Highlights

- High production performance of Atlantic salmon fed 3% fishmeal and appetite stimulating nitrogenous compounds.
- Increased feed intake by free Leu and Phe or nucleotide dietary supplementation lead to less efficient body growth demonstrated by increased FCR and decreased ADC of dietary energy.
- Higher body growth correlates positively with liver lipid levels, which in turn show a positive correlation with plasma total cholesterol, free cholesterol, triglycerides and phospholipids.

Physiological responses of Atlantic salmon (*Salmo salar* L.) fed very low (3%) fishmeal diets supplemented with feeding-modulating crystalline amino acid mixes as identified in krill hydrolysate

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Abstract

Crystalline amino acids and nucleotides, previously identified as potential feed-intake modulators in krill hydrolysate (KH), were mixed into low fish meal diets for Atlantic salmon in five combinations: A1) Arg, A2) Arg+Ala+Pro, A3) Arg+Ala+Pro+Leu+Phe, A4) Arg+Ala+Pro+Leu+Phe + nucleotides (AMP, GMP, CMP, IMP), and A5) Arg+Ala+Pro+Leu+Phe+ nucleotides + rest free amino acids as in KH. Each compound mix

was added to one of five otherwise identical 3% fishmeal diets. A 15% fishmeal (MFM) diet and a 3% fishmeal diet (LFM) served as positive and negative controls, respectively. The experimental diets were fed to seven triplicate populations of 60 salmon smolts for a period of 83 days. The initial mean body weight of the fish was 130 g while the final weights for the different treatments ranged between 500 and 560 g, with feed efficiency ratio (FCR) values of 0.8 or lower. The compound mixes were efficient in modulating feed intake rates, A1 negatively and A3, A4 and A5 positively, and resulted in a complex matrix of differential physiological responses related to growth, apparent nutrient digestibility, plasma and liver lipids and appetite-regulating neuropeptide relative gene expression, which are analysed in this paper.

Keywords: Feed intake; krill hydrolysate free amino acids; Atlantic salmon, appetite regulation.

Running title: Salmon feeding and physiology modulation by free amino acids as in krill hydrolysate

1 Introduction

Feed intake is often suppressed in farmed salmon fed diets high in plant meals (Opstvedt et al., 2003; Mundheim et al., 2004; Aksnes et al., 2006c). Inclusion of feeding stimulants in an otherwise balanced low-fishmeal diet may increase both feed intake and growth. The raw materials used as feeding attractants for aquatic organisms are typically marine extracts or hydrolysates from fish, squid, krill etc. (Mackie and Mitchell, 1985; Berge & Storebakken, 1996; Yilmaz, 2005). Water-soluble proteins (WSPs) from fish protein hydrolysate or stickwater stimulate feed intake, utilization and growth in Atlantic salmon, rainbow trout and Atlantic cod (Aksnes et al., 2006abc; Berge & Storebakken 1996; Espe et al., 2006; Espe et al 2007; Kousoulaki et al., 2009; Kousoulaki et al., 2012; Kousoulaki et al., 2013; Refstie et al.,

2004). WSPs also affect lipid utilization and deposition (Espe et al., 2012; Kousoulaki et al., 2013). Chemical attractants such as free amino acids (FAA), nucleotides (Ishida & Hidaka, 1987; Rumsey et al, 1992; Hara, 1994ab; Kubitza et al., 1997; Lin et al., 2009; Li & Gatlin, 2006) and choline chloride or betaine have also been evaluated as attractants with variable results (Dias et al. 1997; Yilmaz 2005; Tiril et al. 2008). Besides the attractant properties, marine low molecular weight N-compounds possess several other bioactive functions related to stimulation of growth, feed utilization, osmoregulation, antioxidant functions, the immune system and modulation of neuro- and antimicrobial activity and modulation of biological transport systems (Dias et al., 1997; Stapelon et al., 1997; Refstie et al., 1998; Refstie et al., 2000; Burrells et al., 2001ab; Gil & Rueda 2002; Murray et al., 2003; Aksnes, 2005; Yilmaz, 2005; Romarheim et al., 2006; Tiril et al. 2008; Aksnes et al., 2006abc; Li & Gatlin 2006; Liang et al., 2006; Kousoulaki et al., 2009; Bakke et al., 2010; Kousoulaki et al., 2012). Gene expression of neuropeptides associated with appetite regulation indicates that some of the crude soluble concentrates improve appetite and digestive regulation (Kousoulaki et al., 2013). It is however difficult to distinguish which of the specific compounds present in marine (soluble) raw materials are responsible for complex mechanisms such as growth and feeding stimulation.

Of the chemical compounds, amino acids are the most studied gustatory stimuli for fish. Salmonids belong to a group of fish that respond in terms of feed intake to only a few specific amino acids (Hara et al., 1993; 1999). Hara & Marui suggested based on their experiments in 1984 that salmonids detect AA by at least three independent receptor types: (1) proline (proline, hydroxyproline and alanine), (2) betaine (betaine and 2-amino-3-guanidinopropionic acid or Agp) and (3) leucine (leucine and phenylalanine). Hara (1994ab) suggested a classification of fish into two groups based on their gustatory responses, i.e. those responding to a wide spectrum of naturally occurring amino acids and those responding only a few AA, with salmonids

belonging to the latter. Electrophysiological studies by Hara (2005) and Yamashita et al. (2006) suggested that salmonids possess gustatory receptors that are sensitive only to proline-alanine, leucine and a few other amino acids, but that they can also detect mainly cysteine, arginine and glutamate with three distinct olfactory receptors. Furthermore, Hara (2006) verified that in naïve fish including salmonids, single amino acids and closely related chemicals can initiate feeding behaviour by olfaction, at water concentrations of 10^{-6} M. It is believed that the ability of salmon to imprint and develop memory for single amino acids, such as proline, to which they respond at a later stage, is important for their ability to return to their native streams. In sockeye salmon (*Oncorhynchus nerka*) imprinting is efficient if exposure to proline lasts around 14 days before the stage of parr-smolt transformation, when changes in the expression of the salmon olfactory imprinting-related gene also occur (Yamamoto et al., 2010).

We have previously shown that diets with a similar FAA profile, formulated with either 3.5% whole krill (Antarctic) hydrolysate or with crystalline FAA, choline chloride and adenosine monophosphate (AMP) balanced to the same level as in the krill hydrolysate, have similar physiological effects (Kousoulaki et al., 2013). The observed effects included higher feed intake and significantly higher plasma phospholipids and cholesterol compared with diets containing a higher level of fishmeal and marine solubles derived from fish and not krill. Krill hydrolysate contains high levels of FAA with appetite regulatory function in fish and in particular in salmonids, such as alanine, proline, arginine, glutamine, leucine and glycine (Hara et al., 1994; Li et al., 2009). Krill is an abundant and sustainably exploited marine animal organism (Nicol & Endo, 1999), and is widely used as a fishmeal replacement and attractant for fish, including salmonids (Storebakken, 1988).

