Tissue Expression**

Synganglion, salivary gland, midgut, and ovaries from unfed females were dissected to understand baseline tissue expression profiles of IsILPs. IsILP1 and IsILP5 had significantly higher expression in synganglion. IsILP5 expression was limited to synganglion. IsILP3 and IsILP4 expression was significantly higher in salivary glands. However, both expressed in all tissues examined.

IsILP1 expression was reduced after a blood meal in all tissues except for ovaries where no difference was noted in expression before or after feeding. IsILP3 expression was highest in unfed females in all tissues examined whereas IsILP4 expression was higher in midgut 7 days post detachment from the host. IsILP5 expression was highest in the synganglion of the unfed females. We noted some expression in ovaries; however, the Ct values were too high to accurately measure expression (36–38 out of 40 cycles) suggesting very little transcript was available. IsILP5 expression was higher in a few midgut and salivary gland samples at 7 day post detachment (Figure 4).

DISCUSSION

To date, little examination has been made of ILPs in arachnids and our characterization of ILPs in *I. scapularis* provides a foundation for further exploration of the roles of these neuropeptides in this medically important arthropod. The discovery of four ILPs is consistent with the pattern seen in insects of multiple ILPs. Some of the *I. scapularis* ILP sequences we characterized have been previously noted as ILPs in the literature or as automated annotations in online databases. For instance, putative transcripts for IsILP1 were noted from *I. scapularis* (34) that were highly similar to an A-chain fragment from *D. variabilis* (21). However, this is the first study to specifically focus on IsILPs, and IsILPs 3 and 4 are identified for the first time in our study. It is of interest that only IsILP5 (ISCW002549, partial fragment annotated as ILP4), and not IsILP1, was initially annotated in the *I. scapularis* genome (26). Also, an ILP sequence was recently recognized from *A. americanum* that is highly similar to IsILP4, yet this did not map to the *I. scapularis* genome (23). This highlights the large and scattered nature of the current *I. scapularis* genome and the challenge of assembling and annotating a genome with extensive repetitive sequences and expansive intron regions.

The IsILPs we identified fit the characteristic hallmarks of ILPs in other organisms, containing signal peptides, B and A chains, and dibasic cleavage sites for excision of the C-peptide. Conserved cysteine residues are predicted to form disulfide bridges between B and A chains or internally in the A chain (Figures 1A, 2A). Insulin-like growth factors (IGFs) have a similar structure, yet retain a short C-peptide that is not cleaved (11). None of the IsILPs in this study appear to have a short C-peptide indicative of an IGF-like structure. Some other commonalities for ILPs include basic (Arg, Lys, and His) and amino acids with aromatic rings (Tyr and His)

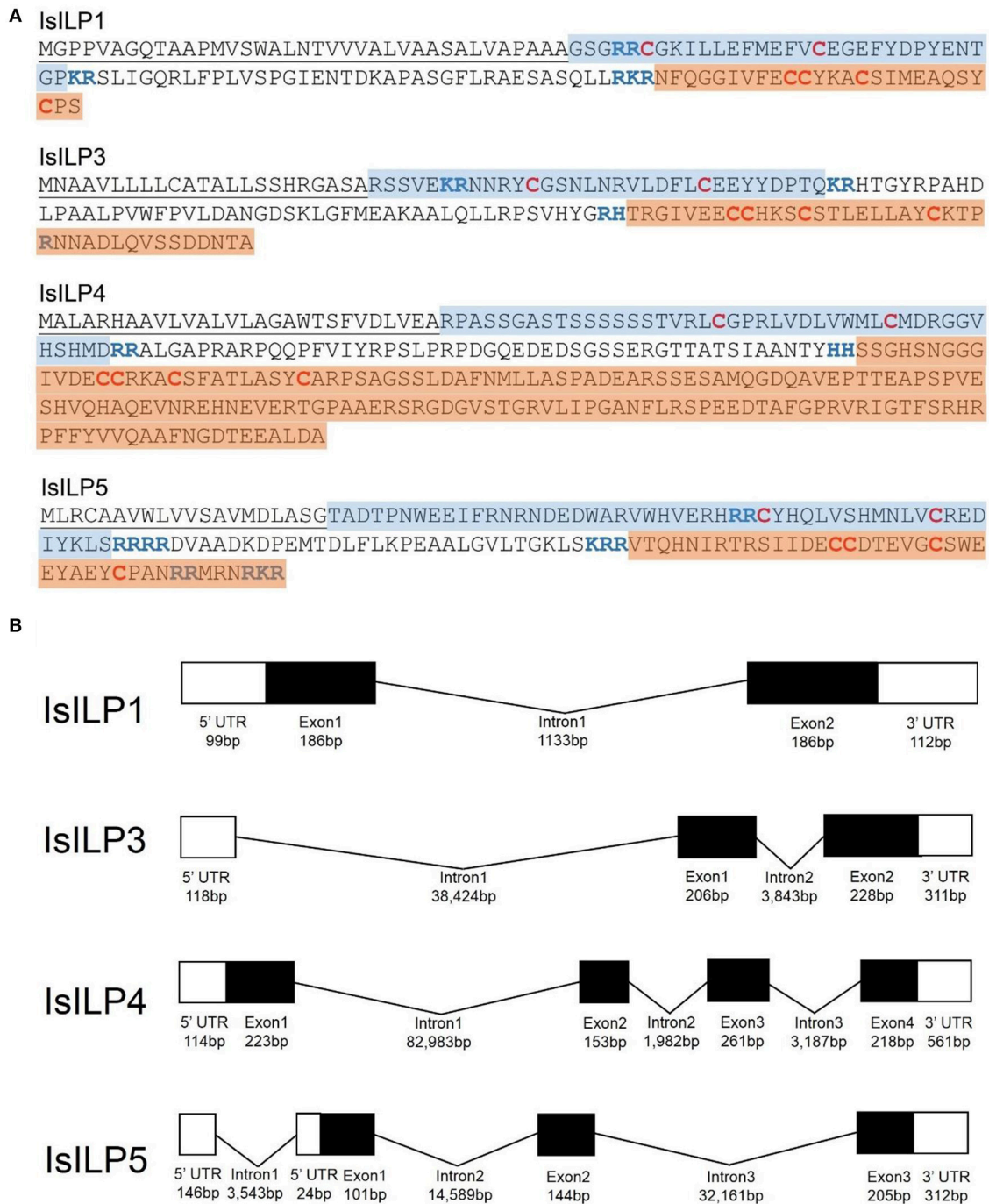
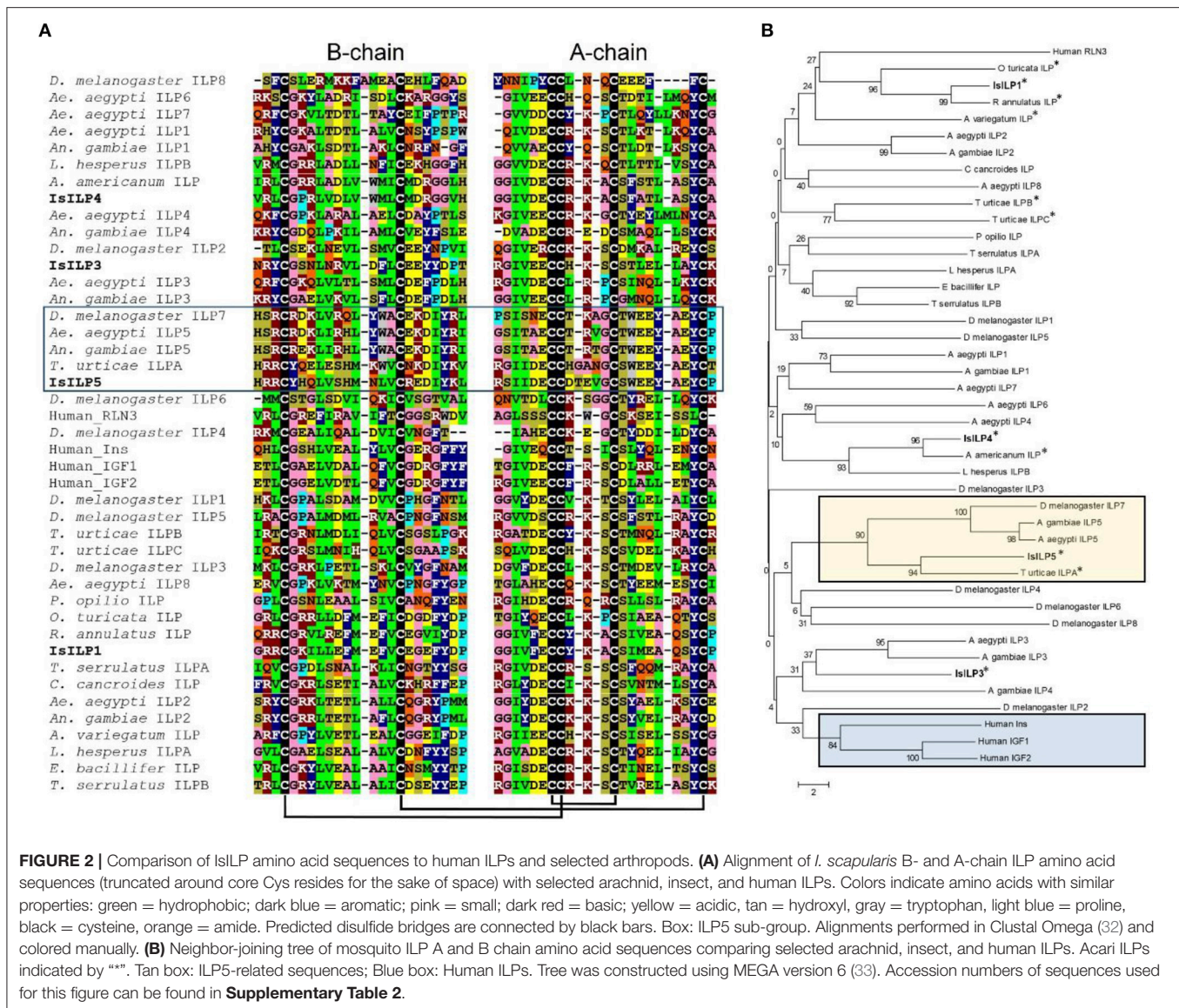


FIGURE 1 | Structural features of *I. scapularis* ILPs. **(A)** Amino acid translations and structural predictions of *I. scapularis* ILP sequences confirmed by Sanger sequencing. Cysteine disulfide bridges in bold red text. Predicted di-basic cleavage sites in bold blue text; B-chain: Blue highlight; A-chain: Orange highlight; Signal peptide underlined. Genbank accession numbers: IsILP1: MK649814; IsILP3: MK649815; IsILP4: MK649816; IsILP5: MK649817; **(B)** Predicted gene models of IsILPs based on alignment of confirmed sequences to the *I. scapularis* genome (IsILP1, IsILP3: Contig: PKSA02008623, IsILP4: Contig: PKSA02013804, IsILP5: Contig: PKSA02002782). Intron and exon lengths in the diagram are not proportional.

upstream of the first Cys of the B-chain, and consistently spaced hydrophobic (particularly Leu) or charged residues (frequently Glu, but also Asp). IsILP5, AgILP5, AaILP5, and DmILP7 are

unusual in that they have additional amino acids between Cys 2 and 3 of the A-chain compared to other ILPs, where there are consistently three amino acids (**Figure 2A**). These particular



ILPs share considerable amino acid sequence conservation in comparison to other ILPs, particularly in the A-chain. Outside of this particular sub-group, IsILPs vary greatly in their similarity to each other and to those of other arthropods (Figure 2). As has been the case with other invertebrate ILPs, amino acid sequence similarity alone provides only limited information as to their potential functions when comparing between species. It is likely that the secondary structure imposed by disulfide bonds and as yet unverified key amino acid residues may preserve the functionality of these molecules in binding to their cognate IRs. Unfortunately, the chemical synthesis of invertebrate ILPs has been a challenge and has so far limited the study of structure-activity relationships with techniques such as alanine screens (3, 15).

