Effect of dietary macronutrients on the expression of cholecystokinin, leptin, ghrelin and neuropeptide Y in gilthead sea bream (*Sparus aurata*)

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Abstract

Endocrine factors released from the central nervous system, gastrointestinal tract, adipose tissue and other peripheral organs mediate the regulation of food intake. Although many studies have evaluated the effect of fed-to-starved transition on the expression of appetite-related genes, little is known about how the expression of appetite-regulating peptides is regulated by the macronutrient composition of the diet. The aim of the present study was to examine the effect of diet composition and nutritional status on the expression of four peptides involved in food intake control in gilthead sea bream (*Sparus aurata*): neuropeptide Y (NPY), ghrelin, cholecystokinin (CCK) and leptin. Quantitative real-time RT-PCR showed that high protein/low carbohydrate diets stimulated the expression of CCK and ghrelin in the intestine and leptin in the adipose tissue, while downregulation of ghrelin and NPY mRNA levels was observed in the brain. Opposite effects were found for the expression of the four genes in fish fed low protein/high carbohydrate diets or after long-term starvation. Our findings indicate that the expression pattern of appetite-regulating peptides, particularly CCK and ghrelin, is modulated by the nutritional status and diet composition in *S. aurata*.

Keywords: Appetite-regulating peptides; neuropeptide Y; cholecystokinin; leptin; ghrelin; *Sparus aurata*.

1. Introduction

Food intake is a primary regulator of growth that is governed by a complex regulatory system involving post-ingestive signals and nutrient-sensing neurons in the hypothalamus and throughout the brain. Dietary nutrients trigger changes in neural and endocrine pathways, energy intake, the release of stored nutrients, nutrient utilisation, and even macronutrient choice interaction (Blouet and Schwartz, 2010; Kulczykowska and Sánchez Vázquez, 2010; Levin et al., 2011; MacKenzie et al., 1998). In addition to the fact that a variety of neurohormones cooperate to regulate appetite, fish exhibit different feeding behaviour depending on diet composition and nutritional status (Kulczykowska and Sánchez Vázquez, 2010). Many fish species undergo fasting periods during their life cycle as a result of migration, reproduction and food availability (Day et al., 2014). Depending on the fish species and its habits, different strategies have evolved for adjusting the intermediary metabolism to food deprivation. For instance, glycogen levels are rapidly depleted in the liver of some fish species on starvation, while they are hardly affected by food restriction for up to several months in other species (Caruso and Sheridan, 2011; Takahashi et al., 2011).

In vertebrates, peripheral hormones controlling appetite include satiety signals such as cholecystokinin (CCK) and leptin. CCK is secreted by the gastrointestinal (GI) tract as well as by neurons in the brain and enteric nervous system. It exerts a variety of physiological actions, but functions primarily as a satiety signal in mammals. By binding to receptors in the GI tract and central nervous system, CCK causes satiation through endocrine and/or neural mechanisms such as enhancing gastric distention signals (Cummings and Overduin, 2007). Progress has been made in studying the effect of starvation and hormone administration on circulating and tissue expression levels of appetite-regulating peptides in a number of fish species. However, fish are

phylogenetically diverse and relatively few species have been studied to date (Hoskins and Volkoff, 2012). In goldfish, both intracerebroventricular and intraperitoneal injections of CCK suppress food intake (Volkoff et al., 2003), while treatment of trout with CCK antagonists increases food intake (Gelineau and Boujard, 2001).

It has been proposed that the most important role for CCK in food intake regulation might be synergistic interaction with long-term adiposity signals, such as leptin (Cummings and Overduin, 2007). In mammals, leptin is synthesised and secreted predominantly by the adipose tissue. It has a major role in the regulation of body fat mass, by decreasing food intake and inducing weight loss. Leptin acts as a lipostatic and satiety signal by inhibiting hypothalamic orexigenic pathways and stimulating anorexigenic peptides (Doyon et al., 2001). In addition to energy homeostasis, mammalian leptin regulates glucose and lipid metabolism as well as immune function, among other physiological processes (Izadi et al., 2014; Park and Ahima, 2014). In humans and rodents, leptin levels decrease dramatically with starvation (Ahima et al., 1996; Izadi et al., 2014). As in mammals, leptin seems to act as an anorexigenic factor in fish (Volkoff, 2015). However, the liver seems to be the main site of leptin production in fish species (Gorissen and Flik, 2014; Salmerón et al., 2015). Indeed, leptin signalling in zebrafish regulates glucose homeostasis, but not adipostasis (Michel et al., 2016). In goldfish, both intraperitoneal and intracerebroventricular injections of murine leptin accentuate the anorexigenic effects of CCK and amphetamine-regulated transcript (CART), and decrease food intake and neuropeptide Y (NPY) mRNA levels in the brain. These findings suggest that, as in mammals, leptin acts primarily in the brain of fish to control energy homeostasis and inhibit food intake (Volkoff and Peter, 2001; Volkoff et al., 2003).