In the present study, we divided the previously used amino acid mix resembling the FAA in a krill hydrolysate (Kousoulaki et al. 2013) into five groups of potentially appetite-inhibiting or -stimulating amino acids for salmonids, consisting of nucleotides and single, pair or larger

groups of FAA. In a 12-week long feeding trial with Atlantic salmon smolts we evaluated the physiological effects of gradual dietary supplementation of those components. We assessed feed intake rates (FI), gene expression of appetite-controlling neuropeptides, growth, feed efficiency, lipid-energy deposition in body tissues, plasma lipid class and glucose levels.

2 Materials and methods

2.1 Experimental feed ingredients and feeds

Crystalline amino acids arginine (Arg), alanine (Ala), proline (Pro), leucine (Leu), phenylalanine (Phe) and nucleotides (AMP, guanosine monophosphate (GMP), cytidine monophosphate (CMP), inosine monophosphate (IMP)) were purchased from Sigma Aldrich (Oslo, Norway) and used singly or mixed in combination at levels similar to those in krill hydrolysate (KH) (Kousoulaki et al., 2013):

A1) Arg

A2) Arg+Ala+Pro

A3) Arg+Ala+Pro+Leu+Phe

A4) Arg+Ala+Pro+Leu+Phe + nucleotides (nu)

A5) Arg+Ala+Pro+Leu+Phe + nu + rest FAA as in KH

Each mix was added to one of five otherwise identical low fishmeal (3%) diets. A 15% fishmeal (MFM) diet and a 3% fishmeal diet (LFM) served as positive and negative control diets, respectively. The experimental diets' formulation, approximate composition and physical quality, in addition to their total and FAA composition, are presented in Tables 1-3.

2.2 Fish feeding trial

The experimental diets were fed to seven triplicate populations of 60 salmon smolts each, of 130.3 g mean starting body weight (0.1027 standard error of mean), for a period of 83 days.

Before the trial start, the fish were acclimatised for 6 weeks whilst fed a commercial diet (450-480 g kg^{-1} crude protein; 280-300 g kg^{-1} crude fat; 24.2 MJ kg^{-1} gross energy). Thereafter, the fish were starved for 2 days and then counted, weighed in groups and distributed into 21 experimental tanks (1 m^3) in Nofima's indoor tank facilities at Sunndalsøra, Norway. Individuals belonging to the 10% lower and higher body size range were excluded. The initial mean fish body weight was 130 g and the final mean body weights of fish at different treatments ranged between 500 and 560 g. Fish were fed continuously using automatic feeders 7 days a week. Unconsumed feed was collected daily. Based on the amount of feed dispensed and the uneaten feed collected, the amount of feed fed each day was adjusted to about 20% in excess of fish appetite. The fish tanks had constant illumination and were supplied with seawater pumped from a depth of 50 m at a rate of 80 L min^{-1} (flow-through system). The water oxygen levels in the tanks were measured daily and was maintained above 7.5 mg L^{-1}. The mean water temperature during the experiment was 10.0 ± 0.8 $^{\circ}$C, and salinity was stable at 32 g L^{-1}.

At the end of the experiment all fish in each tank were bulk weighed. All but the ten sample fish were stripped to collect faeces, which were separated from urine and kept in a box per tank frozen at –20ºC until further analysis. Ten fed fish per tank, i.e. containing feed in both stomach and intestine, were used for tissue sampling. Blood was collected from five anaesthetised (MS 222) fish per tank and thereafter all the sampled fish were killed by a blow to the head before tissue sampling. From 5 fish per tank, fork length, body weight and liver weight measurements were taken for determination of fish condition factor (CF), dress-out percentage (D%) and hepatosomatic index (HSI). Additionally, small samples of the brains were collected, immediately immersed in liquid nitrogen, transferred to and transported in dry ice and eventually stored at –80ºC until performing the gene expression analyses. The other five fish were frozen at –20ºC, and later measured for gutted body, gastrointestinal tract (GIT) and liver

159 composition analyses. In these fish, undigested feed were removed from their digestive tracts.
160 The analyses were performed on pooled and homogenized samples of five fish from each tank.
161
162 **2.3 Analytical methods**
163 **2.2.1 Chemical analyses**
164 Chemical analyses were carried out by accredited laboratories. Crude protein in the fish tissues
165 was assessed by the combustion method (ISO/DIS 16634, 2004) and in the diets, whole body
166 and feces samples by the Kjeldahl method (N x 6.25) (ISO 5983-1997). Moisture (ISO 6496-
167 1999) and ash (ISO 5984-2002) were determined gravimetrically after drying pre-weighed
168 samples in porcelain cups for 4.5 hours at 103 ± 1 °C followed by incineration of the dried
169 samples at 550°C ± 20 °C for 16 h. Total lipid in the raw materials, the diets and the body tissues
170 was quantified by the Soxhlet method (AOCS Ba 3–38). Dietary gross energy was determined
171 in a Parr adiabatic bomb calorimeter. Yttrium was determined by inductively couple plasma
172 atomic emission spectroscopy (ISO 11885-1996). For total amino acid profile determination,
173 samples were hydrolysed in 6 M HCl for 22 h at 110 °C and analysed by HPLC using a
174 fluorescence technique for detection (Cohen and Michaud, 1993). FAA, taurine and anserine
175 were analysed as described by Bidlingmeyer et al. (1987). Total P were determined
176 spectrophotometrically (430nm) after ashing and acid digestion in 6 M HCl (ISO 6491-1998).
177 All chemical analyses were performed in duplicates. In case of differences between parallels
178 exceeded the standardised values, new duplicate analyses were carried out according to
179 accredited procedures.
180
181 **2.2.1 Pellet physical properties**
182 Pellet water stability was determined by stirring the feed samples in a water bath for 120 min,
183 then sieved, weighed, dried and weighed again (Bæverfjord et al., 2006 modified). Pellet

hardness was measured by a texture analyzer (TA-HDi®, Stable Micro Systems Ltd, Surrey, UK) which consists of a load arm, equipped with a cylindrical flat-ended aluminum probe (70 mm diameter). The pellets were broken individually between the probe and the bottom plate, and the major break of the pellet (the peak force) was measured and presented in Newton (N). Measurements were conducted for 20 individual pellets from each one of the seven experimental diets and the average values are reported.

2.2.1 Plasma lipids, glucose and leptin

Plasma lipids were measured enzymatically on a Hitachi 917 system (Roche Diagnostics GmbH, Mannheim, Germany) using the triacylglycerol (GPO-PAP) and cholesterol kit (CHOD-PAP) from Roche Diagnostics, the free fatty acid (FFA) kit from DiaSys Diagnostic Systems GmbH (Holzheim, Germany), and the phospholipid kit from bioMerieux SA (Marcy l'Etoile, France). The plasma glucose (gluco-quant -glucose/HK) level was also determined enzymatically on the Hitachi 917 system. Salmon plasma leptin hormone levels was measured using Fish (salmon) leptin (LEP) ELISA kit (Cusabio, Hubei Province, China) following the manufacturer's protocol. Absorbance was measured at 450 nm in a Spectrostar Nano microplate reader from BMG LabTech GmbH (Ortenberg, Germany).