The IsILPs we identified in this study had from two to four exons (Figure 1B), comparable to other arthropod examples (A.

gambiae: 1–3 exons; *Ae. aegypti*: 2–3 exons; *D. melanogaster*: 1–3 exons) (35, 36). Curiously, the ILP with the most conserved amino acid structure among the arthropods we examined, ILP5 (DmILP7), exhibited high variability in intron number between organisms (AgILP5: 0 introns; AaILP5: 1 intron; DmILP7: 2 introns; IsILP5: 3 introns). The gene structure of IsILP1 was consistent with previous predictions in *I. scapularis* of a single intron (21). IsILPs 3, 4, and 5 are predicted to have massive introns, ranging from 32,161 to 82,983 bp. However, this is not inconsistent with observed intron lengths in other organisms such as IGF1 in humans, which has a 55,952 bp intron (35). Both IsILP1 and IsILP3 were found on the same contig (PKSA02008623), but curiously the entire IsILP1 gene sequence was found within intron 1 of the IsILP3 sequence. We encountered another contig containing both IsILP1 and IsILP3 (PKSA02013879) with a similar gene structure, but with different

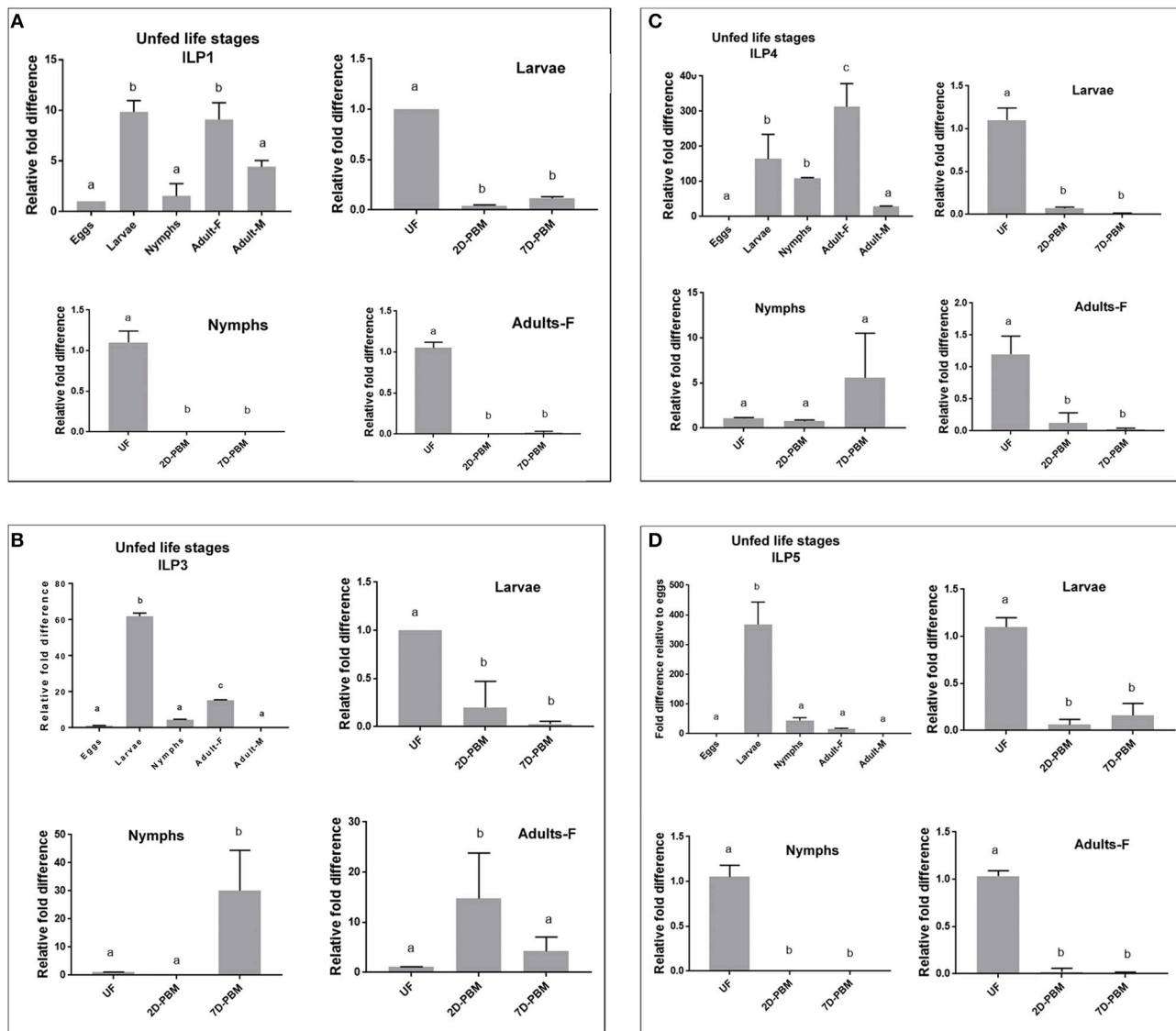


FIGURE 3 | Expression of IsILP transcripts in *I. scapularis* life stages, as determined by quantitative RT-PCR. F, female; M, male; UF, unfed; 2D-PBM, 2 days post engorgement and host detachment; 7D-PBM, 7 days post engorgement and host detachment. Fold expression levels comparing life stages were scaled to expression in eggs. Feeding trials were examined within life stages by scaling expression to the unfed state. Treatments were examined by one-way ANOVA followed by Dunnett's multiple comparison test. **(A)** Expression of IsILP1 in unfed developmental stages and larvae, nymphs, and adult females post detachment. All unfed stages, $F_{(4,5)} = 29.27$, $P = 0.001$; Larvae: $F_{(2,3)} = 4583$, $P < 0.0001$; Nymphs: $F_{(2,3)} = 121$, $P = 0.001$; Adults: $F_{(2,3)} = 410.2$, $P = 0.0002$. **(B)** Expression of IsILP3 in unfed developmental stages and larvae, nymphs, and adult females post detachment. All unfed stages, $F_{(4,5)} = 1968$, $P < 0.0001$; Larvae: $F_{(2,3)} = 21.46$, $P = 0.01$; Nymphs: $F_{(2,9)} = 16.9$, $P = 0.0001$; Adults: $F_{(2,9)} = 6.774$, $P = 0.01$. **(C)** Expression of IsILP4 in unfed developmental stages and larvae, nymphs, and adult females post detachment. All unfed stages, $F_{(4,5)} = 16.5$, $P = 0.004$; Larvae: $F_{(2,3)} = 111$, $P < 0.001$; Nymphs: $F_{(2,8)} = 3.002$, $P = 0.10$; Adults: $F_{(2,3)} = 24.25$, $P = 0.01$. **(D)** Expression of IsILP5 in unfed developmental stages and larvae, nymphs, and adult females post detachment. All unfed stages, $F_{(4,15)} = 101.9$, $P < 0.0001$; Larvae: $F_{(2,6)} = 99.08$, $P < 0.0001$; Nymphs: $F_{(2,9)} = 264.6$, $P < 0.0001$; Adults: $F_{(2,6)} = 661.4$, $P < 0.0001$.

intron lengths for IsILP3 (Intron 1: 35,843 bp; Intron 2: 3,773 bp). It is unclear whether the current genome assembly accurately reflects the gene structure of these IsILPs. This confusion may be clarified with newer sequencing technologies permitting longer reads allowing more robust genome assemblies.

Perhaps of greater value toward understanding IsILP functions are the expression patterns of ILPs. In specific

body tissues, IsILP5 was almost exclusively expressed in the synganglion suggesting a neurotransmitter or neurohemal role. IsILP1 was also expressed at significantly higher levels in the synganglion compared to other tissues. In contrast, IsILPs 3 and 4 were expressed in all tissues examined with highest expression in the salivary glands. Tick salivary glands undergo a growth phase during feeding in order to increase saliva production and

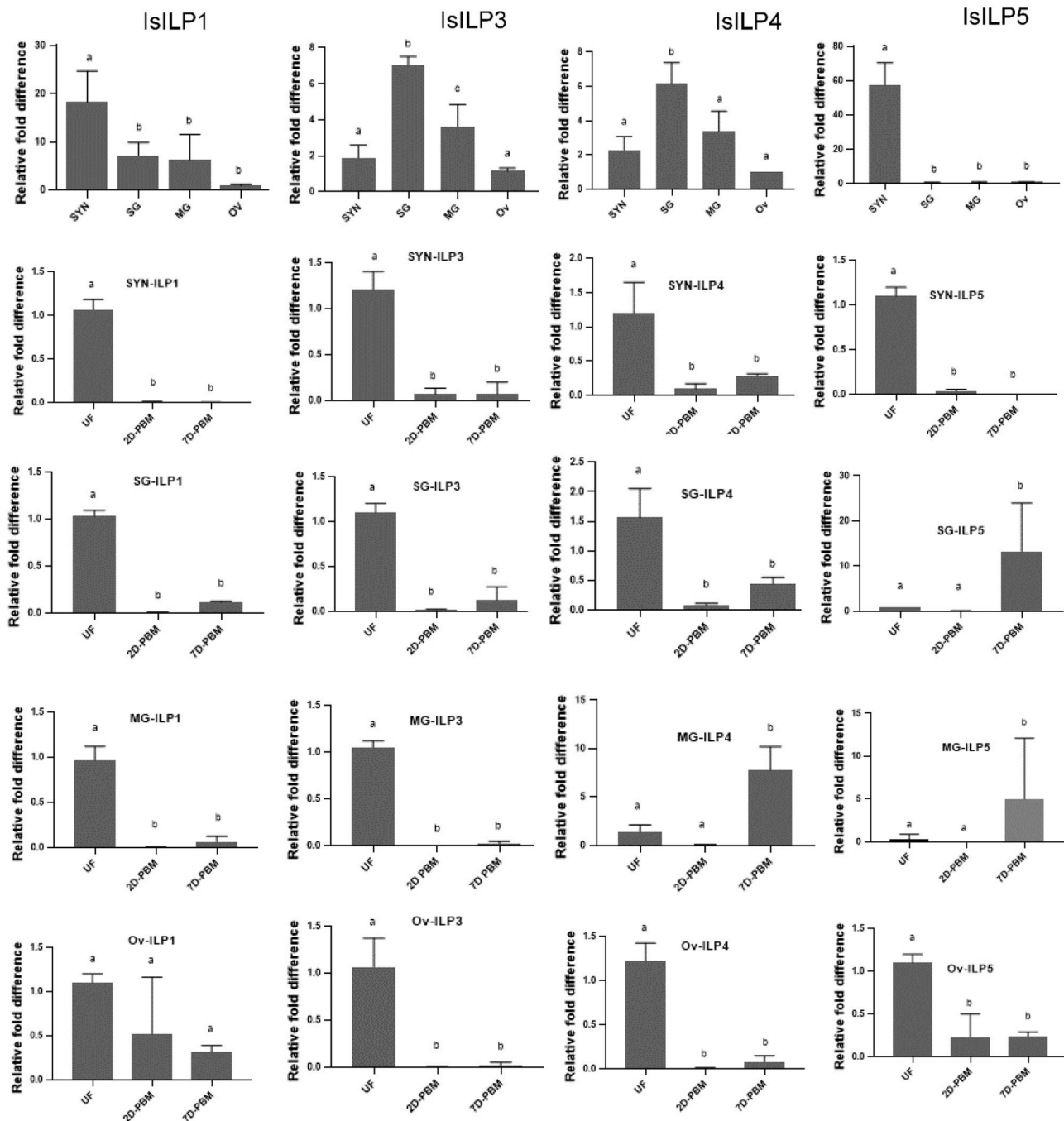


FIGURE 4 | Expression of IsILP transcripts in adult female *I. scapularis* tissues, as determined by quantitative RT-PCR. SYN, synganglion; SG, salivary gland; MG, midgut; Ov, ovaries; UF, unfed; 2D-PBM, 2 days post engorgement and host detachment; 7D-PBM, 7 days post engorgement and host detachment. Top row: Expression of individual IsILPs in unfed adult females compared by body tissue. Fold expression levels were scaled to expression in ovaries and were examined by one-way ANOVA followed by Dunnett's multiple comparison test. IsILP1 unfed tissues, $F_{(3,8)} = 8.156$, $P = 0.008$; IsILP1-Synganglion: $F_{(2,6)} = 252.1$, $P < 0.0001$; IsILP1-salivary glands: $F_{(2,6)} = 836.8$, $P < 0.0001$; IsILP1-midgut: $F_{(2,6)} = 97.47$, $P < 0.0001$; IsILP1-Ovaries: $F_{(2,6)} = 3.491$, $P = 0.09$. IsILP3 unfed tissues, $F_{(3,7)} = 25.42$, $P = 0.0004$; IsILP3-Synganglion: $F_{(2,6)} = 68.23$, $P < 0.0001$; IsILP3-salivary glands: $F_{(2,6)} = 98.08$, $P < 0.0001$; IsILP3-midgut: $F_{(2,6)} = 374.6$, $P = 0.0003$; IsILP3-ovaries: $F_{(2,6)} = 35.26$, $P = 0.0005$. IsILP4 unfed tissues, $F_{(3,7)} = 14.26$, $P = 0.002$; IsILP4-Synganglion: $F_{(2,6)} = 15.17$, $P = 0.004$; IsILP4-salivary glands: $F_{(2,6)} = 21.64$, $P = 0.001$; IsILP4-midgut: $F_{(2,5)} = 28.4$, $P = 0.001$; IsILP4-ovaries: $F_{(2,6)} = 94.22$, $P < 0.0001$. IsILP5 unfed tissues, $F_{(3,8)} = 57.74$, $P < 0.0001$; IsILP5-Synganglion: $F_{(2,6)} = 340.3$, $P < 0.0001$; IsILP5-salivary glands: $F_{(2,7)} = 5.176$, $P = 0.04$; IsILP5-midgut: $F_{(2,5)} = 1.73$, $P = 0.2$; IsILP5-ovaries: $F_{(2,6)} = 26.32$, $P < 0.001$.

then greatly reduce in size after host detachment. As some ILPs act as growth factors, one might speculate higher expression in these tissues may indicate synthesis and storage of these ILPs in preparation for initiation of a feeding event, where their release subsequently stimulates salivary gland growth.

Expression of most ILPs decreased after a blood meal which is similar to a previous study where expression of an ILP (the homolog of IsILP1) dropped during and after a blood meal in *D. variabilis* females (22). In our study, this occurred in most tissues and in most life stages. The importance of a reduction in IsILP transcripts in response to feeding is, as yet, unclear, but may be tied to regulation of energy storage as is the case for ILPs in other organisms. Also of interest are exceptions to this trend, such as the increase in expression of IsILP4 and IsILP5 in the midgut of adult females 7 days post blood meal. The increase in expression in this tissue after a blood meal may suggest a role of IsILPs 4 and 5 in blood digestion, a situation similar to that seen in *Ae. aegypti* where AaILP3 secreted from the brain stimulates the midgut to produce the digestive enzyme trypsin following a blood meal (4). In addition, nymphs increased expression of IsILPs 3 and 4 after detaching from hosts, possibly suggesting a developmental role unique to this life stage. A greater number of timepoints over a longer period may further elucidate the role of IsILPs before, during, and after blood feeding, and during development.