In rodents, ghrelin promotes food intake, body weight gain and adiposity through central and peripheral modes of action (Nakazato et al., 2001; Tschöp et al., 2000). The orexigenic action of ghrelin is mediated by NPY/agouti-related peptide (AgRP) in the brain (Seoane et al., 2003). NPY, one of the more highly conserved neuropeptides in vertebrates, is involved in many physiological functions, such as cardiovascular control, anxiety, sexual behaviour, and feeding (Pedrazzini, 2004). In particular, NPY is considered the most potent orexigenic peptide in the mammalian brain (Woods et al., 1998). Both NPY and ghrelin stimulate food intake and the release of growth hormone from the pituitary gland in goldfish (Matsuda et al., 2011; Peng et al., 1993).

The role exerted by dietary nutrients on the hypothalamic integration of neural inputs and the expression and release of hormones controlling food intake in fish remain largely unknown. To gain insight into endocrine regulation of food intake in gilthead sea bream (*Sparus aurata*), which is currently the most widely cultured marine fish in Europe (FAO Fishery Statistics Web Page), in the present study we analysed the effect of starvation and diet composition on the expression of orexigenic peptides ghrelin and NPY, and anorexigenic peptides CCK and leptin.

2. Materials and methods

2.1. Rearing procedures and feeding trial

S. aurata juveniles (16.9 ± 0.9 g body weight) were obtained from Piscimar (Burriana, Castellón, Spain). After arrival, fish were distributed in 260-L aquaria located in isothermal rooms at 21 ± 0.2 ºC and provided with seawater in a closed circuit with active pump filters and UV lamps. The photoperiod was adjusted to a 12 h: 12 h dark-light cycle. Acclimation to our facilities and maintenance procedures were as previously described (Fernández et al., 2007). To study the effect of diet composition on

the expression of peptide hormones involved in food intake control, three groups of fish were fed for 23 days a ration of 25 g/kg body weight once a day (10 a.m.) with each of the experimental diets shown in Table 1. A fourth group of fish was starved during the same period. The experimental diets were formulated a macronutrient composition (protein and carbohydrates) that was above and below the levels in commercially available diets. The experimental diets were named HLL (high protein, low lipid and low carbohydrate; with a macronutrient composition similar to the natural diet of wild *S. aurata*), MHL (medium protein, high lipid and low carbohydrate; with a composition similar to commercial diets for *S. aurata* culture) and LLH (low protein, low lipid and high carbohydrates; with partial substitution of protein by carbohydrates, compared to diet HLL). Nutrient composition was adjusted to achieve similar energy levels (20 kJ/g for the HLL and LLH diets, and 22 kJ/g for the MHL diet).

2.2. Sampling

At the end of the feeding trial, 24 fish per condition were anaesthetised with tricaine methanesulfonate (MS-222) diluted in seawater (1:12,500) before handling (10 a.m., 24 h after the last meal). Five fish per condition were killed by cervical section, and samples from liver, brain, intestine and adipose tissue were dissected out, immediately frozen in liquid nitrogen, and kept at -80 ºC until RNA isolation. The remaining fish were pooled into three groups per diet (6-7 fish per pool) and stored at -20 ºC to further determine moisture, protein, lipid and ash content. All experimental procedures were approved by the University of Barcelona's Animal Welfare Committee (proceeding #461/16), in compliance with local legislation and directive 2010/63/EU.

2.3. Analysis of food and body composition

Ground samples of feed and fish pools were dried at 70 °C until a constant weight was obtained to calculate the moisture content. The percentage of moisture in the sample was calculated using the following equation: % FW = (A-B) x 100 / A, where % FW = percentage of moisture in the sample, A = weight of wet sample, and B = weight of dry sample. Nitrogen (N) in dried samples was analysed with a Carlo Erba NA 2100 elemental analyser (CE Instruments, Wigan, UK). Crude protein was calculated by multiplying N content by a factor of 6.25. Crude fat was extracted with petroleum ether using a Soxtec HT 1043 (Foss, Hillerød, Denmark), and then weighed to compute its contribution to whole dry weight. Ash content was determined by comparing the weight of the sample before and after incineration in a Hobersal 12PR/300 muffle furnace (Hobersal, Barcelona, Spain) at 450 ºC for 12 h. Each pool of fish was assayed in triplicate.

2.4. Molecular cloning of S. aurata *cholecystokinin, leptin, ghrelin and neuropeptide Y cDNA fragments*

Total RNA was extracted from frozen samples of brain, intestine, liver and adipose tissue using the High Pure RNA Tissue Kit (Roche, Basel, Switzerland). RNA integrity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA Integrity Number (RIN) > 9.2 were used for subsequent studies as a template for RT-PCR and RT quantitative real-time PCR (RT-qPCR). RNA was quantified with a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). *S. aurata* cDNA fragments for CCK, leptin and ghrelin were isolated by RT-PCR. To this end, single-strand cDNA templates for PCR amplification were synthesised from 1 μg of total RNA by incubation with M-MLV RT (Promega, Madison, WI, USA) at 37 °C for 1h in the presence of RNase inhibitor (RNasin;

Promega, Madison, WI, USA) and random hexamer primers. RNA samples from the GI tract (CCK), liver and adipose tissue (leptin), and brain (ghrelin and NPY) were used to isolate cDNA fragments. RT products were subjected to PCR amplification using the primer pairs CC1412/CC1413r (CCK), LP1414/LP1415r (leptin), G1410/G1411r (ghrelin) and N1407/N1408r (NPY) (Table 2). PCR products of the expected size were isolated and ligated into pGEM T easy (Promega, Madison, WI, USA). Three independent clones for each amplified product were fully sequenced on both strands. The identity of isolated sequences was verified using BLAST against the National Center for Biotechnology Information (NCBI) public databases. Sequence analysis was subsequently used to design oligonucleotides to perform qPCR analysis.