2.2.1 Gene expression of appetite controlling neuropeptides

Total RNA from individual salmon brains (n=7 from each treatment) was extracted using TRI reagent (Sigma). Isolated RNA was further purified by DNAse treatment (Turbo DNA-free kit, Ambion) and first-strand cDNA synthesis was performed for RNA (4g) using oligo(dt) primer (Superscript III first-strand synthesis RT-PCR kit, Invitrogen) according to the manufacturer's protocol. mRNA expression levels of appetite regulating genes were quantified using qPCR analysis on the CFX-96 Real-Time PCR detection system platform (Bio-Rad) using a Power

SYBR Green PCR kit, (Applied Biosciences, UK) in a final volume of 25 µl per reaction. qPCR analysis was performed in triplicate reactions for all samples. A total of 12 appetite-regulating genes were analyzed comprising presumed orexigenic (*npy, agrp1, agrp2*) and anorexigenic neuropeptides (*cart*, *cck-l*, *cck-n*, *pyy*, *pomca1*, *pomca2*, *pomca2s*, *pomcb*). The primers and the qPCR conditions were according to previously established methods (Murashita, et al., 2009a; Murashita, et al., 2009b; Murashita, et al., 2011). Absence of primer dimers and non-specific products was verified in every qPCR assay by melting curve analysis (temperature reading every 0.2 °C from 60 °C until 95 °C).

Atlantic salmon elongation factor 1 α (Elf 1α: Genbank accession No. AF321836) was used as a reference gene for normalising mRNA expression. Standard curves (two-fold dilution series of cDNA) were generated for sample genes and eEF1α by plotting the cycle threshold (Ct) obtained in qPCR analysis versus the logarithm of input quantity of RNA, and performing a linear regression. The threshold was consistently set at 0.10 and analyzed in CFX manager software. The data were exported to Microsoft Excel for further analysis. CT values of triplicates were processed using Q-gene.

2.4 Calculations and statistics

Apparent digestibility coefficient (ADC) of dietary nutrients and in the test diets was calculated from the following formula: ADC = 100 – 100 x Yd x Nf / Nd / Yf, were d is diet, f is faeces, Y yttrium content and N nutrient content. Data were tested for normality using a Kolomogorov–Smirnov test and homogeneity of variance using Levene's test, and where necessary, transformed *via* arcsine function. Biological and analytical data were subjected to correlation analyses (ANNEX 1), paired sample T test, one-way analysis of variance (ANOVA) using IBM SPSS statistics 24. When differences among groups were identified, multiple comparisons among means were made using the Duncan's test. Treatment effects were considered at a

significance level of $P<0.05$. Principal component analysis (PCA) was performed using The Unscrambler X 10.4.1. The fish feeding trial setup is designed for performance of ANOVA and t-test analysis studying the difference between single diets and correlations in the dataset, and conclusions are mainly based on these analysis. By the PCA, we looked for patterns in the dataset that cannot be found by correlations between single factors, revealing more of the underlying data structure. However, the extent to which we base our conclusions on the PCA results is limited according to the inherent limitations of the method by certain assumptions made in its derivation such as on the scaling of the variables and the presence of outliers that produce large errors.

3 Results and Discussion

Fish performance (FI, FCR, feed efficiency (FE), thermal growth coefficient (TGC), standard growth rate (SGR) and protein efficiency ratio (PER)), apparent digestibility coefficient (ADC) of dietary nutrients, biometry, body tissue composition, plasma cholesterol, lipids, glucose, leptin and FAA, and gene expression level of appetite-regulating neuropeptides are presented in Tables 4-10.

3.1 Feed physical properties and nutrient ADC

We obtained unintended effects of the supplementation on the physical quality of the feed pellets. Feed pellet hardness and water stability were affected and showed an inverse correlation, the least water-stable feeds being the hardest (Table 1). Diet MFM was that of the diets with the highest fishmeal inclusion level and had high water stability and the lowest pellet hardness of all the experimental diets. The physicochemical properties vary among different fishmeals, and among fishmeal- and protein-rich plant ingredients, which affect the technical quality of extruded feeds with different fishmeals (Samuelsen et al., 2013; 2014), or where raw

materials of plant origin replace fishmeal (Draganovic et al., 2011; Sørensen, 2012; Storebakken et al., 2015). The WSPs in fishmeal, both in terms of inclusion level, as we observed, and degree of hydrolysis, have been shown to act as plasticizers and binders, significantly affecting feed binding and physical pellet quality (Kousoulaki et al., 2013, Samuelsen et al., 2014; Oterhals & Samuelsen, 2015; Samuelsen & Oterhals 2016).

In formulating iso-protein diets with very different levels of fishmeal, the diets with low levels of fishmeal contain higher levels of carbohydrates (starch and soluble and non-soluble non-starch polysaccharides) and antinutritional factors, compared to the high fishmeal feeds, which can affect farmed fish physiology. Generally, salmonids have a poor capacity to digest starch (Krogdahl et al., 2004), due to low intestinal pancreatic α-amylase activity (Frøystad et al., 2006). In this study, however, the binder (horse beans) was kept at the same inclusion level, while the fishmeal was replaced in the 3% fish meal diets by wheat gluten (WG), corn gluten (CG) or soy protein concentrate (SPC), producing less than a 1% increase in the starch level of the diets.

In accordance with previous studies (Aas et al., 2011; Oehme et al., 2014) feed intake was highest in salmon fed the diets with lowest water stability. There was a significant negative correlation between water stability and feed intake rates ($P<0.05$), probably due to a higher release of feed nutrients in the least water-stable diets, among them some of the FAA that stimulate fish feeding activity. However, the fish with the highest feed intake, did not grow accordingly, thereby increasing their feed conversion ratio. Pellet water stability correlated significantly and negatively with FCR ($P<0.05$) but not with fish growth. Soaking the feed in freshwater increased the feed intake in another study, particularly in periods with low feed intake (Oehme et al., 2014). The effect of water stability and moist feed on feed intake may be related to the rate at which the pellet disintegrates and passes through the gut (Aas et al., 2011; Aas et al., 2013; Aas et al., 2014). Although feed intake appears to increase as the gut evacuation

rate increases, the ADC of macronutrients seems to be less efficient as feed intake and gut evacuation rate increases (Aas et al., 2011; Oehme et al., 2014). In our study, water stability correlated negatively with feed intake ($P<0.05$) and had no effect on macronutrient ADC values (Table 6). The ADC of protein was affected by the level of fishmeal or alternative plant material in the diets, indicating that processed plant protein ingredients, such as gluten meals, have similar or higher ADC of protein than the fishmeal, as we have previously observed in Atlantic salmon (Kousoulaki et al. 2009; 2012). On the other hand, apparent digestibility of energy was higher in the MFM diet, mainly due to higher lipid ADC (Table 6) but possibly also due to slightly lower levels of dietary starch, as also observed by Kousoulaki et al. (2012). No correlation between ADC of protein, energy or lipids with salmon growth performance was observed.