In adult *I. scapularis* males, IsILP expression was low for all ILPs except IsILP1. The primary activity for adult males of this species is to survive long enough to find and mate with a female. While this activity is most successful if the males cling to and search a vertebrate host where a prospective female is likely to be attached for feeding, the males themselves do not feed. Considering the major functions of ILPs identified in other animals include storage of nutrients, endocrine cascades resulting in yolk protein synthesis, and growth, the lack of IsILP expression in males may make sense in that they do not engage in these processes in the adult stage.

In addition to ILP expression levels in ticks, binding proteins, which were not investigated in this study, may also play a role in insulin signaling dynamics. For instance, knockdown of IGFBP in *A. americanum* prevented blood-feeding females from feeding to repletion (24). Although they are not ILPs, IGFbps do interact with ILPs and can regulate binding dynamics with the insulin receptor by altering degradation rates and binding kinetics. Exploration of the expression dynamics of IGFbps and their impact on IsILP stability in *I. scapularis* will provide further information on the roles of these signaling

molecules in tick physiology. ILPs also have been implicated in immune responses, and expression of an ILP (homolog of IsILP1) was upregulated in *A. americanum* exposed to *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis (23). Whether similar expression patterns occur in *I. scapularis* in response to pathogens remains to be explored, but it is a subject of particular interest given the role of insulin signaling in immunity in other blood-feeding arthropods (37), and the importance of *I. scapularis* as a vector of disease.

CONCLUSIONS

Ticks represent an extreme lifestyle compared to many animals. They feed exclusively on vertebrate blood, and Ixodid ticks feed only three times in their entire life cycle. From these scant meals, they can endure many months between feedings, yet still manage to deal with the harsh realities of the environment as other arthropods do, but without the luxury of regular food intake. No doubt absorption and conservation of energy is critical to their survival and ILPs are likely to play a key role in this process. We are eager to uncover the role of these signaling molecules with regard to the extreme physiology of these arachnids, and this study provides an important first step in the characterization and role of these neuropeptides.

AUTHOR CONTRIBUTIONS

AN and MG-N wrote the draft manuscript, conceived the experiments, and wrote the final manuscript. RP and PV amplified and sequenced the ILP transcripts. AS and BG carried out the insulin transcript expression study. MG-N performed the statistical analysis.

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The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fendo.2019.00292/full#supplementary-material>

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Neurosecretory Protein GL Induces Fat Accumulation in Chicks

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We recently found a previously unidentified cDNA in chicken hypothalamus which encodes the precursor for neurosecretory protein GL (NPGL). A previous study showed that intracerebroventricular (i.c.v.) infusion of NPGL caused body mass gain in chicks. However, it was not clear which part(s) of the body gained mass. In the present study, we investigated which tissues increased in mass after chronic i.c.v. infusion of NPGL in chicks. We found that NPGL increased the masses of the liver, abdominal fat, and subcutaneous fat, while NPGL did not affect the masses of muscles, including pectoralis major, pectoralis minor, and biceps femoris. Oil Red O staining revealed that fat deposition had occurred in the liver. In addition, the size of the lipid droplets in the abdominal fat increased. Furthermore, we found an upregulation of lipogenesis and downregulation of lipolysis in the abdominal fat, but not in the liver. These results indicate that NPGL is involved in fat storage in chicks.

Keywords: neurosecretory protein, chicken, hypothalamus, fat accumulation, growth

INTRODUCTION

The processes of animal development and growth are regulated by several hormones, genetics, nutrition, and the environment (1). Among these factors, growth hormone (GH), produced by the pituitary gland, and insulin-like growth factor-1 (IGF-1), made by the liver, play important roles in the growth of peripheral tissues, including bone, liver, muscle, and adipose tissue (1, 2). In addition, increases in the masses of peripheral tissues are also influenced by energy states, such as starvation and satiation. For energy homeostasis, bioactive substances, including neuropeptide Y (NPY), glucocorticoids, and leptin from the hypothalamus, adrenal gland, and adipose tissue, can also influence body mass growth in mammals (3–5). These neuropeptides, or circulating steroids and peptide hormones are secreted in response to physiological conditions, and the signals are finally integrated in the hypothalamus to regulate feeding behavior. Owing to the complexity of feeding regulation and growth, the exact mechanism(s) regulating these physiological processes remains to be elucidated.

The efficiency of nutrient utilization and growth rate in domestic animals is very important for the farming/food industry. Particularly significant progress has been made over the last several decades in poultry production following genetic selection (6). Orexigenic and anorexigenic factors act differently in birds as compared to mammals. In mammals, NPY, agouti-related protein (AgRP), melanin-concentrating hormone (MCH), orexin, and ghrelin stimulate food intake (7, 8). In contrast to mammals, MCH and orexin do not affect food intake of chicks, and ghrelin inhibits feeding behavior (9). In addition to the differential effects in birds and mammals, it is likely that other undiscovered factors take part in the regulation of feeding and growth.

In a preceding study, we aimed to find unknown regulatory substance(s) in the chicken hypothalamus that affect neuronal control of feeding behavior and/or growth. Food intake, energy consumption, and increase in body mass are inextricably linked to animal growth. We recently deduced a cDNA from chicken hypothalamus that encodes the unidentified precursor to a small neurosecretory protein (10). The deduced pre-pro-protein contains a signal peptide sequence, a mature secretory protein sequence of 80 amino acid residues, a glycine amidation signal, and a cleavage signal consisting of dibasic amino acids (Lys-Arg) (10). The predicted C-terminal amino acid sequences were Gly-Leu-NH₂, so we termed the protein neurosecretory protein GL (NPGL) (10). *In situ* hybridization revealed that the NPGL mRNA was expressed in the infundibular nucleus (IN) and the medial mammillary nucleus (MM) of the hypothalamus (10). The IN and MM in chicks are known to correspond to the mammalian arcuate nucleus (Arc) and the tuberomammillary nucleus (TMN), respectively; both nuclei are recognized to be involved in regulation of food intake in mammals. In addition, we found that the expression levels of NPGL mRNA were elevated during the post-hatch period (10). Furthermore, we reported that subcutaneous administration of NPGL for 4 days increased body mass gain independently of feeding behavior in chicks (10). Chronic intracerebroventricular (i.c.v.) infusion of NPGL for 2 weeks stimulated intake of food and water, with an associated rise in body mass (11). These data indicate that NPGL may influence growth processes of chicks. However, the specific body tissue(s) that are actually impacted by NPGL administration have not been elucidated. In the present study, we addressed this gap in our knowledge of NPGL action.

MATERIALS AND METHODS

Animals

Male layer chicks (*Gallus domesticus*, 1 day old, $n = 8$ in each group) were obtained from a commercial hatchery (Nihon Layer, Gifu, Japan) and kept in a windowless room at 28°C on a light/dark cycle: 20 h light (4:00–24:00) and 4 h dark (0:00–4:00) according to our previous report (11). The chicks had access to food and water *ad libitum*. Chicks were raised in a group and then separated into individual cages from 4 days of age in order to measure individual food intake. The experimental protocols were in accordance with the Guide for the Care and

Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan).

Production of Chicken NPGL

Chicken NPGL was synthesized with Fluorenylmethyloxycarbonyl (Fmoc) chemistry using a peptide synthesizer (Syro Wave; Biotage, Uppsala, Sweden) according to our previous method (12). The protein was cleaved from the resin with reagent K (trifluoroacetic acid: TFA 82.5%, phenol 5%, thioanisole 5%, H₂O 5%, and 1,2-ethanedithiol 2.5%) for 3 h. The crude protein was purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column (YMC-Pack ProC18, 10 × 150 mm; YMC, Kyoto, Japan) at a flow rate of 1.0 ml/min for 40 min with a linear gradient of 40–60% acetonitrile containing 0.1% TFA. The solvent was evaporated and lyophilized. The protein was dissolved in dimethyl sulfoxide (DMSO) and then diluted to a final concentration of 0.5 mM glutathione disulfide, 5 mM glutathione, 50% acetonitrile, 1 mM EDTA, 10% DMSO, 0.4 M Tris-HCl (pH 8.5). The solvent was rotated for 2 days at room temperature to allow for an intramolecular disulfide bond to form, purified by HPLC and then lyophilized. The purity of the protein was >95%. Lyophilized NPGL was weighed using an analytical and precision balance (AP125WD; Shimadzu, Kyoto, Japan).

i.c.v. Infusion of NPGL for 2 Weeks

NPGL was dissolved in absolute propylene glycol and adjusted to 30% propylene glycol as a vehicle solution. Eight day old chicks were i.c.v. infused with 0 (vehicle) or 15 nmol/day NPGL via an infusion cannula (model 328OP; Plastics One, Roanoke, VA) and an Alzet mini-osmotic pump (model 2002, delivery rate 0.5 µl/h; DURECT Corporation, Cupertino, CA). The dose was determined on the basis of previous studies (11, 13). The infusion cannula tip was implanted into the lateral ventricle: 2.0 mm rostral to lambda, 1.0 mm lateral to midline, and 5.5 mm ventral to the skull surface. Osmotic mini-pumps containing vehicle or NPGL were implanted subcutaneously in the neck according to our previous method (11).

Body mass and food intake were measured daily (between 9:00–10:00) throughout the experiment. After 2 weeks of i.c.v. infusion of NPGL, chicks were killed by decapitation and masses of liver, abdominal fat, subcutaneous fat, pectoralis major muscle, pectoralis minor muscle, and biceps femoris muscle were measured.

Histological Analysis

For Oil Red O staining to detect fat accumulation, the livers from 5 animals of each group were fixed in 4% paraformaldehyde and sliced into 10 µm sections. These were air-dried, rinsed with 60% isopropanol, stained with Oil Red O solution for 15 min at 37°C, and rinsed with 60% isopropanol. The nucleus was counterstained with hematoxylin for 5 min, and the sections were washed in tap water. The slides were mounted in aqueous mounting medium for microscopic examination.

For hematoxylin and eosin staining, fixed abdominal fat was embedded in paraffin and cut into 7 µm sections with a microtome. The sections were then delipidated with acetone.

The nucleus and cytoplasm were stained with hematoxylin and eosin (5 min for each stain), and the sections were washed in tap water. After serial dehydration with alcohol and clearing with xylene, the sections were mounted on slides for microscopic examination using an Eclipse E600 conventional microscope (Nikon, Tokyo, Japan).

Real-Time RT-PCR

The procedures were carried out in a similar manner to our previous report (11). At the end of the NPGL infusions, chicks were killed by decapitation between 13:00–15:00. The liver and abdominal fat were dissected out and snap-frozen in liquid nitrogen. RNA was extracted using TRIzol reagent for liver (Life Technologies, Carlsbad, CA) or QIAzol lysis reagent for abdominal fat (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (1 µg) using a ReverTra Ace kit (TOYOBO, Osaka, Japan).

PCR amplifications were conducted using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the following procedure: 95°C for 20 s, followed by 40 cycles at 95°C for 3 s, and at 60°C for 30 s using a real-time thermal cycler (CFX Connect; BioRad, Hercules, CA). Amplifications of lipogenic and lipolytic enzymes and related factors were performed with the primer sets listed in **Table 1**. We chose acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), malic enzyme (ME) as lipogenic enzymes, peroxisome proliferator-activated receptor γ (PPAR γ) as cell differentiation marker, fatty acid transporter 1 (FATP1) as fatty acid uptake, and PPAR α , carnitine palmitoyltransferase 1a (CPT1a), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and comparative gene identification-58 (CGI-58) as lipolytic enzymes and related factors.

The relative quantification for each expression was determined by the $2^{-\Delta\Delta C_t}$ method (14) using β -actin (ACTB) as the internal control.

Statistical Analysis

Data were analyzed with Student's *t*-test for tissue mass and mRNA expression or one-way repeated measures analysis of

variance (ANOVA) followed by Bonferroni's test for body mass gain and cumulative food intake. The significance level was set at $P < 0.05$. All results are expressed as the mean $\pm$ SEM.

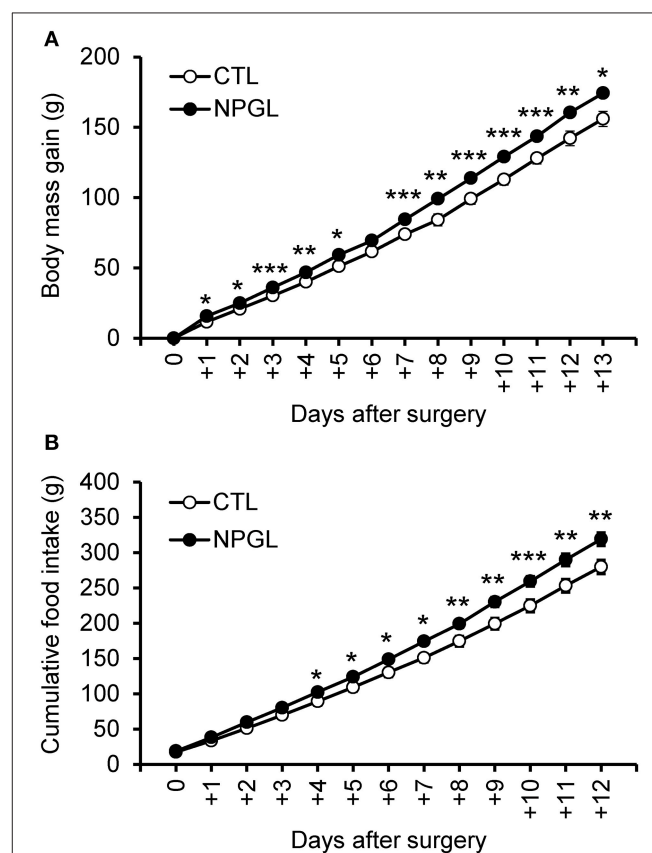


FIGURE 1 | Effect of chronic i.c.v. infusion of NPGL on body mass gain and food intake. The results were obtained by the infusion of the vehicle (control; CTL) and NPGL. The change in the body mass gain after surgery (A). The cumulative food intake (B). Data are expressed as the mean $\pm$ SEM ($n = 8$). Data were analyzed with a one-way repeated measures analysis of variance (ANOVA), followed by Bonferroni's test. An asterisk indicates a statistically significant difference (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

TABLE 1 | Sequences of oligonucleotide primers for real-time PCR.