2.5. Quantitative real-time PCR

The RT product synthesised as described in Section 2.4 was subsequently used for the qPCR analysis. CCK, leptin, ghrelin and NPY mRNA levels were determined in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using 0.4 μM of each primer (Cck-2/Cck-R2, LP1414/LP1415r, G-2/G-R2 and N1407/N1408r for CCK, leptin, ghrelin and NPY, respectively; Table 2), 10 μl of Power SYBR Green (Applied Biosystems, Foster City, CA, USA), and 1.6 μl of cDNA (dilution 1:10 for liver; 1:3 for brain and adipose tissue; and 1:2 for GI tract) in a final volume of 20 μl. Specific primers were designed using the OligoFaktorySE software (Schretter and Milinkovitch, 2006). The mRNA levels were assessed in tissues corresponding to the major sites of CCK, leptin, ghrelin and NPY production in fish: CCK in the intestine; leptin in the liver and the adipose tissue; ghrelin in the GI tract and the brain; and NPY in the brain. Amplification conditions were: initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation for 15 s at 95 °C, and

annealing-extension for 1 min at 62 °C. A dissociation curve was run after each experiment to ensure that there was only one product. The specificity of the amplification was assayed by sequencing the reaction products at least once for each gene. The efficiency of the PCR reaction was checked for each gene by generating standard curves with serial dilutions of a control cDNA sample. The amount of mRNA for the genes of interest in each sample was normalised with *S. aurata* elongation factor 1 α (EF1α) using the primer pair AS-EF1Fw/AS-EF1Rv (Table 2). Amplicon size (113, 175, 171, 110 and 135 bp for CCK, leptin, ghrelin, NPY and EF1α, respectively) was routinely controlled by agarose gel electrophoresis. EF1α was chosen as a housekeeping gene, as it showed stable expression levels that were constitutively expressed, irrespective of the treatment (Kozera and Rapacz, 2013). For each sample, PCR was performed in duplicate and variations in gene expression were calculated by the standard ΔΔCt method (Pfaffl, 2001). The data presented were confirmed in at least two independent runs of qPCR experiments.

2.6. Statistical analysis

Analyses were performed using SPSS software Version 22 (IBM, Armonk, NY, USA). Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances prior to their comparison. Differences between conditions were analysed using the Student´s *t*-test (for paired comparisons to determine differences between initial and final body weight for each experimental condition) or one-way ANOVA (comparisons among three or more groups). When statistical significance was found for ANOVA, the Student-Newman-Keuls (SNK) post-hoc test was used to determine differences among treatments ($P < 0.05$). Linear regression analysis (*F*-test) was performed between body lipid, body protein, dietary protein and the mRNA levels of

CCK, leptin, ghrelin and NPY in *S. aurata* fed with diets HLL, MHL and LLH. A discriminant function analysis, with Wilk's Λ, was used to determine whether mRNA levels of CCK, leptin, ghrelin and NPY enable discrimination of dietary condition in *S. aurata*.

3. Results

To study the impact of diet composition on the gene expression of appetite-regulating hormones in *S. aurata*, three groups of fish were fed the HLL, MHL or LLH diet for 23 days, while a fourth group of animals was deprived of food during the same period. The starved fish were the only group of animals that lost weight during the experimental period (the initial and final body weight expressed as mean ± SD were 29.7 ± 4.2 and 26.3 ± 4.1, respectively; n=24 fish; $P < 0.001$, paired Student's *t*-test). Among fed fish, those fed the LLH diet exhibited significantly lower body weight values than those fed HLL or MHL at the end of the trial (Fig. 1). No significant differences in body weight were found between the groups of fish fed the HLL and MHL diets. The highest values of body protein were found in the fish fed the HLL diet, while feeding the MHL and LLH diets promoted higher body lipid (Table 3).

To date, genomic data and molecular resources to study the impact of dietary nutrients on peptide hormones and genes involved in food intake in *S. aurata* are scarce. Given that no sequences were previously reported for CCK, leptin and ghrelin in this species, we isolated partial cDNA fragments of the corresponding genes by RT-PCR. To this end, we designed primers from highly conserved regions reported for other fish or vertebrate species in public nucleotide databases. Nucleotide sequences of *S. aurata* CCK, leptin and ghrelin were submitted to the GenBank database (accession numbers KP822925, KP822924 and KP822922, respectively). In addition, we isolated a cDNA

fragment of *S. aurata* NPY (GenBank accession no. KP822926). In all cases, the deduced amino acid sequences of the isolated *S. aurata* cDNAs exhibited a high degree of identity with their corresponding orthologues in other fish (CCK: 96 % with CCK 1 from *Diplodus sargus*; leptin: 90 % with leptin a from *Epinephelus coioides*; ghrelin: 97 % with ghrelin from *Acanthopagrus schlegelii*). The isolated nucleotide sequences were used to design oligonucleotides to determine mRNA levels by RT-qPCR in tissues of starved fish or those fed the HLL, MHL and LLH diets.