3.2 Effects on feed intake

In the present study, we observed reduced feed intake with very low supplementation levels of free Arg (A1), and a gradual increase in daily feed intake with the addition of the chemical mixes A3, A4 and A5, as compared to LFM (A4 and A5) and A1 (A3, A4 and A5) (Table 5). These results are supported by the findings of Hara (2006), who tested dissolved FAA in water in the absence of feed, and described suppressed locomotor activity with free Arg, Bet or Quinine·HCl and increased food searching activity in rainbow trout with free Cys, Ala, Lys and Pro, in some cases stronger than the responses elicited by food extracts. In our study, the free dietary Ala+Pro supplementation did not produce a clear feeding stimulation effect, which can be explained by the fact that Ala and Hyp are competitive agonists for the Pro-receptor and that therefore even a low level of Pro alone can initiate a stronger response than when combined with free Ala (Hara *et al.*, 1993). Leu+Phe, both alone (A3) and in combination with the other AA (A5) were apparently more efficient in stimulating feed intake, reaching similar or higher

feed intake and growth rates than the positive MFM control (Table 4). Besides the supplemented putative orexigenic AA Pro, Ala, Leu and Phe, other potential appetite-stimulating FAA may be present in the rest AA mix (A5), such as free Cys (Hara *et al*., 1993). Hara (2006) also observed that a mixture of six amino acids (Cys, Ser, Arg, Lys, Glu and Pro) increased the swimming activity of fish only slightly and less than e.g. Pro alone, and did not elicit any search behaviour. Free Arg was added in all experimental diets (A1-A5), potentially reducing the feeding stimulation effect of the putative attractant FAA in A2-A5 compared to the LFM diet (Hara 2006).

Based on the supplementation level of the different putative orexigenic FAA (Ala+Pro+Phe+Leu-Arg), the water solubility of the feed pellets, the tank volume, the exchange rates, and the total amount of feed distributed per unit of time, we calculated the theoretical relative levels of released FAA in the tank of the different dietary treatments and correlated these with the feed intake rates of the fish. The calculated total amounts of released FAA in our trial (final water concentrations of 10^{-5} - $2.8*10^{-5}$ M) were lower (Figure 1) than the levels used in the study of Yamashita et al. (2006) (10^{-3} M), who however observed that the threshold for the most potent of the stimulants (Pro) can be as low as 10^{-7} to 10^{-8} M. In our trials, the data revealed a highly significant correlation ($P<0.01$) between the dietary amounts of putative feeding regulating FAA and daily feed intake rates (Figure 2). According to the feed formulations, diet A2 contained higher amounts of FAA than A1; however, this was not true for the respective relative released amounts, due to the difference in pellet solubility in A1 and A2, which corresponded better with the lower feed intake rates of fish in A2 than A1.

Diet A4, with the nucleotides added to A3, induced a higher rate of feeding in the present trials. An orexigenic effect of nucleotides has been reported in other studies (Kiyohara et al., 1975; Mackie & Adron, 1978) and in several fish species, the presence of nucleotide receptors in the facial gustatory system of several species of fish has also been described (Hara, 1992).

3.3 Fish performance, biometrics, blood chemistry and correlations between feed intake and production physiology

Fish performance in all the experimental treatments was good, with TGC values above 3.5 and FCR 0.8 or below. There were no significant differences in the performance parameters between the 10% and 3% fishmeal control diets (Table 4). Moreover, no significant differences were found in fish biometrics and body composition, with the exception of liver lipid levels which were significantly higher in A3 fish compared to A2 and A5, as well as in LFM compared to A2 (Table 7). Fish fed the LFM diet had significantly lower LDL cholesterol compared to the fish of all other treatments, and no other significant differences were observed in the plasma lipid classes of the fish in the different experimental treatments (Table 8). Last, fish plasma free Hyp levels were significantly higher in salmon fed the highest fishmeal diet MFM, compared to all other treatments (Table 9), which can easily be explained as dietary Hyp derives in practice only by animal based, in our case fish, ingredients.

Total feed intake (g/fish) correlated significantly and positively with fish growth (TGC) (Figure 3), while the feeding level (feed intake as a percentage of BW) correlated significantly and positively with FCR (Figure 4) and PER. The deterioration of FCR can be explained by the increase in feed intake rate (FI). Consequently, in our trial, the ability of fish to transform additional feed energy into body growth was suboptimal. This may further be explained by high growth rates in all the fish groups, suggesting that the fish were close to their maximum growth and digestion efficiency potential. Alternatively, it may be due to lack or imbalance of essential components necessary to convert the additional feed energy into muscle growth. A series of fishmeal substitution studies performed by the authors of the present study have demonstrated that feed conversion ratio in Atlantic salmon improves as feed intake increases only among relatively poorly performing fish (Figure 5).

The feeds that were consumed most efficiently contained the highest levels of orexigenic FAA, and were less water-stable and harder, except for feed A1, which was among the hardest pellets in this trial. Feed hardness had a significant positive correlation with the expression levels of both orexigenic (*npy*, *agrp)* and anorexigenic neuropeptides (*cck*, *pomcb* and *pyy*), and a direct explanation for this relationship is elusive.

In the current study, growth rates correlated positively with liver lipids ($P<0.01$), but not with gutted fish weight, intraperitoneal lipid levels or slaughter yield (dress-out percentage, D). However, there was a tendency towards a positive correlation between fish growth with both HSI and condition factor ($P<0.10$). Moreover, the condition factor correlated positively with gutted fish lipids ($P<0.01$), but not with intraperitoneal or liver lipids. Slaughter yield thus did not correlate with any of the above-mentioned characteristics. On the other hand, liver lipids correlated positively with plasma total cholesterol (tendency $P=0.075$), free cholesterol ($P=0.048$), triglycerides ($P=0.013$), phospholipids (tendency $P=0.082$), but not with leptin or any of the neuropeptide expression levels. Plasma free cholesterol, FFA and phospholipids correlated significantly and positively with GIT lipids. Plasma lipids (total and free cholesterol and phospholipids) and glucose also showed significant positive correlation with the expression levels of the presumed anorexigenic neuropeptide *pomca2s*. Plasma LDLC also tended to correlate positively with the relative expression of the antioxigenic neuropeptides *pomca1*, *pomca2* and *pomcb*. Plasma FFA too correlated positively with GIT lipids but not with *pomca2s*, whereas there was a significant negative correlation between FFA and the relative expression of the *pomca1*, *pomca2* and *pomcb*. Plasma leptin correlated significantly ($P<0.01$) and positively with HDLC ($P<0.044$) but not with any other plasma metabolites, final body weight or tissue lipid composition parameters we analysed. However, plasma HDLC correlated negatively with plasma phospholipids ($P=0.041$), condition factor (tendency, $P=0.063$) and HSI

(tendency, P <0.063), which in turn correlated positively also with fish body weight. Single and total plasma FAA correlated significantly and positively with growth ($P < 0.05$).