Gene	Forward primer	Reverse primer	Accession no.
ACC	AATGGCAGCTTTGGAGGTGT	TCTGTTTGGGTGGGAGGTG	NM_205505.1
FAS	CCAACGATTACCGTCTCAA	CAGGCTCTGTATGCTGTCCA	NM_205155.2
SCD1	AGTGGTGTTGCTGTGCTTCA	CTAAGGTGTAGCGCAGGATG	NM_204890.1
ME	AGTGCCTACCTGTGATGTTG	GGCTTGACCTCTGATTCTCT	NM_204303.1
PPAR γ	TCAAGCATTTCTTACCACACT	ATTGCATTTGGCAATCCTGG	NM_001001460.1
FATP1	TACAATGTGCTCCAGAAGGG	GTCTGGTTGAGGATGTGACTC	NM_001039602.2
PPAR α	TGCTGTGGAGATCGTCTGGT	AGAGGAAGATATCGTCAGGATGG	NM_001001464.1
CPT1a	TGATCTGAAGAAGAACCCTGAGAT	TCCAAGCGATGAGAATCCG	NM_001012898.1
LPL	CAGTGCAACTTCAACCATACCA	AACCAGCCAGTCCACAACAA	NM_205282.1
ATGL	CACTGCCATGATGGTCCCCTA	CCACAAGGAGATGCTGAAGAA	NM_001113291.1
CGI-58	ACCGTGGTTTATGGAGCACG	GAAACAGTGTGCAACAGAGCC	NM_001278145.1
ACTB	CCAGAGTCCATCACAATACC	AGCCAACAGAGAGAAGATGA	NM_205518.1

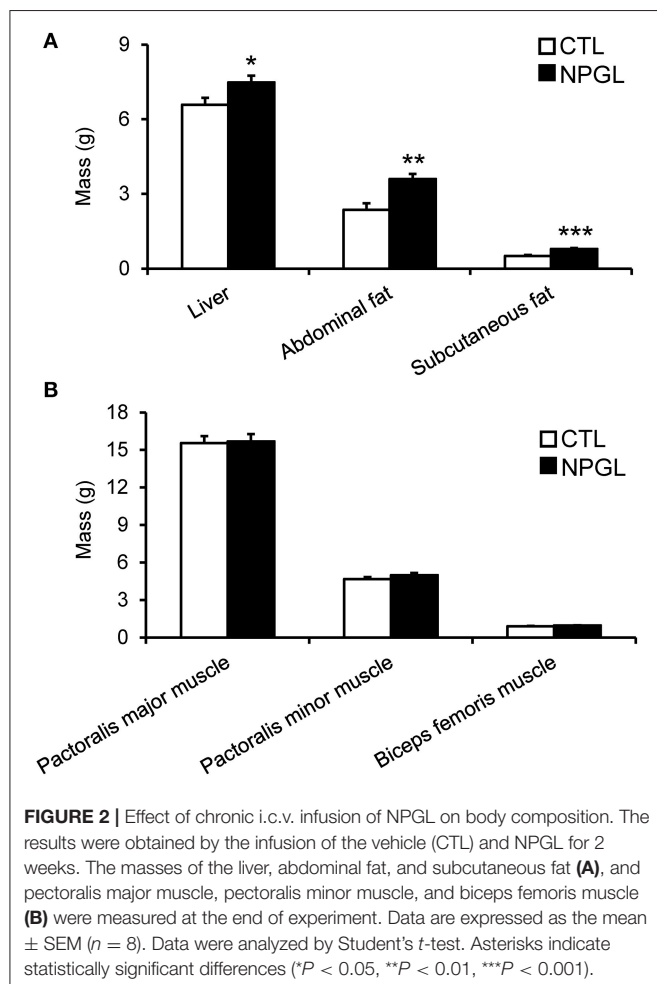
RESULTS

Effect of i.c.v. Infusion of NPGL on Body Mass Gain, Food Intake, and Body Composition

NPGL infusion significantly promoted body mass gain (Figure 1A), and NPGL also increased cumulative food intake (Figure 1B). Furthermore, NPGL increased the mass of the liver, abdominal fat, and subcutaneous fat (Figure 2A), while no change was observed in the muscle mass of the pectoralis major muscle, pectoralis minor muscle, and biceps femoris muscle (Figure 2B).

Effect of i.c.v. Infusion of NPGL on Lipid Deposition in the Liver and Abdominal Fat

Lipid droplets were observed in the liver after chronic i.c.v. infusion of NPGL, increasing its mass (Figure 3). Moreover, abdominal fat accumulated around the gizzard in the NPGL-infused chicks (Figure 4, left in lower panel). The lipid droplets in the abdominal fat of the chicks were substantially larger after chronic NPGL infusion than those in the controls (Figure 4, right in lower panel).

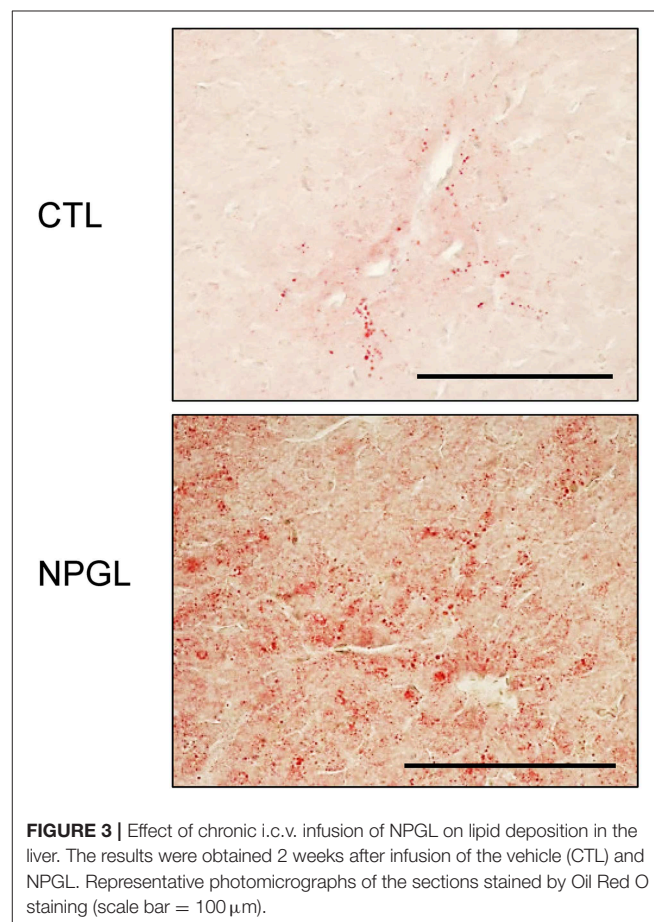


Effect of i.c.v. Infusion of NPGL on the mRNA Expression of Lipogenic and Lipolytic Factors in the Liver and Abdominal Fat

To investigate the gene expression of lipogenic and lipolytic factors in the liver and abdominal fat, we analyzed the mRNA levels of *ACC*, *FAS*, *SCD1*, *ME*, *PPAR γ* , *FATP1*, *PPAR α* , *CPT1a*, *LPL*, *ATGL*, and *CGI-58* after chronic i.c.v. infusion of NPGL. NPGL decreased the mRNA expression of *PPAR α* in the liver (Figure 5) and increased the expression of *FAS*, *SCD1*, and *PPAR γ* in the abdominal fat; whereas, NPGL decreased the *CPT1a* mRNA (Figure 6).

DISCUSSION

The precursor gene for NPGL was identified in the chicken hypothalamus through a cDNA subtractive screen designed to identify novel neuronal substance(s) in the avian brain (10). After sequencing 596 clones from hypothalamic cDNA, we found an unidentified cDNA that encodes an unknown protein. The deduced precursor protein consisted of 182 amino acid residues, containing a putative secretory protein of 80 amino acid residues, and which had Gly-Leu-NH₂ at its C-terminus. Therefore,



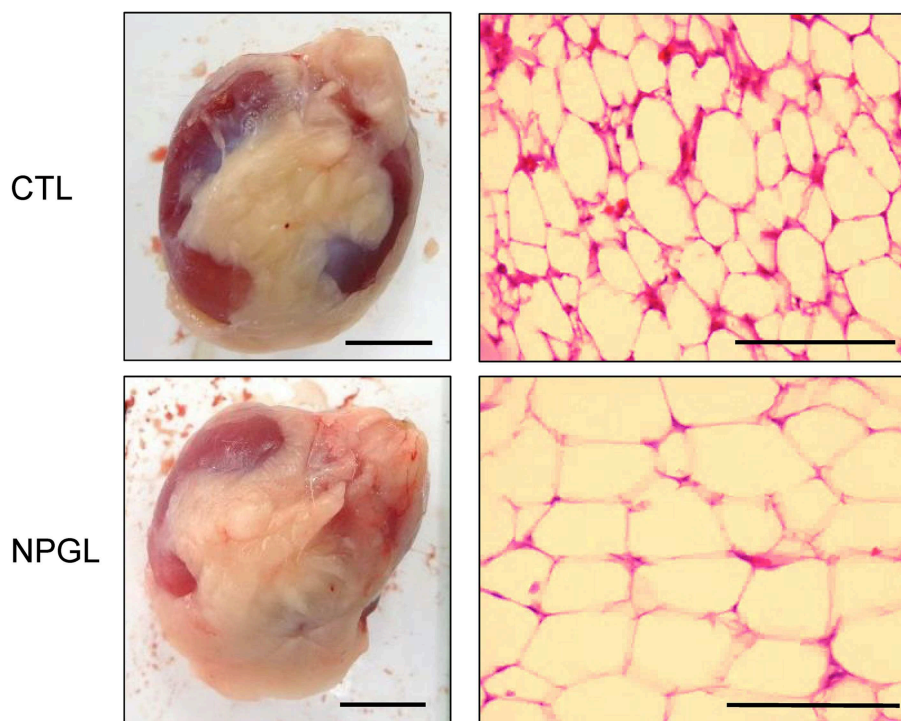


FIGURE 4 | Effect of chronic i.c.v. infusion of NPGL on the lipid droplets in the abdominal fat. The results were obtained 2 weeks after infusion of the vehicle (CTL) and NPGL. Exterior photographs of the abdominal fat around the gizzard (left panel, scale bar = 1 cm), and representative photographs of the sections stained by hematoxylin and eosin staining (right panel, scale bar = 100 μ m).

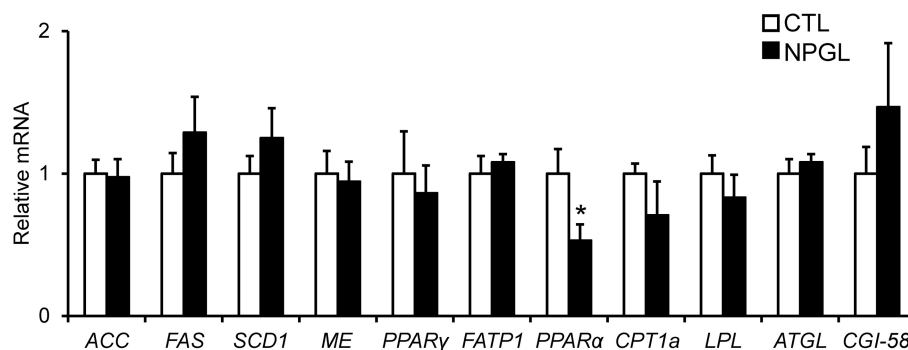


FIGURE 5 | Effect of chronic i.c.v. infusion of NPGL on the mRNA expression of lipogenic and lipolytic factors [acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), malic enzyme (ME), peroxisome proliferator-activated receptor γ (PPAR γ), fatty acid transporter 1 (FATP1), peroxisome proliferator-activated receptor α (PPAR α), carnitine palmitoyltransferase 1a (CPT1a), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and comparative gene identification-58 (CGI-58)] in the liver. The results were obtained 2 weeks after infusion of the vehicle (CTL) and NPGL. Data are expressed as the mean $\pm$ SEM ($n = 8$). Data were analyzed by Student's t -test. An asterisk indicates a statistically significant difference (* $P < 0.05$).

the protein was termed neurosecretory protein GL (NPGL) (10). As NPGL contains two Cys residues, the presence of an intramolecular disulfide bond bridge is likely (10). Subcutaneous administration of NPGL can cause an increase in body mass of chicks without affecting feeding behavior (10). However, the exact body components that are affected by NPGL had not been studied. The aim of this study was determination of the body component(s) that increased after NPGL infusion. We

measured the masses of various tissues, including the liver, fat, and muscles after 2 weeks of i.c.v. NPGL infusion. The results showed that NPGL increased the mass of the liver, abdominal fat, and subcutaneous fat (Figure 2A), while no change was observed in the muscle masses of pectoralis major, pectoralis minor, and biceps femoris (Figure 2B). As we speculated that NPGL stimulates fat accumulation in the liver and adipose tissue, this phenomenon was confirmed using a morphological analysis. The

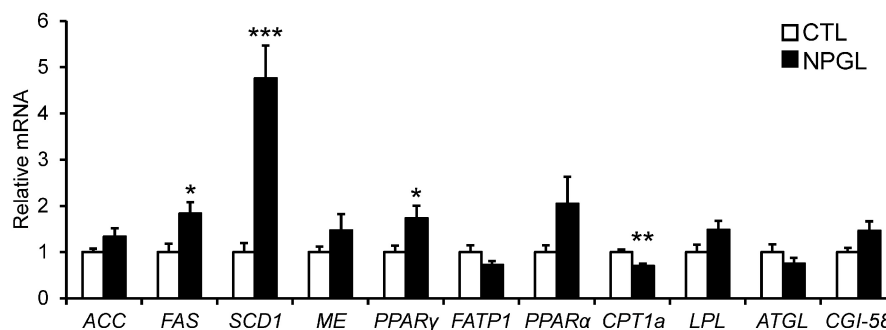


FIGURE 6 | Effect of chronic i.c.v. infusion of NPGL on the mRNA expression of lipogenic and lipolytic factors [acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), malic enzyme (ME), peroxisome proliferator-activated receptor γ (PPAR γ), fatty acid transporter 1 (FATP1), peroxisome proliferator-activated receptor α (PPAR α), carnitine palmitoyltransferase 1a (CPT1a), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and comparative gene identification-58 (CGI-58)] in abdominal fat. The results were obtained 2 weeks after infusion of the vehicle (CTL) and NPGL. Data are expressed as the mean $\pm$ SEM ($n = 8$). Data were analyzed by Student's *t*-test. Asterisks indicate statistically significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

results revealed lipid deposition in the liver and the enlargement of lipid droplets in the abdominal fat (Figures 3, 4).