Gene expression levels of CCK, leptin, ghrelin and NPY were differently affected by the nutritional status and macronutrient composition of the diet in *S. aurata*. The highest CCK mRNA expression level was found in the intestine of fish fed the high protein/low carbohydrate diet (HLL), achieving values 2.8-fold higher than in fish fed the MHL and LLH diets, and 4.5-fold higher than in starved animals (Fig. 2A).

Leptin mRNA levels were determined at the main sites for leptin production in fish: liver and adipose tissue. Leptin mRNA abundance was significantly higher (3.4-fold) in the adipose tissue of *S. aurata* that were fed the HLL diet than in long-term starved fish. Although not significant, the same tendency was observed in the fish fed either high lipid or low protein/high carbohydrate diets (MHL and LLH) (Fig. 2B).

Gene expression levels of ghrelin and NPY in the brain and ghrelin in the GI tract were also analysed. Among the fed fish, higher ghrelin mRNA levels were found in the GI tract of fish supplied with the HLL and MHL diets, while the lowest values corresponded to fish fed the LLH diet. No significant differences were found between the group of fish subjected to long-term starvation and those fed the LLH diet. The mRNA levels of ghrelin and NPY in the brain exhibited a similar trend. The lowest levels of ghrelin and NPY mRNA were found in the brain of fish fed the HLL diet. Compared to fish fed LLH, feeding with the HLL diet resulted in a 2.3- and 1.8-fold

decrease in ghrelin and NPY mRNA levels, respectively. Starved *S. aurata* presented the highest ghrelin and NPY mRNA expression in the brain (Fig. 3).

Linear regression analysis with the highest statistical significance between diet/body composition and the expression of appetite-regulating peptides in *S. aurata* fed the experimental diets are listed in Table 4. When considered relationships with $p < 0.05$ (*F*-test), significant positive correlations were found between leptin mRNA levels in the liver and body protein, as well as for NPY mRNA abundance in the brain and body lipid. A negative correlation was found between ghrelin mRNA in the brain and the protein content of the diet.

A discriminant function analysis using the expression data represented in Figures 2 and 3 succeeded to classify 100 % of fish into the four nutritional conditions assayed (starvation and feeding with HLL, MHL or LLH diet). Two canonical discriminant functions (function 1: Wilk's $\Lambda = 0.003$, $\chi^2 = 47.077$, $P < 0.001$, accounting for 80.3 % of total variance; and function 2: Wilk's $\Lambda = 0.091$, $\chi^2 = 19.153$, $P < 0.05$, accounting for 19.0 % of total variance) significantly enabled the discrimination of experimental fish according to the dietary condition.

4. Discussion

The effect of diet composition and nutritional status on the expression of orexigenic and anorexigenic peptides is not conclusive and appears to be species-dependent. In the present study, long-term starvation decreased mRNA levels of anorexigenic peptides in *S. aurata*: CCK in the intestine, and leptin in the liver and the adipose tissue. Differences were more pronounced when compared to data obtained from fish fed the highest protein/lowest carbohydrate diet (HLL), whose macronutrient composition is close to the natural diet of wild *S. aurata*. At present, substitution of dietary protein by

cheaper nutrients with a reduced environmental impact, such as carbohydrates, is a challenging trend for sustainable aquaculture (Naylor et al. 2009). However, teleost fish are considered to be glucose intolerant and exhibit prolonged hyperglycemia following a glucose load or a high carbohydrate meal (Moon, 2001). We previously showed that *S. aurata* tolerates partial substitution of dietary protein by carbohydrates through a metabolic adaptation involving increased glycolysis and the pentose phosphate pathway in the liver. Nevertheless, carbohydrate levels over 20 % have a negative impact on growth performance (Fernández et al., 2007; Metón et al., 1999). Bearing in mind that the low protein and high carbohydrate content of the LLH diet (which is far from the composition of natural diets of wild *S. aurata*) resulted in less weight gain than feeding with the HLL and MHL diets, it is not surprising that the mRNA levels for anorexigenic genes in *S. aurata* fed the LLH diet were closer to those found in starved fish. Similarly as in *S. aurata*, starvation decreases CCK expression in the intestine of most fish species studied (Babichuk and Volkoff, 2013; Feng et al., 2012; Ji et al., 2015; Koven and Schulte, 2012; MacDonald and Volkoff, 2009a; Murashita et al., 2006; Yuan et al., 2014; Yuan et al., 2016). However, upregulation of CCK has been reported in skate (MacDonald and Volkoff, 2009b) and no changes in CCK mRNA abundance were reported in the intestine of *Pygocentrus natteteri* (Volkoff, 2014).

Discrepancies in the expression of orexigenic and anorexigenic peptides among fish species may result from species-specific and tissue-specific differences in the expressional response to food deprivation. At present, limited availability of data and methodological variations among fish studies do not allow the formulation of general relationships between nutritional modulation of appetite-regulating peptides and phylogenetic diversity of fish species, feeding habits or habitat. Considering the impact of diet composition on the gene expression of appetite-regulating peptides in *S. aurata*,

it cannot be ruled out that differences in composition of diets used in previous studies might lead to apparently contradictory results.