Following correlation and PCA analysis of all trial data (Figure 6 and 7), we were able to assign the trial sample fish to three groups with distinct combinations of physiological characteristics and performance (Figure 8). In terms of feed intake *vs* growth performance those were (Figure 9): group (a) fish that consumed less feed and grew relatively less (A1 and A2), group (b) fish that consumed more feed and grew more (MFM, A3 and to some extend LFM), and group (c) fish that consumed more feed and grew relatively less (mainly A4, but also to some extend A5). PCA, including all parameters, gives an indication that the LFM group may be closer to group (c), and that A5 could be placed between groups (b) and (c); as both treatments showed relatively high feed intake, medium growth, and medium/high FCR. A1 fish belonging in group (a) (A2 was not analysed), and A4 and A5 of group (c) displayed increased levels of expression of the putative anorexigenic peptides *cart*, *pomca1*, *pomca2* and *pomcb*, whereas Kousoulaki et al. (2013) observed this effect only in the treatments that promoted the highest feed intake. We were unable to demonstrate a consistent correlation between feed intake and mRNA expression of orexigenic or anorexigenic neuropeptides comparable to that observed in mammals.

In fish, as in all vertebrates, central signals arising in the hypothalamus are crucial for the control of food intake, and this brain area produces both orexigenic and anorexigenic factors. This area also receives input about metabolic status and changes in energy homeostasis as well as hunger and satiety signals from the digestive tract (See reviews by Volkoff et al., 2005; Volkoff and Peter, 2006; Volkoff 2011; 2016; Rønnestad et al., 2017). The brain interprets and integrates these signals and responds with efferent signals that affect appetite, feed intake and energy balance. The neuropeptides targeted in this study all play key roles in the control of appetite in vertebrates as well as in Atlantic salmon (Murashita et al., 2009a;b). Murashita et

al. (2011) observed changes in the brain expression of both *npy*, *agrp*, *pomc* and *cart* mRNA after intraperitoneal slow-release administration of leptin (a hormone with a documented anorexigenic effect in mammals) suggesting similar mechanisms for these neuropeptides to those demonstrated in mammals (Korner et al., 2001, Volkoff, 2016). However, the lack of a clear and consistent correlation between feed intake and gene expression for these neuropeptides (Kousoulaki et al., 2013) and in the present study may indicate that the differences in the experimental treatments were too small to provide consistent differences in gene expression, or alternatively, that any differences may have been masked, since some of these neuropeptides also serve other signaling purposes in other brain areas. Also, since the fish were sampled when feed was available and with feed in the digestive tracts this would have caused some satiation, which would have stimulated some of the anorexigenic signaling pathways from the gut. Lastly, there is not necessarily always a proportional relationship between mRNA and protein expressions (Haider et al. 2013), and care should be taken when the physiological functions of these neuropeptides are discussed based on gene expression data. However, our results show significant positive correlations between the expression of different putative anorexigenic neuropeptides (*cart vs cckn*, *pyy* and *cckl*) but, apparently contradictorily, also between the putative orexigenic *npy* and *cart* (Figures 9 A-D).

Like the results of Kousoulaki et al. (2013), the full FAA mix (A5) added in the low fishmeal diet stimulated feed intake, but not to the same extent in growth relative to the fish fed the low FM control diets (LFM). However, the growth rates in our study were higher in all treatments, which may explain the lack of additional effects on growth effect in A5.

In this study, the nucleotide mix led to increased feed intake rates as was also found in a study of largemouth bass (*Micropterus salmoides (*Kubitza et al., 1997). However, it did not lead to increased growth or FCR improvement. Several studies suggest that there is no growth effect in farmed fish fed nucleotide supplements in their diet (Glencross & Rutherford, 2010;

Kousoulaki et al., 2013), while growth promotion by nucleotide products has also been reported in Atlantic salmon (Burrells 2001b), rainbow trout (Adámek et al., 1996; Tahmasebi-Kohyani et al., 2010) and red drum (Li et al., 2005; 2009). Most reported dietary nucleotide effects are related to immune responses and originate mainly from mammalian studies (in Burrells 2001b), whereas there are very few studies in farmed fish (Ramadan et al., 1994; Ringø et al., 2011). High immune responses do not necessarily result in increased growth rates in farmed fish unless a challenge is present. Nevertheless, again in mammals, nutritional nucleotide effects on lipid metabolism, such as increases in certain blood lipoproteins (Sánchez-Pozo et al., 1986), have been reported, which if present in salmon could contribute to better growth performance. In the present study, no effects on effects of dietary nucleotide supplementation on plasma lipids were observed.

4 Conclusions

The control of feeding behaviour in fish is very complex and diverse and is influenced by a range of abiotic and biotic factors (Jones, 1992; Lamb, 2001; Kasumyan & Døving, 2003). We observed that Atlantic salmon fed diets with addition of certain crystalline amino acids increased feed intake, which in turn correlated significantly with growth, compared to other free AA or combinations thereof that did not have this effect. No data capable of explaining the impaired feed utilisation and growth in response to the enhanced feed intake is made available by this study. However, it may be explained by excess feeding stimulation activity in fish (overfeeding), imbalanced diets, hormonal interactions or inefficient digestion. Further detailed studies are needed to validate all these potential explanations. The current study provides strong indications that putative feeding inhibiting and stimulating FAA for salmon may function when added in small amounts to low fishmeal diets.

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699 **Tables and Figures**

700

701 Table 1. Experimental diet formulation, chemical composition and pellet physical quality.

	MFM	LFM	A1	A2	A3	A4	A5
Fishmeal (Biomar) (%)	15.00	3.00	3.00	3.00	3.00	3.00	3.00
SPC (%)	21.77	24.11	24.11	24.11	24.11	24.11	24.11
Corn gluten (%)	21.70	24.00	24.00	24.00	24.00	24.00	24.00
Wheat gluten (%)	2.00	7.18	7.18	7.18	7.18	7.18	7.18
Horse beans (%)	16.00	16.00	16.00	16.00	16.00	16.00	16.00
Fishoil (%)	13.92	14.15	14.15	14.15	14.15	14.15	14.15
Rape seed Oil (%)	4.64	4.72	4.72	4.72	4.72	4.72	4.72
Soya lecithin, Inositol and Choline chloride (%)	0.93	0.93	0.93	0.93	0.93	0.93	0.93
Arg (%)			0.0385				
Arg+pro+ala mix (%)				0.0860			
Arg+pro+ala+leu+phe mix (%)					0.1347		
Arg+pro+ala+leu+phe+nucleotide mix (%)						0.1597	
Full amino acid mix as in krill faa + nucleotide mix (%)							0.3486
Lys (%)	0.93	1.54	1.54	1.54	1.54	1.54	1.54
Met (%)	0.25	0.36	0.36	0.36	0.36	0.36	0.36
Thr (%)	0.07	0.21	0.21	0.21	0.21	0.21	0.21
His (%)		0.14	0.14	0.14	0.14	0.14	0.14
Monocalciumphosphate (%)	2.97	3.66	3.66	3.66	3.66	3.66	3.66
Vitamin - Mineral premix (Biomar) (%)	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Anti-moulding agent (%)	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Lucantin Pink CWD 10% (%)	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Yttrium oxide (%)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Feed analysed chemical and gross energy composition as fed							
Crude Protein (%)	44.7	44.2	44.7	43.6	42.7	43.7	43.7
Crude Fat (%)	23.0	21.4	22.2	21.3	21.8	21.7	22.9
Moisture (%)	6.9	7.0	5.3	6.6	6.6	6.0	6.0
Crude Ash (%)	6.8	6.0	5.8	5.9	6.2	5.8	6.1
Carbohydrates (%) *calculated*	18.6	21.4	22	22.6	22.7	22.8	21.3