In the experiments to survey the gene expression related to lipogenic and lipolytic factors, we found a decrease in the PPAR α mRNA in the liver, increases in FAS, SCD1, and PPAR γ , and a reduction of CPT1a in the abdominal fat. It is known that target genes of PPAR α participate in fatty acid β -oxidation in the liver (15, 16). FAS and SCD1 are lipogenic enzymes, and CPT1a is lipolytic enzyme (17). It has been reported that PPAR γ plays important roles in the process of adipogenic differentiation (18). Taken together with these findings, the present study suggests that NPGL inhibits fatty acid oxidation in the liver and induces *de novo* lipogenesis in adipose tissue. It has been demonstrated previously in birds that *de novo* fatty acid synthesis takes place predominantly in the liver (19). The present data indicate the novel possibility of *de novo* lipogenesis in the adipose tissue of birds.

In mammals, *de novo* lipogenesis is the process of transforming non-lipid precursors into fatty acids and triglycerides for energy storage (20). Although *de novo* lipogenesis in adipose tissue is important for the maintenance of metabolic homeostasis, *de novo* lipogenesis in non-adipose tissues, such as the liver and muscle, results in ectopic lipid deposition, lipotoxicity, and metabolic stress disorder (21, 22). Lipogenesis and lipolysis in adipose tissue are controlled by the hypothalamus via the sympathetic nervous system (23, 24). Therefore, it is likely that NPGL takes part in the sympathetic control of fat accumulation in chicks, although there may be other pathways that mediate its actions. Future studies are necessary to classify the target regions and neuronal networks regarding the NPGL neurons to elucidate the exact mechanism of *de novo* lipogenesis in birds.

The present study also showed that i.c.v. infusion of NPGL stimulated food intake. As mentioned above, subcutaneous infusion of NPGL did not affect the feeding behavior of chicks (10). The results from the present study suggest that NPGL may act on the brain to stimulate feeding behavior, although we have found that NPGL did not change the mRNA expression

of various hypothalamic ingestion-related neuropeptides, i.e., NPY, AgRP, proopiomelanocortin (POMC; precursor of α -melanocyte-stimulating hormone), glucagon-like peptide (GLP-1), and cholecystokinin (CCK) (11). Future studies are needed to elucidate the target neurons of NPGL that alter feeding behavior.

After the identification of the NPGL precursor gene in the chicken hypothalamus, we searched for related genes in the genome database (TBLASTN) using the amino acid sequence of the NPGL precursor. We found an orthologous gene in humans and rats (10). In addition, the NPGL precursor gene is conserved in mouse, turtle, frog, and fish (25). In fact, we cloned the cDNA encoding NPGL in the mouse and rat hypothalamus and found that the mature NPGL amino acid sequence was 85% similar between chicken and rodent (26, 27). In mice, we demonstrated that acute i.c.v. injection of NPGL increased food intake from 2 to 10 h after administration (26). Although we also performed acute injection of NPGL in chicks in our preliminary experiments, we did not observe a significant effect on feeding behavior. The data suggest that the mechanisms of action of NPGL on food intake differ between chicks and mice. The present data show that chronic infusion of NPGL stimulated feeding behavior as mentioned above. We need to elucidate the reasons for differential effects of acute and chronic administration of NPGL on food intake in future studies.

Recently, we also investigated the biological actions of NPGL in rats by overexpressing the NPGL precursor gene in the hypothalamus and chronic i.c.v. infusion of NPGL peptide, similar to the present study. The results showed that NPGL induced a significant rise in the mass of adipose tissue and the magnitude of adipocytes in rats (27). Next, we investigated the mRNA expression of lipogenic and lipolytic enzymes in the adipose tissue and liver of rats and found that the mRNA expression levels of the lipogenic enzymes in the adipose tissue significantly increased after NPGL overexpression and i.c.v. infusion of NPGL, but no differences were detected in the liver (27). Thus, NPGL-induced *de novo* lipogenesis does not occur in the liver, but is restricted to adipose tissue. To our knowledge, the previous study in rats was the first report of

an endogenous neuronal substance that can regulate peripheral *de novo* lipogenesis in animals (27). In the present study, we also found that peripheral *de novo* lipogenesis was induced by chronic i.c.v. infusion of NPGL in chicks. These results suggest that NPGL-induced *de novo* lipogenesis in adipose tissue is a conserved property in birds and rodents.

In conclusion, a chronic i.c.v. administration of NPGL stimulated food intake, increased the masses of the liver and adipose tissue, and finally, caused an increase in body mass gain in developing chicks. Thus, NPGL is a positive regulator of feeding and growth post-hatch. This is the first report describing the upregulation of *de novo* lipogenesis from chronic i.c.v. infusion of NPGL in birds. The cognate receptor for NPGL has yet to be characterized in any animal, but this will be necessary in order to elucidate the precise roles of NPGL action in the brain. Regulation of feeding behavior and fat storage are vital for survival and for the transition into specific life-history stages, such as pregnancy, puberty, aging, migration, and hibernation. Future comparative analyses using other animal models will help elucidate the unity and diversity of the physiological functions of NPGL.

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ETHICS STATEMENT

The experimental protocols were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan).

AUTHOR CONTRIBUTIONS

KU conceived and designed the experiments. All authors performed the experiments and analyzed the data. KU, KS, EI-U, and GB wrote the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Insight Into Mosquito GnRH-Related Neuropeptide Receptor Specificity Revealed Through Analysis of Naturally Occurring and Synthetic Analogs of This Neuropeptide Family

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Adipokinetic hormone (AKH), corazonin (CRZ), and the AKH/CRZ-related peptide (ACP) are neuropeptides considered homologous to the vertebrate gonadotropin-releasing hormone (GnRH). All three *Aedes aegypti* GnRH-related neuropeptide receptors have been characterized and functionally deorphanized. Individually they exhibit high specificity for their native ligands, prompting us to investigate the contribution of ligand structures in conferring receptor specificity for two of these receptors. Here, we designed a series of analogs based on the native ACP sequence and screened them using a heterologous system to identify critical residues required for ACP receptor (ACPR) activation. Analogs lacking the carboxy-terminal amidation, replacing aromatics, as well as truncated analogs were either completely inactive or had very low activities on ACPR. The polar threonine (position 3) and the blocked amino-terminal pyroglutamate are also critical, whereas ACP analogs with alanine substitutions at position 2 (valine), 5 (serine), 6 (arginine), and 7 (aspartate) were less detrimental including the substitution of charged residues. Replacing asparagine (position 9) with an alanine resulted in a 5-fold more active analog. A naturally-occurring ACP analog, with a conserved substitution in position two, was well tolerated yet displayed significantly reduced activity compared to the native mosquito ACP peptide. Chain length contributes to ligand selectivity in this system, since the endogenous octapeptide Aedae-AKH does not activate the ACPR whereas AKH decapeptides show low albeit significant activity. Similarly, we utilized this *in vitro* heterologous assay approach against an *A. aegypti* AKH receptor (AKHR-IA) testing carefully selected naturally-occurring AKH analogs from other insects to determine how substitutions of specific residues in the AKH ligand influence AKHR-IA activation. AKH analogs having single substitutions compared to Aedae-AKH revealed position 7 (either serine or asparagine) was well tolerated or had slightly improved activation whereas changes to position 6 (proline) compromised receptor activation by nearly 10-fold. Substitution of position 3 (threonine) or analogs with combinations of substitutions were quite detrimental with a significant decrease in AKHR-IA activation. Collectively,

these results advance our understanding of how two GnRH-related systems in *A. aegypti* sharing the most recent evolutionary origin sustain independence of function and signaling despite their relatively high degree of ligand and receptor homology.

Keywords: GnRH-related neuropeptides, G protein-coupled receptor, structure activity relationships, adipokinetic hormone (AKH), corazonin (CRZ), AKH/CRZ-related peptide (ACP)

INTRODUCTION

In invertebrates there exist three neuropeptide systems of which the mature peptides as well as their cognate G protein-coupled receptors (GPCRs) are structurally similar, and collectively, they are related to the vertebrate gonadotropin releasing hormone (GnRH) system and suggested to form a large peptide superfamily (1–4). These neuropeptide systems are called the adipokinetic hormone (AKH)/red pigment-concentrating hormone (RPCH) family, the corazonin (CRZ) family, and the structurally intermediate adipokinetic hormone/corazonin-related peptide (ACP) family. AKHs are primarily or exclusively produced in neurosecretory cells of the corpus cardiacum, and a major function is mobilization of energy reserves stored in the fat body, thus providing an increase of the concentration of diacylglycerols, trehalose, or proline for locomotory active phases in the haemolymph. In accordance with this function, AKH has been shown to activate the enzymes glycogen phosphorylase and triacylglycerol lipase and transcripts of the AKHR are most prominently found in fat body tissue [see reviews by (5–7)]. As with most endocrine regulatory peptides, additional functions such as inhibition of anabolic processes (protein and lipid syntheses), involvement in oxidative stress reactions and egg production *inter alia* are known making AKH a truly pleiotropic hormone [see reviews by (8, 9)]. This is also true for CRZ which is mainly synthesized in neuroendocrine cells of the pars lateralis of the protocerebrum and released via the corpora cardiaca (10). Although originally described as a potent cardiostimulatory peptide (11), it does not generally fulfill this role but is known for many additional functions such as involvement in the (i) release of pre-ecdysis and ecdysis triggering hormones, (ii) reduction of silk spinning rates in the silk moth, (iii) pigmentation events (darkening) of the epidermis in locusts during gregarization and (iv) regulation of caste identity in an ant species (12–15). The functional role of ACP is less clear. All previous studies had not found a clear-cut function for this peptide until work by Zhou and colleagues claimed that ACP in the cricket *Gryllus bimaculatus* regulates the concentration of carbohydrates and lipids in the haemolymph (16).

Experiments in the current study have been conducted with the mosquito species *Aedes aegypti* for a number of reasons. Firstly, *A. aegypti* is an infamous disease vector for pathogens such as Yellow and Dengue fever, Chikungunya and, as latest addition, Zika arboviruses; summarily these are responsible for affecting approximately 50–100 million people per year (17–19). Secondly, knowledge on the interaction of the ligands with their respective receptor are thought to be very helpful for drug research using specific GPCRs as targets for development of selective biorational insecticides (20–22). Another reason was

that each of the three neuropeptide systems has already been partially investigated, thus, this study could build on previous research data. With respect to the peptides, these were first predicted from the genomic work on *A. aegypti* (23). The presence of mature AKH and corazonin, but not of ACP, was shown by direct mass profiling (24). The AKH and ACP precursors have been cloned (25) as have one CRZ receptor (CRZR) and multiple transcript variants of the AKHR and ACPR (25–27). Each receptor is very selective and responds only to its cognate peptide, thus there are indeed three completely separate and independently working neuroendocrine systems active in this mosquito species. Here, we hone in on two of these systems, specifically ACP/ACPR and AKH/AKHR, which share the closest evolutionary relationship (2, 28–30), and examined structural features of each ligand important for conferring receptor specificity and activation.

With respect to the ACP system in *A. aegypti*, expression studies revealed transcripts of the major ACPR form as well as the ACP precursor were enriched during development in adults, specifically during day one and four after eclosion (25, 27). On the tissue level, ACP precursor transcripts are mainly present in the head/thorax region (25) and, more specifically, within the brain and thoracic ganglia (27) and the ACPR transcripts are mainly detected in the central nervous system with significant enrichment in the abdominal ganglia, particularly in males (27). The AKH precursor transcripts are present in head/thorax region of pupae and adult females and in the abdomen of males (25). Expression of AKHR was shown in all life stages (25), but similar to the ACPR and ACP transcripts, AKHR transcript variants were found to be significantly enriched in early adult stages (26). On the tissue level, highest expression in adults was found in thorax and abdomen with low levels detected in ovaries (25). Similar expression profiles were determined recently by Oryan et al. (26) using RT-qPCR: expression in head, thoracic ganglia, accessory reproductive tissues, and carcass of adult females as well as in abdominal ganglia and specific enrichment in the carcass and reproductive organs of adult males.