Downregulation of leptin mRNA levels in the liver and the adipose tissue of *S. aurata* subjected to long-term starvation is consistent with the notion that leptin might act as a long-term negative feedback signal to suppress appetite and food intake. Leptin circulating levels and mRNA abundance in other fish species have been reported to have been decreased (Nieminen et al., 2003; Tian et al., 2015; Volkoff, 2015; Won et al., 2012; Yuan et al., 2014), increased (Frøiland et al., 2012; Fuentes et al., 2012; Gambardella et al., 2012; Trombley et al., 2012; Zhang et al., 2013), or not affected by starvation (Huising et al., 2006; Shpilman et al., 2014; Tinoco et al., 2012). Consistent with a coordinated anorexigenic action of CCK and leptin in *S. aurata*, leptin mRNA levels (remarkably in the adipose tissue) followed the same pattern of dietary regulation as CCK in the intestine: increased mRNA abundance in fish fed high protein/low carbohydrate diets and decreased values in starved fish. These results argue for a role of CCK and leptin in the regulation of energy balance in the group of fish that exhibited the greatest weight gain. Indeed, *S. aurata* fed the HLL diet had the highest body protein content and the lowest body lipid composition. Besides, leptin mRNA levels in liver showed a positive correlation with body protein content in *S. aurata* fed the experimental diets.

In humans, evidence supports the fact that high protein diets increase leptin activity, either by increasing leptin levels in serum or leptin sensitivity (Kozłowska et al., 2004; Sullivan et al., 2009; Weigle et al., 2005). Moreover, leptin expression and secretion is enhanced by circulating glucose and insulin, but suppressed by free fatty acids (Izadi et al., 2014). Given that amino acids have strong insulinotropic activity in fish (Andoh, 2007), upregulation of leptin mRNA expression in the adipose tissue of *S. aurata* fed

high protein/low carbohydrate diets may result from enhanced insulin secretion. Consistent with this hypothesis, insulin increases leptin secretion in isolated adipocytes from rainbow trout (Salmerón et al., 2015). The metabolic features of carnivorous fish and their low ability to utilise dietary carbohydrates may determine less dependence of leptin mRNA expression on dietary carbohydrates in *S. aurata*.

Our findings show that nutritional status and diet composition affected orexigenic peptides ghrelin and NPY at mRNA level in *S. aurata*. Food deprivation increased ghrelin mRNA levels in the intestine and brain of most fish species studied (Murashita et al., 2009; Nieminen et al., 2003; Unniappan et al., 2004; Volkoff, 2015). Either upregulation or no effect of long-term starvation on ghrelin brain expression has been reported in zebrafish (Amole and Unniappan, 2009; Eom et al., 2013). However, starvation decreased plasma ghrelin levels in trout (Jönsson et al., 2007), and did not affect stomach ghrelin mRNA levels in Atlantic cod (Xu and Volkoff, 2009). Interestingly, the effect of long-term starvation on ghrelin mRNA expression was tissue-specific in *S. aurata*. Higher ghrelin mRNA levels were observed in the GI tract of fish fed high or medium protein/low carbohydrate diets (HLL and MHL), while feeding low protein/high carbohydrate diets (LLH) and starvation resulted in similar ghrelin mRNA abundance. The opposite trend was observed in the brain. Tissue specificity and duration of food deprivation may have major impacts on the regulation of ghrelin expression. Consistently, the time course of ghrelin upregulation in the intestine and brain of starved grass carp and blunt snout bream differed according to the starvation period. Ghrelin mRNA levels reached maximal values in the intestine after 7 days of food deprivation, while the highest ghrelin mRNA expression in the brain was found after 15 days of starvation. Besides, ghrelin mRNA levels in the intestine of 15

day-starved fish fell to about 50-80 % of the values observed after 7 days of starvation (Feng et al., 2013; Ji et al., 2015).

Compared with fed fish, a trend to present higher NPY mRNA levels was observed in the brain of starved *S. aurata*. Significant differences were found when starved *S. aurata* were compared with those fed the high protein/low carbohydrate diet (HLL). As in *S. aurata*, starvation increased NPY mRNA expression in the whole brain and hypothalamus of several fish species (Campos et al., 2010; Ji et al., 2015; MacDonald and Volkoff, 2009a; Narnaware et al., 2000; Silverstein et al., 1999). Furthermore, hyperglycaemia downregulated NPY mRNA levels in the hypothalamus of rainbow trout (Conde-Sieira et al., 2010). However, no effect was observed in cod forebrain (Kehoe and Volkoff, 2007) and tilapia whole brain (Riley et al., 2008). Three weeks of starvation decreased NPY mRNA abundance in the telencephalon of cunner, although no significant changes were found in the hypothalamus (Babichuk and Volkoff, 2013).

The effect of diet composition on ghrelin and NPY expression in fish remains unclear. High protein diets and diets supplemented with starch decreased NPY expression in the rainbow trout hypothalamus (Figueiredo-Silva et al., 2012). However, in the omnivorous goldfish, dietary carbohydrates and fat, but not protein, affected NPY expression in the brain (Narnaware and Peter, 2002). In the present study, downregulation of ghrelin and NPY mRNA levels was observed in the brain of *S. aurata* fed with the highest protein/lowest carbohydrate diet (HLL). Conceivably, increased leptin circulating levels together with a low expression of ghrelin in the brain may determine NPY expression in *S. aurata* fed the HLL diet. Consistent with our findings, intracerebroventricular administration of ghrelin modulated hypothalamic fatty acid sensing in rainbow trout, leading to increased expression of orexigenic peptides AgRP and NPY (Velasco et al., 2016a; Velasco et al., 2016b). Indeed, NPY mRNA

expression showed a positive correlation with body lipid, while dietary protein correlated negatively with ghrelin mRNA levels in the brain of fed *S. aurata*.