Gross Energy (kJ/g)	21.8	21.6	22.5	22.2	22.4	22.4	22.5
Soluble protein (% protein)	12.8	12.3	12.3	12	13.4	12.6	14.5
Total P (%)	1.40	1.30	1.30	1.30	1.45	1.34	1.38
Soluble P (%)	1.0	1.0	1.0	1.0	1.1	1.1	1.1
Pellet physical properties							
Hardness (N)	69	78	84	70	76	87	84
Water stability (% recovered)	92.3	91.9	90.5	92.8	91.1	90.0	88.1

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704 Table 2. Experimental diet total amino acid profile in g/100 g protein.

Amino acid (g/100g protein)	MFM	LFM	A1	A2	A3	A4	A5
Aspartic acid	8.91	8.39	8.33	8.43	8.49	8.38	8.38
Glutamic acid	18.45	20.53	20.39	20.64	20.72	20.85	20.40
Hydroxyproline	0.33	0.00	0.00	0.00	0.00	0.00	0.00
Serine	5.14	5.20	5.15	5.12	5.19	5.10	5.14
Glycine	4.37	3.78	3.82	3.80	3.82	3.79	3.83
Histidine	2.65	2.59	2.61	2.62	2.67	2.61	2.65
Arginine	5.88	5.25	5.34	5.31	5.14	5.19	5.33
Threonine	3.97	3.97	3.96	3.96	3.99	3.96	4.15
Alanine	5.85	5.56	5.98	5.89	5.79	5.58	5.63
Proline	5.83	6.70	6.62	6.63	6.56	6.64	6.61
Tyrosine	3.89	3.68	3.61	3.58	3.56	3.70	3.69
Valine	4.78	4.54	4.53	4.52	4.47	4.49	4.52
Methionine	2.65	2.42	2.42	2.43	2.45	2.49	2.38
Isoleucine	4.52	4.33	4.32	4.28	4.23	4.25	4.28
Leucine	10.61	10.69	10.54	10.52	10.43	10.48	10.40
Phenylalanine	5.38	5.47	5.41	5.33	5.31	5.36	5.33
Lysine	6.79	6.89	6.95	6.94	7.16	7.13	7.25

705

706 Table 3. Experimental diet free amino acid levels (FAA) in g/100g diet. In bold are marked the
707 putatively appetite modulating ones that were differentially supplemented in the diets (i.e.
708 besides the essential Lys, Thr, Met and His, which were also differentially supplemented among
709 the control MFM and the rest low fish meal diets).

FAA (g/100g diet)	MFM	LFM	A1	A2	A3	A4	A5
Arginine	0.127	0.127	**0.150**	**0.164**	**0.125**	**0.132**	**0.133**
Proline	0.025	0.022	0.023	**0.049**	**0.052**	**0.056**	**0.061**
Alanine	0.083	0.064	0.062	**0.057**	**0.059**	**0.069**	**0.07**
Phenylalanine	0.029	0.024	0.025	0.023	**0.038**	**0.038**	**0.041**
Leucine	0.053	0.044	0.043	0.04	**0.067**	**0.069**	**0.072**
Creatinine	0.202	0.079	0.079	0.077	0.065	0.056	**0.06**
Aspartic acid	0.017	0.018	0.014	0.018	0.019	0.022	**0.023**
Glutamic acid	0.047	0.031	0.028	0.027	0.024	0.027	**0.037**
Serine	0.019	0.014	0.012	0.013	0.012	0.013	**0.022**
Asparagine	0.029	0.033	0.03	0.033	0.028	0.032	**0.04**
Glycine	0.017	0.01	0.009	0.01	0.009	0.01	**0.025**
Glutamine	0.003	0.002	0.002	0.002	0.002	0.003	**0.004**
3-amino-propionic acid	0.001	0.001	0.001	0.001	0.001	0.001	**0.019**
Taurine	0.098	0.021	0.021	0.019	0.018	0.016	**0.044**
Histidine	0.091	0.109	0.111	0.115	0.119	0.106	**0.119**
4-amino-butanic acid	0.02	0.024	0.023	0.022	0.021	0.023	**0.023**
Threonine	0.083	0.267	0.26	0.202	0.215	0.218	**0.236**
Carnosine	0.03	0.023	0.073	0.007	0.008	0.012	**0.024**
Tyrosine	0.009	0.003	0.003	0.004	0.003	0.004	**0.015**
Valine	0.023	0.013	0.013	0.015	0.016	0.017	**0.032**
Methionine	0.289	0.443	0.442	0.432	0.442	0.434	**0.459**
Isoleucine	0.016	0.01	0.01	0.01	0.009	0.01	**0.024**
Tryptophan	0.003	0.002	0.002	0.002	0.002	0.002	**0.004**
Ornithine	0.012	0.014	0.01	0.016	0.012	0.013	**0.013**
Lysine	0.7	1.21	*0.991*	1.233	1.219	1.295	**1.277**
Total FAA	2.026	2.609	2.284	2.591	2.585	2.678	2.877

710

711 Table 4. Fish performance. Values are mean (n=3) ± standard deviation.

	MFM	LFM	A1	A2	A3	A4	A5	One-way
Start fish number	60	60	60	60	60	60	60	
Final mortality (%)	0	0	0	0	0	0	0	
start mbw (g)	130±0.5	130±0.5	130±0.8	130±0.3	130±0.3	130±0.5	130±0.8	ns
final mbw (g)	536±21.1	533±23.8	519±3.9	525±3.9	539±13.1	521±17.9	533±5.4	ns
total feed (kg)	18788±830	18620±1347	17734±563	17802±691	18815±893	18723±578	19000±712	ns
total feed/fish (g)	313±13.8	310±22.5	296±9.4	295±14.2	314±15.0	312±9.6	318±10.3	ns
FI[1] (% mbw/day)	1.30^{abc}±0.02	1.30^{abc}±0.06	1.26^{ab}±0.03	1.25^{a}±0.04	1.30^{abc}±0.04	1.32^{bc}±0.02	1.33^{c} ±0.04	0.05
FCR[2]	0.77^{AB}±0.01	0.77^{AB}±0.01	0.76^{AB}±0.02	0.75^{A}±0.03	0.77^{AB}±0.01	0.80^{B}±0.02	0.79^{B}±0.03	0.1
FE[3]	1.30±0.02	1.30±0.02	1.32±0.03	1.34±0.06	1.30±0.02	1.25±0.02	1.26±0.05	ns
SGR[4]	1.70±0.05	1.70±0.06	1.67±0.00	1.68±0.01	1.71±0.03	1.67±0.04	1.70±0.00	ns
TGC[5] (*1000)	3.67±0.13	3.65±0.15	3.57±0.01	3.60±0.02	3.69±0.08	3.58±0.11	3.65±0.02	ns
PER[6]	2.90^{a}±0.04	2.94^{ab}±0.04	2.95^{ab}±0.08	3.07^{b}±0.14	3.05^{b}±0.05	2.86^{a}±0.06	2.89^{a}±0.10	0.05