Thus, although quite a substantial body of information on the three signaling systems in *A. aegypti* is known, we lack knowledge of how the various ligands interact with their cognate receptor. For instance, which amino acid residue in the ligand is specifically important for this interaction? By replacing each residue successively with simple amino acids such as glycine or alanine one can probe into the importance of amino acid side chains and their characteristics in relation to facilitating receptor specificity. Such structure-activity studies have been conducted on the AKH system in a number of insects and a crustacean either by measuring physiological actions *in vivo* (31, 32) or in a cellular mammalian expression system *in vitro* (33–35). In conjunction

with nuclear magnetic resonance (NMR) data on the secondary structure of AKHs (36, 37) and the knowledge of the receptor sequence, molecular dynamic methods can devise models how the ligand is interacting with its receptor (38, 39). Such a comprehensive study carried out recently on the locust AKH receptor demonstrated that all three endogenous AKH peptides, including two octa- and one decapeptide, interact with similar residues and share the same binding site on their receptor (40).

Since no such data is available for any ACP system, the objective of the current study was to fill this knowledge gap by determining critical amino acids of the ACP neuropeptide necessary for activation of its cognate receptor, ACPR. We therefore designed a series of analogs based on the endogenous *A. aegypti* ACP sequence and screened them using an *in vitro* receptor assay system to determine the crucial residues for ACPR activation. Given the closer evolutionary relationship between the ACP and AKH systems in arthropods (2, 28–30), a second aim of the current study was to advance our knowledge and understanding of critical residues and properties of uniquely positioned amino acids necessary for the specificity of the AKH ligand to its receptor, AKHR-IA. Given the extensive earlier studies examining AKH structure activity relationships, which established that aromatics at positions 4 and 8 and blocked N- and C-terminal ends are critical, we took advantage of naturally occurring AKHs from other insects to examine other positions with amino acid substitutions and determine the consequences onto AKHR-IA activation using the same *in vitro* heterologous assay. Collectively, determining indispensable amino acid residues of these two GnRH-related mosquito neuropeptides that are necessary for activation of their prospective receptors will help clarify how these evolutionarily-related systems uphold specific signaling networks avoiding cross activation onto the structurally related, but functionally distinct signaling system.

MATERIALS AND METHODS

ACP and AKH Receptor Expression Construct Preparation

A mammalian expression construct containing the ACPR-I ORF (hereafter denoted as ACPR) with Kozak translation initiation sequence had been previously prepared in pcDNA3.1⁺ (27); however, this earlier study revealed that coupling of the ACPR with calcium mobilization in the heterologous expression system was not very strong, even following application of high concentrations of the native ACP ligand. Therefore, to improve the bioluminescent signal linked to calcium mobilization following receptor activation, which was required in order to provide greater signal to noise ratio when testing ACP analogs with substitutions leading to intermediate levels of activation of the ACPR, we created a new mammalian expression construct using the dual promoter vector, pBudCE4.1. Specifically, we utilized a cloning strategy as reported previously (41) whereby the ACPR was inserted into the multiple cloning site (MCS) downstream of the CMV promoter in pBudCE4.1 using the sense primer *SalI*-KozakACPR, 5'-GGTGCAGCCAC

CATGTATCTTTTCGG-3' and anti-sense primer *XbaI*-ACPR, 5'-TCTAGATTATCATCGCCAGCCACC-3', which directionally inserted the ACPR within the MCS downstream of the CMV promoter. Secondly, the murine homolog (Gα15) of the human promiscuous G protein, Gα16, which indiscriminately couples a wide variety of GPCRs to calcium signaling (42) was inserted into the MCS downstream of the EF1-α promoter in pBudCE4.1 using the sense primer *NotI*-Gα15, 5'-GCGGCCGCCACCATGGCCCGGTCCCTGAC-3' and anti-sense primer *BglII*-Gα15, 5'-AGATCTTCACAGCAGGTTGATCTCGTCCAG-3', which directionally inserted the murine Gα15 within the MCS downstream of the EF1-α promoter.

The AKHR mammalian expression construct used in the current study, specifically AKHR-IA which was the receptor isoform that exhibited the greatest sensitivity to its native AKH ligand, was prepared previously in pcDNA3.1⁺ (26) and coupled strongly with calcium signaling in the heterologous system so there was no need utilize the dual promoter vector containing the promiscuous Gα15 as was necessary for ACPR. For each construct, an overnight liquid culture of clonal recombinant bacteria containing the appropriate plasmid construct was grown in antibiotic-containing LB media and used to isolate midiprep DNA using the PureLink Midiprep kit (Life Technologies, Burlington, ON). Midiprep samples were then quantified and sent for sequencing as described previously (26, 27).

Cell Culture, Transfections, and Calcium Bioluminescence Reporter Assay

Functional activation of the AedaeACPR and AedaeAKHR-IA receptors was carried out following a previously described mammalian cell culture system involving a Chinese hamster ovary (CHO)-K1 cell line stably expressing aequorin (43). Cells were grown in DMEM:F12 media containing 10% heat-inactivated fetal bovine serum (Wisent, St. Bruno, QC), 200 µg/mL Geneticin, 1x antimycotic-antibiotic to approximately 90% confluency, and were transiently transfected using either Lipofectamine LTX with Plus Reagent or Lipofectamine 3000 transfection systems (Invitrogen, Burlington, ON) following manufacturer recommended DNA to transfection reagent ratios. Cells were detached from the culture flasks at 48 h post-transfection using 5 mM EDTA in Dulbecco's PBS. Cells were then prepared for the receptor functional assay following a procedure described previously (27). Stocks of peptide analogs of ACP and AKH were used for preparation of serial dilutions all of which were prepared in assay media (0.1% BSA in DMEM:F12) and loaded in quadruplicates into 96-well white luminescence plates (Greiner Bio-One, Germany). Cells were loaded with an automatic injector into each well of the plate containing different peptide analogs at various concentrations as well as negative control (assay media alone) and positive control wells (50 µM ATP). Immediately after injection of the cells, luminescence was measured for 20 s using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Calculations, including determination of EC₅₀ values, were conducted in GraphPad Prism 7.02 (GraphPad Software, San Diego, USA) from dose-response curves from 3 to 4 independent biological replicates.

TABLE 1 | Primary structure of the synthetic ACP analogs designed, a natural ACP analog along with extended AKH decapeptides that were tested on the *A. aegypti* ACP receptor (AedaeACPR).

Peptide/ Analog	Primary structure of analog	Activity on <i>A. aegypti</i> ACPR (EC ₅₀)	Fold-reduction in activity relative to AedaeACP
Aedae-ACP	pQVTFSRDWNAamide	2.394E-08	–
Aedae-ACP (A1)	A VTFSRDWNAamide	1.576E-06	411.1
Aedae-ACP (A2)	pQ A TFSRDWNAamide	7.052E-07	69.8
Aedae-ACP (A3)	pQVT A FSRDWNAamide	2.26E-05	550.8
Aedae-ACP (A4)	pQVT A SRDWNAamide	ND	>1,000
Aedae-ACP (A5)	pQVT F ARDWNAamide	1.038E-06	44.9
Aedae-ACP (A6)	pQVTFS A DWNAamide	6.872E-07	23.3
Aedae-ACP (A7)	pQVTFSR A WNAamide	3.863E-07	15.9
Aedae-ACP (A8)	pQVTFSRD A NAamide	ND	>1,000
Aedae-ACP (A9)	pQVTFSRDW A Aamide	4.96E-09	0.2
Aedae-ACP (-OH)	pQVTFSRDWNA- OH	ND	>1,000
Aedae-ACP (A1b)	Acetyl-A VTFSRDWNAamide	5.272E-07	43.6
Aedae-ACP (1-9)	pQVTFSRDWN-amide	4.153E-06	434.2
Aedae-ACP (1-8)	pQVTFSRDW-amide	2.156E-06	410.1
Aedae-ACP (1,3-10)	pQ-TFSRDWNAamide	ND	>1,000
Aedae-ACP (+G)	pQVTFSRDWNA G amide	1.113E-07	5.6
Grybi-ACP	pQITFSRDWNAamide	3.255E-07	13.6
Bommo-AKH	pQLTFPGWGWQamide	ND	>1,000
Lacol-AKH	pQLTFSSWGWGamide	ND	>1,000
Helze-AKH	pQLTFSSWGNamide	ND	>1,000

Using heterologous expression of ACPR, the half maximal effective concentration for each of the analogs was determined as well as the corresponding reduction in activity, highlighting how a particular residue substitution or other modification is tolerated in this system. ND = represents no determined EC₅₀ value due to minimal or no detectable activity of the analog when tested up to 10 μ M.

Synthetic Peptides and Analogs

Peptide analogs based on the native ACP sequence were designed and synthesized by Pepmic Co., Ltd. (Suzhou, China) at a purity of over 90%. All synthetic peptides were initially prepared as stock solutions at a concentration of 1 mM in dimethyl sulfoxide. All stocks of arthropod AKH analogs were prepared similarly to the ACP synthetic peptide analogs and were commercially synthesized by either Pepmic Co., Ltd. (Suzhou, China) or by Synpeptide Co. (Shanghai, China) at a purity of over 90% verified by HPLC and mass spectrometry. Specific sequence information for each of the peptides (and analogs) used on the ACP and AKH receptors examined in this study are provided in **Tables 1, 2**, respectively.

RESULTS

ACP Synthetic Analog Activity on Mosquito ACPR

Analogues of native ACP with single alanine substitutions at each amino acid were synthesized and evaluated by examining their functional activation of the *A. aegypti* ACP receptor

(ACPR) expressed using a heterologous system. Carboxy-terminal amidation of ACP was found to be critical for bioactivity as were the two aromatic residues of ACP, including the phenylalanine in the fourth position or tryptophan in the eighth position, since analogs modifying these key features demonstrated no activation of ACPR up to the highest tested concentration of 10 μ M (**Figure 1A**, **Table 1**). Analysis of single residue alanine substitutions revealed that the next most critical residues for retaining ACPR functional activation were the polar threonine in position three and the blocked pyroglutamate (pGlu) at the amino terminus, which upon replacement with alanine resulted in a $\sim$ 551- and $\sim$ 411-fold reduced activity, respectively, compared to native ACP (**Figure 1A**, **Table 1**). Alternative blocking of the amino terminus, using N-Acetyl-alanine instead of pGlu, revealed this analog was 10-times more active than the non-blocked one, having only $\sim$ 44-fold reduced activity compared to native ACP (**Figure 1A**). Substitutions with analogs containing alanine at the second (valine), fifth (serine), sixth (arginine), and seventh (aspartic acid) positions, had noticeably less impact on the reduction of activity (**Figure 1B**), by $\sim$ 70, $\sim$ 45, $\sim$ 23, and $\sim$ 16-fold, respectively. The analog containing an alanine substitution for the asparagine in position nine resulted in a more potent ACPR activation, leading to a $\sim$ 5-fold greater activity compared to the native ACP peptide (**Figure 1B**). C-terminally truncated analogs, which lacked either the alanine residue in the tenth position alone or both asparagine and alanine in positions nine and ten, were almost completely inactive, demonstrating $\sim$ 434 and $\sim$ 410-fold reduced ACPR activation compared to native ACP (**Figure 1C**). A C-terminally extended analog, an undecapeptide (i.e., polypeptide with 11 amino acids residues), ending with a glycine amide on the C-terminus elicited a small decrease of activity by about $\sim$ 6-fold (**Figure 1C**). The internally truncated ACP analog lacking a valine in the second position also was without any effect, similar to the two aromatic residue substituted analogs or the non-amidated ACP analog (**Figure 1C**). An ACP analog from the cricket *Gryllus bimaculatus* (Grybi-ACP) was used to further examine the importance of the second position valine in Aedae-ACP, which is replaced by an isoleucine in *G. bimaculatus*. Grybi-ACP was quite active compared to Aedae-ACP but nonetheless demonstrated nearly $\sim$ 14-fold reduced activity and failed to reach full ACPR activation even when the highest concentration was tested (**Figure 2A**). As shown previously, the endogenous octapeptide Aedae-AKH did not activate the ACPR at all; however, a subset of the three naturally-occurring insect AKH decapeptides tested here caused some activation, albeit very weak with Lacol-AKH and Helze-AKH leading to between 15 and 20% ACPR activation at the highest tested concentrations (**Figure 2B**).

Activity of Natural Arthropod AKH Analogs on Mosquito AKHR-IA

Taking advantage of the variety of natural AKH analogs in arthropods, we examined a subset of insect AKH peptides (see **Table 2**) with unique substitutions compared to the endogenous mosquito AKH peptide and determined their activity on the

TABLE 2 | Primary structure of select naturally occurring AKH analogs from insects and their activity on the *A. aegypti* AKHR-IA receptor (AKHR-IA).

Peptide/Analog	Species origin	Primary structure of analog	Activity on <i>A. aegypti</i> AKHR-IA (EC ₅₀)	Fold-reduction in activity relative to Aedae-AKH
Aedae-AKH	<i>Aedes aegypti</i>	pQLTFTPSWamide	2.159E–09	–
Lacol-AKH-7mer	* <i>Laconobia oleracea</i>	pQLTFTSS-amide	ND	> 10,000
Grybi-AKH	<i>Gryllus bimaculatus</i>	pQVNFSTGWamide	2.23E–06	1031.5
Libau-AKH	<i>Libellula auripennis</i>	pQVNFTPSWamide	1.18E–06	547.9
Pyrp-AKH	<i>Pyrrhocoris apterus</i>	pQLNFTPNWamide	7.269E–07	336.7
Erysi-AKH	<i>Erythemis simplicicollis</i>	pQLNFTPSWamide	6.69E–07	309.9
V ² -PeramCAH-II	* <i>Periplaneta americana</i>	pQVTFTPNWamide	1.485E–07	68.8
Bommo-AKH	<i>Bombyx mori</i>	pQLTFTPGWGQamide	2.362E–08	10.9
Hipes-AKH-I	<i>Hippotion eson</i>	pQLTFTSSWamide	1.647E–08	7.6
Peram-CAH-II	<i>Periplaneta americana</i>	pQLTFTPNWamide	2.226E–09	1.0
Tabat-AKH	<i>Tabanus atratus</i>	pQLTFTPGWamide	1.522E–09	0.7

Using heterologous expression of AKHR-IA, the half maximal effective concentration for each of the analogs was determined as well as the corresponding reduction in activity, highlighting how a particular single residue substitution, a combination of substitutions or other modifications are tolerated in this system. ND = represents no determined EC₅₀ value due to minimal or no detectable activity of the analog when tested up to 10 μ M.