In conclusion, we provide evidence that dietary macronutrient composition regulates the gene expression of orexigenic and anorexigenic peptides in *S. aurata*. Furthermore, a discriminant function analysis confirmed that the dietary condition modulates the expression of appetite-regulating peptides (particularly CCK and ghrelin) in *S. aurata*. Changes in appetite-regulating peptides as a response to diet composition will help to improve understanding of food intake control and diet formulation in aquaculture.

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Figure legends

Fig. 1. Effect of starvation and diet composition on body weight in *S. aurata*. Three groups of fish were fed for 23 days a daily ration of 25 g/kg body weight with HLL, MHL or LLH diet. A fourth group of animals was starved for the same period. Values corresponding to body weight at the beginning of the experiment (initial body weight, BW_0) and after the feeding trial (final body weight, BW_1) are presented as means (g). Error bars represent SD (n = 24 fish). Different capital letters indicate significant differences among dietary conditions ($p < 0.05$; one-way ANOVA with SNK post-hoc test). $^{**}p < 0.01$ and $^{***}p < 0.001$ denote significant differences between initial and final body weight for each dietary condition (paired Student's *t*-test).

Fig. 2. Effect of starvation and diet composition on CCK and leptin expression levels in *S. aurata*. Three groups of fish were fed for 23 days a daily ration of 25 g/kg body weight with HLL, MHL or LLH diet. A fourth group of animals was starved for the same period. (A) CCK mRNA levels in the intestine of *S. aurata*. (B) Leptin mRNA abundance in the liver and adipose tissue from *S. aurata*. Expression levels for each gene was normalised using EF1α as a housekeeping gene. Results are presented as means, and error bars represent SD (n = 4 fish for starvation and 5 fish for the other conditions); a.u.: arbitrary units. Different letters (lowercase for CCK; capital letters for leptin) indicate significant differences among conditions ($p < 0.05$; one-way ANOVA with SNK post-hoc test).

Fig. 3. Effect of starvation and diet composition on ghrelin and NPY expression levels in *S. aurata*. Three groups of fish were fed for 23 days a daily ration of 25 g/kg body weight with HLL, MHL or LLH diet. A fourth group of animals was starved for the

same period. (A) Ghrelin mRNA levels in the GI tract and brain of *S. aurata*. (B) NPY expression in brain from *S. aurata*. Expression levels for each gene was normalised using EF1α as a housekeeping gene. Results are presented as means, and error bars represent SD (n = 4 fish for starvation and 5 fish for the other conditions); a.u.: arbitrary units. Different letters (lowercase for GI tract ghrelin and NPY; capital letters for brain ghrelin) indicate significant differences among conditions ($p < 0.05$; one-way ANOVA with SNK post-hoc test).

Table 1

Formulation and chemical composition of experimental diets.

	HLL	MHL	LLH
Formulation (%)			
Fish meal *	81.6	67.5	54.3
starch ‡	15.0	16.7	37.1
Fish oil †	0.8	13.1	6.0
Carrageenan §	1.5	1.5	1.5
Mineral mixture ¶	0.9	0.9	0.9
Vitamin mixture ‖	0.2	0.2	0.2
Chemical analysis (% dry weight, dw)			
Crude protein	58.0	48.0	38.6
Crude lipid	9.9	20.7	12.1
Carbohydrates**	15.0	16.7	37.1
Ash	15.4	12.9	10.5
Gross energy (kJ/g dw) ††	20.1	22.0	20.0

*Corpesca S.A. Super-Prime fish meal (Santiago de Chile, Chile).

‡ Pregelatinised corn starch from Brenntag Química S.A. (St. Andreu de la Barca, Barcelona, Spain).

† Fish oil from A.F.A.M.S.A. (Vigo, Spain).

§ Iota carrageenan (Sigma-Aldrich).

¶ Mineral mixture provided (mg/Kg): $CaHPO_4.2H_2O$, 7340; MgO, 800; KCl, 750; $FeSO_4.7H_2O$, 60; ZnO, 30; MnO_2, 15; $CuSO_4.5H_2O$, 1.7; $CoCl_2.6H_2O$, 1.5; KI, 1.5; Na_2SeO_3, 0.3.

‖Vitamin mixture provided (mg/Kg): choline chloride, 1200; myo-inositol, 400; ascorbic acid, 200; nicotinic acid, 70; all-rac-tocopherol acetate, 60; calcium pantothenate, 30; riboflavin, 15; piridoxin, 10; folic acid, 10; menadione, 10; thiamin-HCl, 8; all-trans retinol, 2; biotin, 0,7 cholecalciferol, 0.05; cyanocobalamin, 0.05.

** Carbohydrates were calculated by difference.

†† Calculated from gross composition (protein 24 kJ/g, lipids 39 kJ/g, carbohydrates 17kJ/g).

Table 2

Primers used in the present study.