712 Values on the same line followed by different uppercase letters are significantly different
713 ($P<0.05$) or there is an indication of difference ($P<0.1$) following Duncan test.
714 [1] FI is the mean feed consumption per fish per day as a % of the daily fish body weight (bw).
715 The daily fish bw was calculated using daily SGR values equal to the overall SGR of each tank.
716 [2] FCR was feed consumed/biomass increase
717 [3] FE was biomass increase/feed consumed
718 [4] SGR(%) = $(\ln w_2 - \ln w_1) \times 100$/ feeding days, where w_1 and w_2 are initial and final fish weights,
719 respectively, and *ln* the natural logarithm.
720 [5] TGC = $(w_2^{1/3} - w_1^{1/3}) \times 1000 / \sum$(t x feeding days), where $\sum$(t x feeding days) is the sum of
721 water temperatures (^{o}C) for every feeding day in the experiment Cho (1992)
722 [6] PER was fish weight gain/protein consumption
723 ns: non-significant

724

725 Table 5. Comparisons of daily feed intake rates (% of body weight/day) with paired samples T-
726 test (P values when <0.1 or ns when P>0.1).

Treatment	MFM	LFM	A1	A2	A3	A4
LFM	ns					
A1	0.009	0.008				
A2	0.001	0.002	ns			
A3	ns	ns	0.001	0.000		
A4	ns	0.062	0.000	0.000	ns	
A5	0.056	0.030	0.000	0.000	0.015	ns
Significant effects	MFM>A1	LFM>A1	A1<A3	A2<A3	A3<A5	
	MFM>A2	LFM>A2	A1<A4	A2<A4		
	MFM<A5	LFM<A4	A1<A5	A2<A5		
		LFM<A5				

727
728

729 Table 6. Diet apparent digestibility coefficient. Values are mean (n=3) ± standard deviation.

Diet	MFM	LFM	A1	A2	A3	A4	A5	One-way ANOVA (P<)
$ADC_{Protein}$	88.1^{a}±0.51	90.6^{e}±0.13	90.4^{de}±0.10	89.9^{cd}±0.28	89.25^{b}±0.19	90.0^{cde}±0.41	89.67^{bc}±0.37	0.001
ADC_{Lipid}	90.3^{b}±0.39	89.5^{b}±0.51	89.2^{b}±0.70	89.0^{b}±0.72	86.58^{a}±2.45	89.2^{b}±0.61	89.42^{b}±0.44	0.05
ADC_{Energy}	78.3^{d}±1.09	77.0^{c}±0.42	77.6^{cd}±0.09	75.9^{b}±0.74	74.8^{a}4±0.32	77.0^{c}±0.40	77.83^{cd}±0.42	0.001

730 Values on the same line followed by different uppercase letters are significantly different
731 (P<0.05) following Duncan test.
732

733 Table 7. Fish biometrics and tissue composition. Values are mean (n=3 tanks, mean of 5 fish
734 per tank) ± standard deviation.

Diet	MFM	LFM	A1	A2	A3	A4	A5	One-way ANOVA (P<)
Sample fish weight (g)	574.1±8.3	581.3±35.6	536.0±33.4	575.8±28.8	582.2±25.5	543.9±35.2	548.7±30.8	ns
Biometrics								
condition factor	1.57±0.01	1.55±0.08	1.56±0.03	1.55±0.06	1.59±0.04	1.5±0.02	1.6±0.01	ns
D (%)	87.2±1.64	87.6±0.84	88.5±1.96	88.4±1.01	86.9±0.58	87.7±0.64	87.7±0.39	ns
HSI (%)	1.22±0.04	1.25±0.03	1.25±0.07	1.28±0.02	1.32±0.04	1.24±0.04	1.24±0.04	ns
Gastrointestinal tract (GIT) composition								
dry matter (%)	44.43±0.90	42.17±2.75	44.23±0.58	41.50±2.31	43.33±1.07	41.57±1.91	43.83±0.29	ns
lipid (%)	31.07±0.51	27.40±2.36	29.57±2.19	27.17±2.87	29.43±0.55	27.50±2.91	29.37±1.65	ns
Gutted fish composition								
dry matter (%)	32.23±0.45	32.17±0.47	32.27±0.51	32.37±0.58	32.50±0.17	32.47±0.61	32.70±0.36	ns
lipid (%)	12.93±0.59	12.73±0.38	12.63±0.21	13.03±0.57	13.20±0.35	12.77±0.65	13.17±0.29	ns
Liver composition								
dry matter (%)	25.0±0.3	24.8±0.45	25.0±0.23	25.1±0.49	25.4±0.12	25.0±0.17	25.1±0.46	ns
lipid (%)	4.70^{abc}±0.17	4.87^{bc}±0.32	4.73^{abc}±0.12	4.40^{a}±0.10	5.00^{c}±0.10	4.63^{abc}±0.25	4.57^{ab}±0.21	0.05

735 Values on the same line followed by different uppercase letters are significantly different
736 (P<0.05) following Duncan test.
737 ns: non-significant

738

739 Table 8. Fish plasma cholesterol, lipid, glucose (mml l^{-1}) and leptin (ng/ml) levels. Values are
740 mean (n=3 tanks, mean of 5 fish per tank) ± standard deviation.*

	MFM	LFM	A1	A2	A3	A4	A5	***SEM***	One-way ANOVA (P<)
Total cholesterol	10.93	9.95	10.88	10.48	10.73	10.49	10.14	***0.161***	ns
HDL cholesterol	3.19	4.42	2.52	3.39	2.35	2.60	3.50	***0.310***	ns
LDL cholesterol	1.39^{b}	1.13^{a}	1.44^{b}	1.40^{b}	1.46^{b}	1.34^{b}	1.56^{b}	***0.035***	0.05
Free cholesterol	3.41	3.10	3.41	3.32	3.46	3.28	3.28	***0.053***	ns
Esterified cholesterol	7.52	6.86	7.48	7.16	7.27	7.21	6.86	***0.121***	ns
non-HDL cholesterol	7.74	5.53	8.37	7.09	8.38	7.89	6.64	***0.412***	ns
Triglycerides	5.30	4.13	4.42	4.18	5.48	4.85	3.73	***0.224***	ns
Phospholipids	14.9	13.8	14.8	14.5	14.7	14.3	14.1	***0.174***	ns
Free fatty acids	0.117	0.083	0.090	0.107	0.093	0.087	0.097	***0.005***	ns
Glucose	5.067	5.533	5.033	5.067	5.133	4.867	5.200	***0.068***	ns
Leptin	76.7	72.6	66.0	77.2	71.7	77.2	68.2	***3.201***	ns