*Denotes a synthetic analog that is based on an endogenous peptide from this insect.

AKH receptor, *A. aegypti* AKHR-IA. From this analysis, the least effective analog tested was the C-terminally truncated peptide, Lacol-AKH-7mer, with only ~7% AKHR-IA activation at the highest tested concentration (10 μ M), which represents a reduced efficacy of at least five orders of magnitude (**Figure 3A**). The next least active AKH analog was the cricket peptide, Grybi-AKH, which has five residues substituted over the length of the octapeptide compared to Aedae-AKH (see **Table 2**), and the activity of this analog on mosquito AKHR-IA is compromised by over three orders of magnitude (1,032-fold reduced activity) as a result of these combined substitutions (**Table 2** and **Figure 3A**). The AKH from the dragonfly, *Libellula auripennis* (Libau-AKH), with substitutions at positions two and three only, had a nearly 2-fold improved activation over that achieved by Grybi-AKH; however, the activity of this peptide on the *A. aegypti* AKHR-IA was still reduced by 548-fold compared to Aedae-AKH (**Table 2**). The AKH from the firebug *Pyrrhocoris apterus* (Pyrp-AKH) that has two substitutions in positions three and seven, both asparagine, is better tolerated than Libau-AKH and Grybi-AKH but is still less active on the *A. aegypti* AKHR-IA being compromised by 337-fold relative to Aedae-AKH (**Figure 3A**). To better understand the importance of N-terminal residues, we tested an AKH analog from the dragonfly *Erythemis simplicicollis* (Erysi-AKH), which is only different to Aedae-AKH at position three (threonine to asparagine; see **Table 2**); Erysi-AKH had 310-fold less activity compared to Aedae-AKH (**Figure 3A**). The peptide named V²-Peram-CAH-II, which is a synthetic analog of one of the two endogenous peptides of the American cockroach, *Periplaneta americana*, does not occur in this species and is characterized by a second position substitution of valine for leucine along with the seventh position substitution of asparagine for serine (see **Table 2**). AKHR-IA activation by V²-Peram-CAH-II was found much improved, albeit still 69-fold less effective compared to the native *A. aegypti* AKH (**Figure 3B**). If the C-terminus is extended by two amino acids as implemented by

utilizing the AKH analog from the domestic silkworm, *Bombyx mori* (Bommo-AKH), having glycine at position nine, glutamine at position 10 and a glycine at position seven (instead of the serine in Aedae-AKH), revealed activation of AKHR-IA was reduced by only 11-fold (**Figure 3B**). Finally, examining natural analogs of this peptide family containing single substitutions in the C-terminal region, at positions six or seven, it was demonstrated that these changes result in only a slight reduction of activation. The cockroach AKH, Peram-CAH-II (seventh position serine substituted with asparagine) had near identical efficacy to the native mosquito AKH while the AKH analog from the sphingid moth, *Hippotion eson* (Hipes-AKH-I; serine substitution for proline in the sixth position), had only an 8-fold reduced activity (**Figure 3B**). The AKH analog from the black horse fly, *Tabanus atratus* (Tabat-AKH; glycine substitution for serine at position seven), had an activation that partially exceeded (~30% improved efficacy) the activity of the native mosquito AKH (see **Table 2**; **Figure 3B**).

DISCUSSION

The current study set out to examine for the first time the ligand structure-activity relationship for an insect ACP receptor. This is of utmost importance in order to gain insight on the specific structural features of the ACP system given that extensive studies have been carried out previously on AKH receptors in a variety of species using natural and synthetic analogs of this neuropeptide family (32, 33, 44–57). For comparative reasons, we also studied the AKH receptor of *A. aegypti* but did not examine all residues since a plethora of previous research had clearly identified critical residues, for example the aromatics in positions four and eight (see references above). By examining the activity of a variety of ACP analogs on the activation of the *A. aegypti* ACP receptor (ACPR), including single alanine substitutions along with truncated or extended analogs, a few

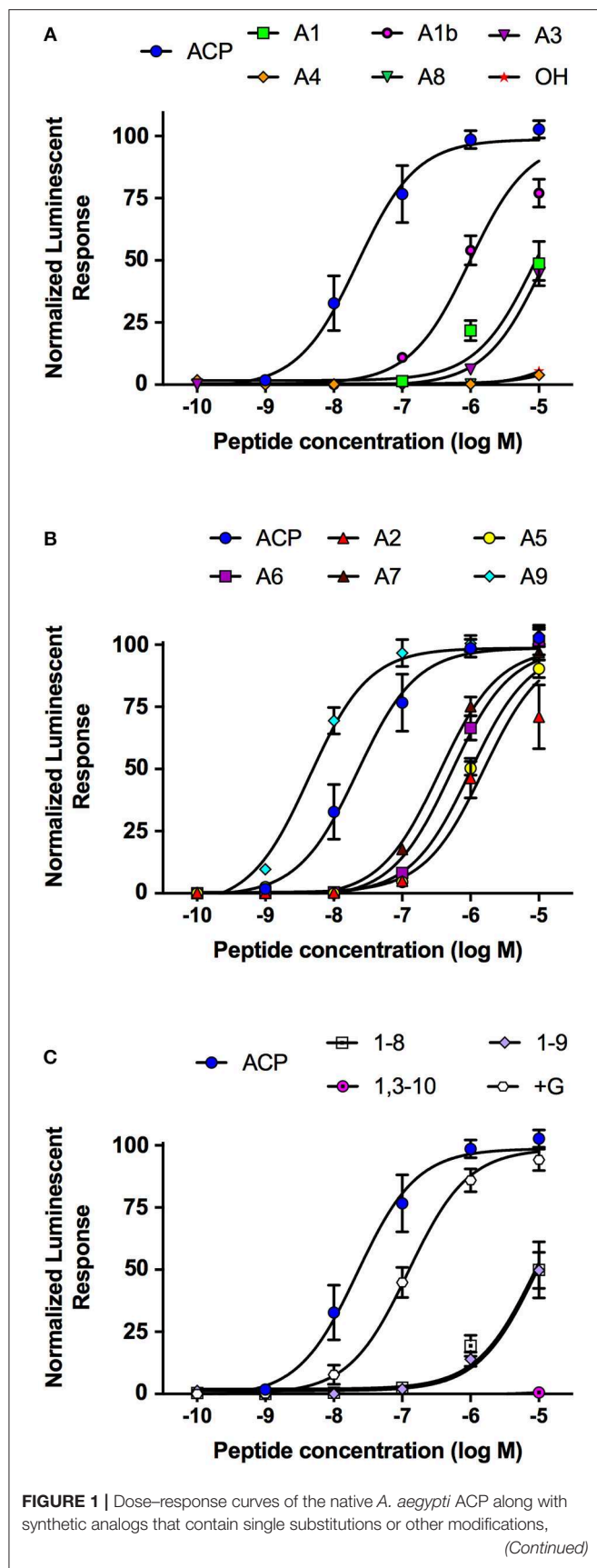


FIGURE 1 | which were tested for their activity on the ACP receptor (ACPR) using a cell-based bioluminescent assay. **(A)** Single amino acid substitutions with alanine or modifications to the normally blocked N- or C-termini resulting in a significant reduction to their activity on the ACPR. **(B)** Single amino acid substitutions with alanine to the native ACP sequence having minimal effects to the activity of the tested analogs indicating these substitutions are well tolerated. **(C)** Analogs with internal or terminal truncations or extension (i.e., +Gly analog) relative to the native ACP sequence conferring significant reductions to their activity on the ACPR. The half maximal effective concentration (EC_{50}) for each analog along with the corresponding change in activity relative to native *A. aegypti* ACP is provided in **Table 1**. Luminescence is plotted relative to the maximal response achieved when 10^{-5} M Aedae-ACP was applied to the ACPR. Data represent mean $\pm$ standard error of three independent biological replicates.

observations can be highlighted. Firstly, the overall charge of the peptide does not appear to play a role with regards to its influence on receptor activation since substitution of the basic (arginine) or acidic (aspartic acid) residues did not lead to a highly detrimental effect. Specifically, one would assume that the ACP receptor may prefer a neutral ligand given that the native ACP has no net charge (i.e., is neutral) since it has both a single basic and acidic residue. However, the modified analogs which replace the basic (position 6) or acidic (position 7) residues with alanine, create negatively or positively charged molecules, respectively, with both being quite active and so neither proved to be detrimental substitutions, suggesting that the extra carboxy- or amino group is not involved in forming a salt bridge with the receptor. Secondly, with the exception of the aromatic tryptophan residue in the eighth position, the C-terminal residues of ACP do not appear to be highly important since alanine substitutions in positions five through seven only marginally impacted activity between ~ 16 to 45-fold whereas alanine substitution in position nine resulted in this analog having 5-fold increased activity. Such a compound may be a good lead substance for the development of a superagonist in future studies, with the eventual goal to design an active non-peptidergic mimetic. Amidation of the C-terminal residue was critical since the free acid analog exhibited no activity; however, it is noteworthy that the C-terminally extended peptide having an amidated glycine was also quite effective with ACPR activation compromised by only 6-fold. Previous studies examining free acid analogs of AKH (including both *in vitro* and *in vivo* assays) have demonstrated that in some cases the absence of a blocked C-terminus is less critical (33, 51, 58), whereas other investigations have revealed the presence of an amidated C-terminus to be very important with poor or no responses with analogs lacking this feature (31, 59). These observed differences in response to key structural elements, including the normally amidated C-terminus of AKH, may reflect the occurrence of inter- and intra-species receptor subtypes which may be differentially sensitive to these modifications (31, 58), and could also point to the well documented phenomenon seen for AKH receptors that are more promiscuous whereas others are very strict in the structural characteristics of their particular ligand (33, 34, 59). This latter scenario is more in-line with observations for the

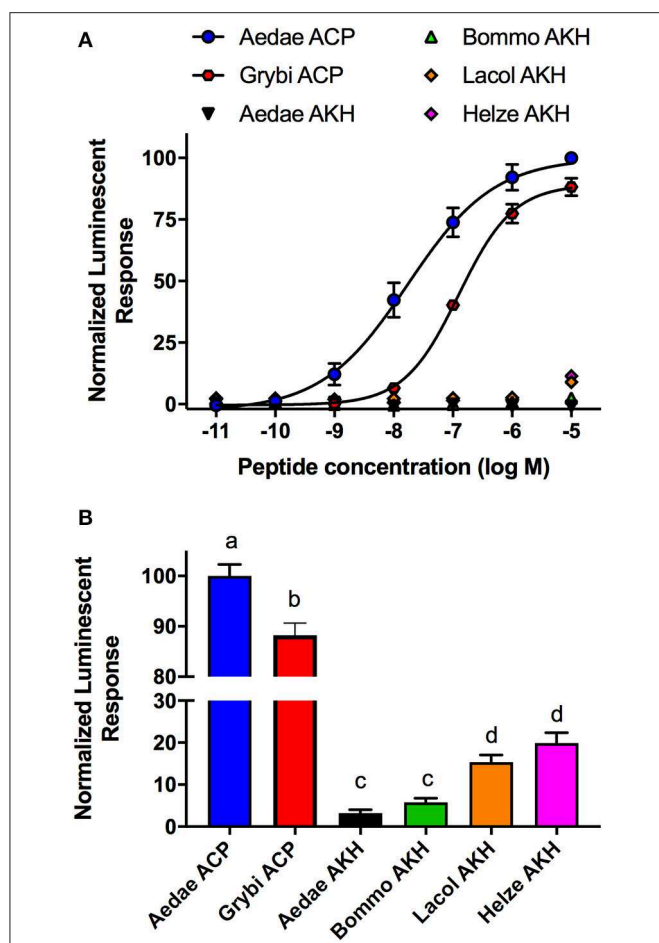


FIGURE 2 | Activity of native ACP analogs from *A. aegypti* and *G. bimaculatus* along with selected naturally-occurring AKH decapeptides tested for their activity on the ACP receptor (ACPR) using a cell-based bioluminescent assay. **(A)** Dose-response curves of natural ACP analogs and selected insect AKH decapeptides on the *A. aegypti* ACPR. **(B)** Analysis of the mosquito ACP receptor activation at the highest tested dose (10 μ M) indicating activity of Grybi-ACP is significantly reduced as a result of the single substitution at position two. The native Aedae-AKH along with Bommo-AKH were inactive on *A. aegypti* ACPR while Lacol-AKH and Helze-AKH showed low activity. The half maximal effective concentration (EC_{50}) for each analog along with the corresponding change in activity relative to native *A. aegypti* ACP is provided in **Table 1**. Luminescence is plotted relative to the maximal response achieved when 10^{-5} M Aedae-ACP was applied to the ACPR. Data represent mean $\pm$ standard error of three independent biological replicates. In **(B)**, different letters denote significant difference ($p < 0.01$) as determined by ANOVA and Tukey post-test.