Gene	Accession no. (GenBank)	Name of primer	Sequence (5' to 3')
CCK	KP822925	CC1412	GATGAATGCAGGGCTGTGTG
		CC1413r	CTGCGGCGGCCGAAGTCCATCCA
CCK	KP822925	Cck-2	CTGTGTACGAGCTGTTTGGGG
		Cck-R2	AGCCGGAGGGAGAGCTTT
Leptin	KP822924	LP1414	TCTCTTCGCTGTCTGGATTCCTGGAT
		LP1415r	CTCCTTCTTGCTCTGTAGCTCTT
Ghrelin	KP822922	G1410	GGCTCCAGCTTCCTCAGCCC
		G1411r	GCTGCAGGATCTCCTGCAGC
Ghrelin	KP822922	G-2	GGAAAGTCTTCCAGGGTCGG
		G-R2	CGCATAGTCCTCTTCTGTCATGGAG
NPY	KP822926	N1407	AAACCGGAGAACCCCGGGGAGG
		N1408r	CTGGACCTTTTTCCATACCTCTG
EF1α	AF184170	AS-EF1Fw	CCCGCCTCTGTTGCCTTCG
		AS-EF1Rv	CAGCAGTGTGGTTCCGTTAGC

Table 3

Whole body composition of *S. aurata* fed HLL, MHL and LLH diets.

	Diet		
	HLL	MHL	LLH
Moisture (% fw)	72.28 ± 0.47[b]	70.09±0.37[a]	70.62±0.23[a]
Protein (% dw)	60.23±1.11[c]	52.18±0.49[a]	55.42±1.00[b]
Lipid (% dw)	22.40±1.20[a]	30.43±0.62[c]	28.79±1.08[b]
Ash (% dw)	12.66±0.75[b]	11.29±0.16[a]	12.17±0.42[b]

Moisture, protein, lipid and ash content are expressed in % of fresh weight (fw) or dry weight (dw) as mean ± SD (n = 3 tanks). Different superscript letters indicate significant differences among treatments ($p < 0.05$).

Table 4

Linear regression analysis of relationships between body lipid, body protein, dietary protein and the expression of CCK, leptin, ghrelin and NPY in 23-day fed *S. aurata* on diets HLL, MHL and LLH.

Variable 1	Variable 2	Regression equation	r^2	p
CCK	Body lipid	$y = -0.29\,x + 10.00$	0.961	0.124
Leptin (adip.)	Body lipid	$y = -0.07\,x + 2.83$	0.947	0.145
Leptin (liver)	Body lipid	$y = -0.02\,x + 1.38$	0.912	0.184
Leptin (liver)	Body protein	$y = 0.02\,x + 0.02$	0.993	0.045
Ghrelin (GI tract)	Dietary protein	$y = 0.14\,x - 4.23$	0.985	0.079
Ghrelin (brain)	Dietary protein	$y = -0.14\,x + 9.73$	0.987	0.044
NPY	Body lipid	$y = 0.05\,x - 0.20$	0.999	0.022
NPY	Body protein	$y = -0.05\,x + 4.12$	0.966	0.117

Linear regression analysis was performed using mean arbitrary units (n = 5) of expression level values for each diet and gene of interest.

Figure 1

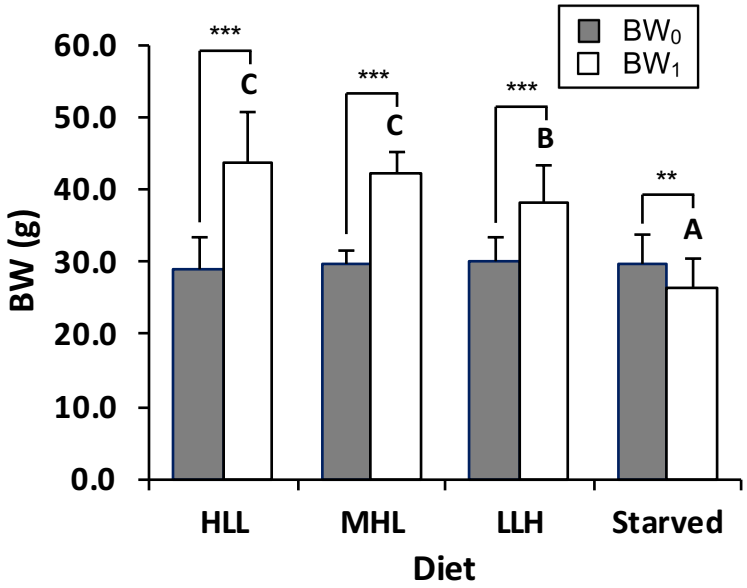
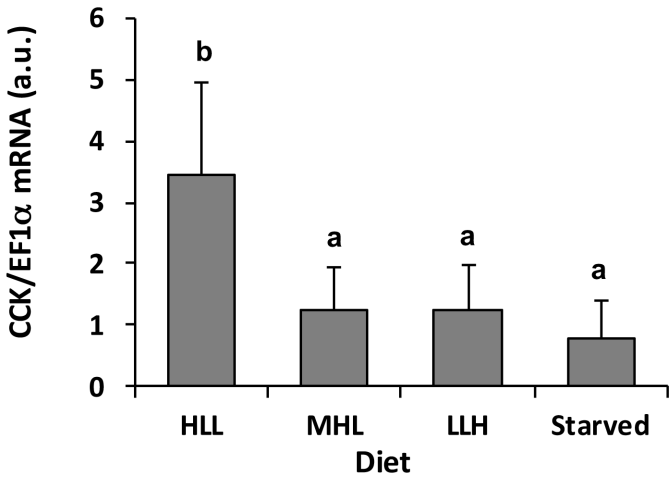

BW0
BW1
60.0
50.0
40.0
30.0
20.0
10.0
0.0
BW (g)
***
C
***
C
***
B
**
A
HLL
MHL
LLH
Starved
Diet

Figure 2

A


CCK/EF1α mRNA (a.u.)
6
5
4
3
2
1
0
b
a
a
a
HLL
MHL
LLH
Starved
Diet

B

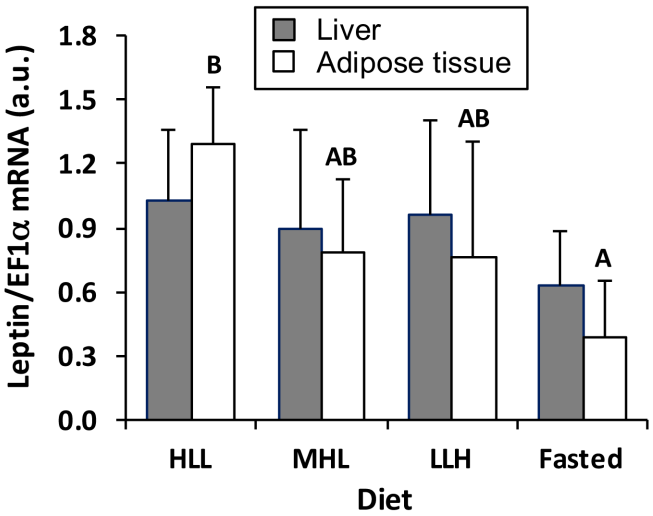
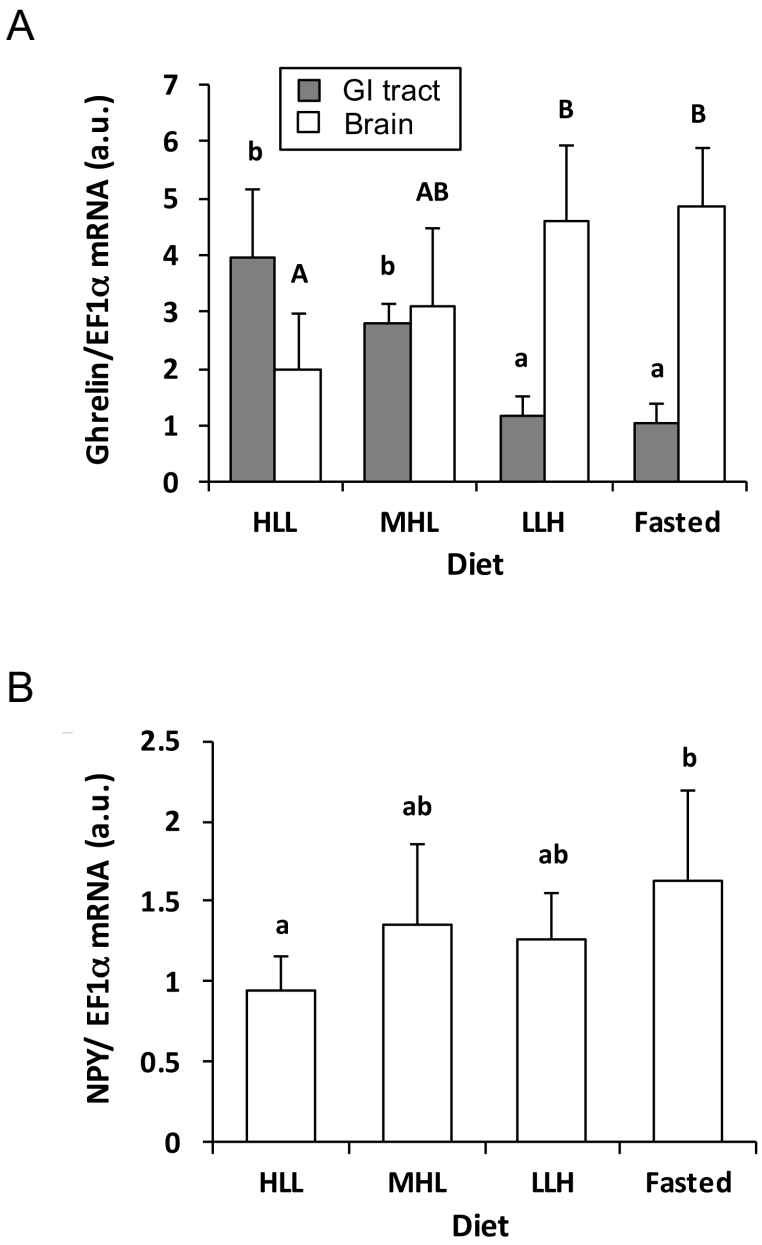

Leptin/EF1α mRNA (a.u.)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Liver
Adipose tissue
B
AB
AB
A
HLL
MHL
LLH
Fasted
Diet

Figure 3


A
GI tract
Brain
Ghrelin/EF1α mRNA (a.u.)
7
6
5
4
3
2
1
0
b
A
b
AB
a
B
a
B
HLL
MHL
LLH
Fasted
Diet
B
NPY/ EF1α mRNA (a.u.)
2.5
2
1.5
1
0.5
0
a
ab
ab
b
HLL
MHL
LLH
Fasted
Diet