A. aegypti ACP system where only a single functional receptor variant occurs (27), which we show herein does not tolerate the absence of the C-terminal amidation and is thus of high importance for the ACP system. Thirdly, both aromatic residues were essential for activity on the ACPR while N-terminal residues of the peptide, including mainly threonine and pyroglutamic acid, were also highly critical since alanine substitutions were not tolerated leading to significantly reduced activity of these analogs relative to the native ACP peptide. However, alternatively

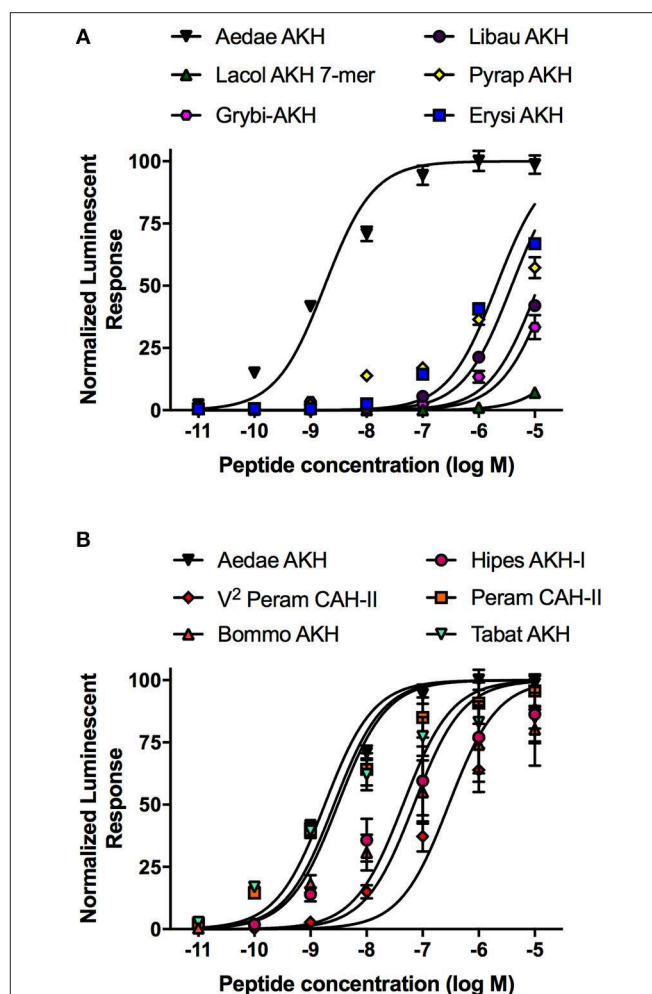


FIGURE 3 | Dose-response curves of the native *A. aegypti* AKH along with mostly naturally occurring analogs from a variety of insects that contain substitutions or other modifications, which were tested for their activity on the AKHR-IA receptor using a cell-based bioluminescent assay. **(A)** Naturally occurring insect AKH analogs having critical substitutions that are not well tolerated by the AKHR-IA, with moderately compromised activity relative to the native *A. aegypti* AKH. **(B)** Naturally occurring insect AKH analogs, or a synthetic analog (V^2 -Peram-CAH-II), having substitutions that are well tolerated by the AKHR-IA, with marginally compromised or slightly improved activity relative to the native *A. aegypti* AKH. The half maximal effective concentration (EC_{50}) for each analog along with the corresponding change in activity relative to native *A. aegypti* AKH is provided in **Table 2**. Luminescence is plotted relative to the maximal response achieved when 10^{-5} M Aedae-AKH was applied to the AKHR-IA. Data represent mean $\pm$ standard error of three independent biological replicates.

blocking this alanine substitution on the N-terminus through acetylation improved the activity of this analog by 10-fold. Similar observations have been made with AKH analogs tested using both *in vivo* and *in vitro* assays whereby complete removal of the N-terminal pyroglutamate abolishes activity of the AKH analog whereas the alternatively blocked N-terminus (i.e., N-Acetyl-Ala), or even simply glutamate or glutamine that can spontaneously undergo cyclization (60), were shown to have improved activity, albeit lower than the native AKH

(31, 54, 58). Interestingly, alanine substitution for valine in the second position, thus both hydrophobic but the former having a shorter side chain, led to a relatively minor effect on ACPR activation (~70-fold reduced activity). Comparatively, replacing valine at position two with another aliphatic amino acid with a hydrophobic but longer side chain as in Grybi-ACP (isoleucine at position two) had only a minor effect on ACPR activation with a ~14-fold reduction compared to Aedae-ACP, indicating that side chain length at this position may be important for ligand-receptor interaction. The internally truncated analog omitting valine within the N-terminal region elicited no ACPR activation, indicating that the spacing between the more critical residues, namely pyroglutamate and polar threonine, is essential for ACP peptide activity on the ACPR. Thus, perhaps in common with AKH structural features, the switch between adjacent hydrophobic and hydrophilic residues is necessary in order to properly “fit” and bind with its particular receptor (31) so that not only is the ACP analog shorter by one amino acid (a non-peptide), but moreover, the entire structural configuration of the peptide is disrupted and the whole sequence is no longer in sync with its binding pocket on the receptor. This corroborates with studies that have examined the conservation of the ACP primary structures across insects from different orders (including Diptera, Lepidoptera, Coleoptera, Hymenoptera and Hemiptera) as well as from various species within the same order (seventeen species of Coleoptera), where the N-terminal pentamer sequence, when the ACP system is present, is nearly completely conserved across these various species with a consensus motif of pQVTFS-, whereas significant sequence variability occurs within the C-terminus of the ACP sequence with deca-, nona- and even dodecapeptides reported (2, 61).

Our studies on the importance of specific amino acids of the ligand Aedae-AKH for activating the mosquito AKH receptor were driven by a number of previous studies on AKH receptors utilizing *in vitro* heterologous assays as well as *in vivo* bioassays monitoring lipid- and/or carbohydrate-mobilizing actions of AKH analogs. For instance, the blocked N- and C-termini were mostly quite important and the aromatics at positions 4 and 8 were absolutely essential (46, 47, 53, 54). Furthermore, it was demonstrated earlier that certain AKH receptors tested *in vitro* were found to be quite promiscuous reacting well to ligands that had many substitutions, for example the AKH receptors from the desert locust *Schistocerca gregaria* and the pea aphid *Acyrtosiphon pisum* (34). On the other hand, a group of AKH receptors from other insects, including the fruit fly *Drosophila melanogaster* and the mosquito *A. aegypti*, were found to be highly specific, tolerating only minor substitutions (33, 34). However, since only a few modifications to the natural AKH ligand were tested on the *A. aegypti* receptor, in the present study we focused on the importance of residues at positions two and three at the amino end and five through seven at the carboxyl end by utilizing naturally-occurring AKH bioanalogues containing all or a subset of these substitutions. Starting with Grybi-AKH, which differs from Aedae-AKH in all the variable positions except the prototypical features of any insect AKH

such as pyroglutamic acid at the first position, phenylalanine and tryptophan at positions four and eight, respectively, and an amidated C-terminus, we demonstrated a large reduction in activation of the Aedae-AKHR-IA by over three orders of magnitude. This gave us the opportunity to tease out which of the five positions are the main drivers for this observed low activity. Previous studies using modifications at positions five and seven together had shown that such ligands resulted only in a slight loss of receptor activation (33, 34, 58). In line with this observation, position six was not found to be essential for AKHR-IA activation since a single amino acid change from the uniquely shaped imino acid proline to a serine residue (as found in Hipes-AKH-I), resulted in <10-fold loss in activity on AKHR-IA. Here, we clearly established that position seven, although changed either from a polar side chain in serine to a much larger polar side chain in asparagine (Peram-CAH-II), or to the shortest amino acid glycine (Tabat-AKH), is not essential for activation of AKHR-IA since these analogs had comparable activity to the native Aedae-AKH. In fact, to our surprise, Taba-AKH may serve as a potential lead substance for design of a superagonist since this natural AKH analog elicited 30% improved activity relative to the native mosquito AKH. It appears that the more flexible and non-polar glycine residue allows the ligand a tighter binding to the receptor than the polar and neutral serine. This is particularly interesting given the permissiveness of substitutions in the vicinity of the C-terminus, which may allow a greater variety of lead compounds to be designed and tested that could interfere with this neuropeptide system and perturb its normal functioning vital for mobilizing energy substrates in insects. Interestingly, Bommo-AKH retained strong activity on the *A. aegypti* AKHR-IA, which in common with Taba-AKH has a glycine substitution in position seven, but also has an extended C-terminus containing glycine and glutamine following after tryptophan in the eighth position. This indicates that the extended nature of this AKH ligand does not compromise AKHR-IA activation and follows with the earlier observations on the overall permissiveness of the C-terminal region of the AKH ligand.

To corroborate that the substitutions in positions two to three were of greatest importance, we examined the activity of a natural AKH analog from *L. auripennis* (Libau-AKH), whose sequence matches that of native Aedae-AKH except for positions two to three (valine and asparagine) that are instead shared with Grybi-AKH. Indeed, our results confirmed that the substitutions in this N-terminal region are much more critical and are not well tolerated by AKHR-IA in *A. aegypti* since Libau-AKH displayed only a marginal improvement (i.e., nearly 2-fold) compared to Grybi-AKH. Supporting the notion that N-terminal residues are most important for AKHR-IA activation, two additional natural AKH analogs were examined having substitutions in either the third position alone (Erysi-AKH) or both the third and seventh position residue (Pyrap-AKH). In comparison to Aedae-AKH, Erysi-AKH had a similar reduction in activity as Pyrap-AKH indicating that the residue in the third position is essential within the natural AKH ligand since it is established that a seventh position substitution has a

negligible effect. In light of this evidence indicating substitutions in positions two and three are not well tolerated by the *A. aegypti* AKHR-IA, we aimed to discern which of these two sites were the absolute most critical. Comparing the activity V²-PeramCAH-II with that of Pyrap-AKH demonstrates that the valine substitution for leucine in the second position is far better tolerated compared to the asparagine substitution for threonine in the third position, indicating this latter position is indeed the most critical.

Taken together, these findings corroborate recent analysis of select dipteran AKH analogs where structure-activity analyses showed the least tolerated substitutions were localized to the N-terminal region, proving crucial for activation of *D. melanogaster* and *A. gambiae* AKH receptors, which the authors described was due to this core region of the peptide forming a predicted β -strand necessary for receptor interaction (33). Further, this supports our findings that the C-terminal region of the AKH octapeptide, excluding the aromatic tryptophan in the eighth position, is not as critical for activity. As a putative reason for this, it is argued that the side chains of a subset of these amino acids may be buried in the assumed β -turn formed by the residues in the C-terminal region of AKH neuropeptides (31, 33, 46), which is now supported by molecular modeling and nuclear magnetic resonance spectroscopy studies that have confirmed the β -turn configuration in the AKH secondary structure (36, 37, 62, 63). Indeed, a comprehensive structural analysis of the AKH system in the desert locust, *Scistocerca gregaria*, recently confirmed that all three of the native AKH neuropeptides despite exhibiting differences in amino acid composition and in the length of the peptide (i.e., both octa- and decapeptide analogs), nonetheless exhibit a β -turn structure and interact with the same residues within the single locust AKH receptor (40).

Integrating the novel data from the present study analyzing structural characteristics of the two most recently diverged GnRH-related family members in arthropods, we can infer with some degree of confidence how these ligands act only upon their respective receptors. The ACP sequence in many insect species often contain charged residues in positions six, seven or nine, which in instances where only a single basic or acidic residue occurs, provide an overall charge to the peptide (2, 61). However, our results revealed that the overall charge of the ACP analog did not largely compromise activity since alanine substitutions of these charged residues were quite tolerated by the ACPR. Comparatively, AKH receptors do not like charges associated with their ligands since no basic residues occur and only a few natural AKH family members containing an acidic residue (e.g., aspartic acid at position seven) are known, which have been shown to be not well tolerated by AKH receptors in *L. migratoria* and *P. americana* (47, 64, 65). Another feature differentiating the AKH and ACP peptides in *A. aegypti* is the presence of an alternating pattern of hydrophobic and hydrophilic residues over the entire length of the Aedae-AKH, which is shared with other AKH family members (31), whereas the ACP contains a motif of three hydrophilic residues at its core residues 5–7, which

may lead to incompatibility with the AKHR-IA. Additionally, clearly the valine substitution in position two is not tolerated well by the *A. aegypti* AKHR-IA, and so this residue which is present in position two of the ACP sequence may also contribute toward the inactivity of this peptide on the AKHR-IA. On the other hand, since the AKH peptide from *B. mori* (a decapeptide) was quite active on the *A. aegypti* AKHR-IA, the longer length of ACP is unlikely to be the cause of its inactivity on the AKHR-IA.

Our findings also set the framework toward understanding why the *A. aegypti* AKH has no activity on the ACP receptor. As we saw with the AKHR-IA, substitution of valine for leucine in position two led to nearly a 70-fold reduction in receptor activity. On the other hand, the removal of valine and its replacement with alanine in the ACP sequence led to a similar 70-fold reduction in activity on the ACPR. In contrast, substitution of valine in position two with another branched-chain amino acid (i.e., isoleucine) found in the Grybi-ACP analog, only marginally reduced activity. Thus, the presence of valine in position two is needed for optimal activity for ACPR whereas its absence in the same position (but on the AKH peptide) is necessary for optimal activation of AKHR-IA. Finally, one clear finding from these studies is that ACP analogs that are too short (nonapeptide or octapeptide), even if they contain all the hallmark features, as demonstrated by the C-terminally truncated analogs for example, fail to activate ACPR. Thus, the length of the ligand is most relevant and the binding pocket of ACPR requires at least a decapeptide leaving the *A. aegypti* AKH, an octapeptide, too short for ACPR binding and activation, while comparatively some decapeptide AKH analogs demonstrated low (albeit significant) levels of activity on ACPR.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author (J-PP: paluzzi@yorku.ca).

AUTHOR CONTRIBUTIONS

GG and J-PP designed the synthetic analogs and wrote the manuscript. AW performed all the experiments and AW, J-PP, and GG analyzed the data. All authors have contributed toward the revisions and have granted approval of the final manuscript submitted for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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