741 Values on the same line followed by different uppercase letters are significantly different
742 (P<0.05) following Duncan test.
743 ns: non-significant
744 *same sample fish as in table 7.
745

746 Table 9. Fish plasma free amino acid levels (mg l^{-1}). Values are means and standard error of
747 mean (SEM) (N=21 tanks, 5 fish per tank).*

	MFM	LFM	A1	A2	A3	A4	A5	***SEM***	One-way ANOVA (P<)
Aspartic acid	2.3	2.7	3.0	3.0	2.7	2.7	2.3	***0.14***	ns
Glutamic acid	23.3	22.7	23.7	23.7	26.0	21.7	26.7	***0.87***	ns
Hydroxyproline	26.0^{b}	19.3^{a}	16.0^{a}	17.3^{a}	18.7^{a}	17.3^{a}	20.0^{a}	***0.80***	0.01
Serine	27.3	31.0	24.0	24.7	24.7	23.0	27.0	***1.18***	ns
Glycine	35.0	35.7	25.0	23.7	30.0	25.0	31.0	***1.63***	ns
Glutamine	232.7	297.0	233.0	242.7	243.5	229.0	266.0	***10.60***	ns
3-amino-propionic acid	7.3	16.7	8.3	11.0	19.7	12.3	12.3	***1.39***	ns
Taurine	95.0	81.0	75.7	80.0	89.0	81.3	118.3	***5.14***	ns
Histidine	11.0	14.3	11.7	12.3	14.0	11.7	13.0	***0.62***	ns
Citrulline	2.7	2.0	2.0	2.3	2.7	2.3	2.7	***0.13***	ns
Threonine	35.0	42.7	27.0	34.0	36.3	39.0	42.0	***2.85***	ns
Alanine	58.7^{A}	86.0^{B}	50.3^{A}	52.7^{A}	60.3^{A}	53.7^{A}	72.0^{AB}	***3.67***	0.1
Arginine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	***0.00***	ns
Proline	31.7	30.3	25.0	29.0	26.0	28.3	32.7	***1.23***	ns
Anserine	31.3	49.3	29.0	34.0	36.7	38.3	40.0	***2.91***	ns
Creatinine	7.3	22.3	8.0	11.0	27.3	13.0	12.3	***2.32***	ns
Tyrosine	17.0	12.3	8.0	8.7	9.7	14.3	11.0	***1.30***	ns
Valine	49.0	53.0	47.0	46.7	44.3	45.7	56.3	***1.48***	ns
Methionine	63.3	60.7	49.0	51.7	50.7	49.0	59.7	***3.00***	ns
Isoleucine	56.0	44.7	42.7	44.0	40.7	39.0	44.0	***2.07***	ns
Leucine	33.0	37.0	25.3	26.7	29.7	30.0	31.0	***1.77***	ns
Phenylalanine	104.7	117.3	92.7	100.0	103.0	106.0	103.7	***4.60***	ns
Tryptophane	51.7	60.3	43.3	53.7	52.7	51.7	57.7	***2.20***	ns
Ornithine	5.3	5.7	4.7	4.3	4.7	4.3	6.0	***0.23***	ns
Lysine	1.7	1.3	1.0	1.7	1.3	1.0	1.7	***0.15***	ns
Total plasma free amino acids	41.0	43.3	37.0	48.3	42.7	43.7	46.7	***1.77***	ns

748 Values on the same line followed by different uppercase letters are significantly different
749 (P<0.05) or there is an indication of difference (P<0.1) following Duncan test.

750 ns: non-significant

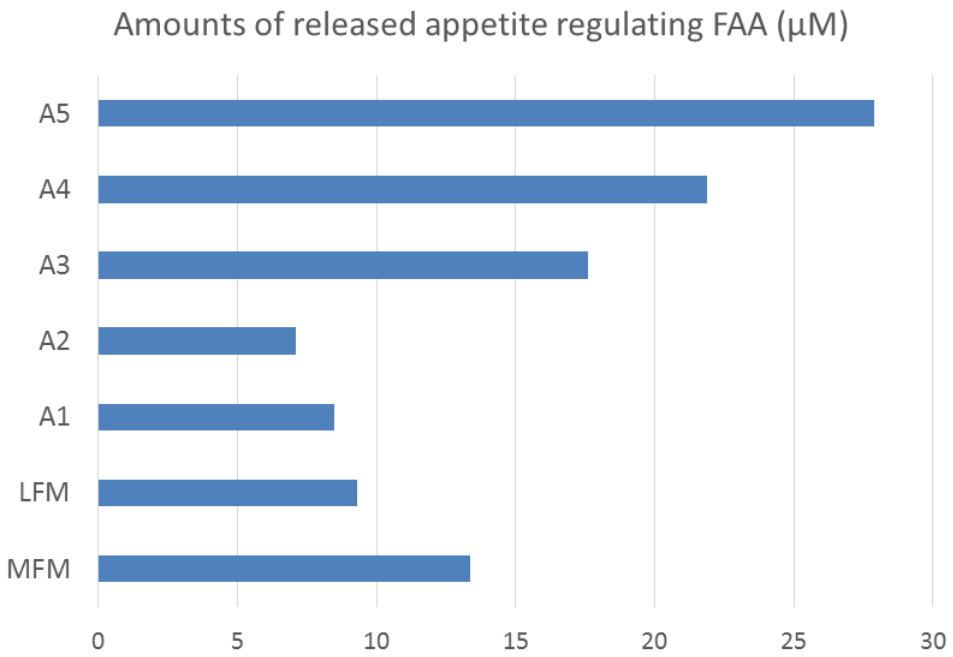
751 *same sample fish as in table 7.

752

753 Table 10. Brain neuropeptide gene expression (%). Values are means and standard error of
754 mean (SEM) (N=15 tanks, 5 fish per tank).*

	MFM	A1	A3	A4	A5	***SEM***	One-way ANOVA (P<)
BW (g)	**574**	**536**	**582**	**544**	**549**		
cart	0.817	0.820	0.823	0.835	0.829	***0.003***	ns
cckl	0.716^{a}	0.716^{a}	0.720^{a}	0.737^{b}	0.737^{b}	***0.003***	0.05
cckn	0.744^{A}	0.756^{AB}	0.757^{AB}	0.772^{B}	0.767^{B}	***0.004***	0.1
pomca1	0.582	0.666	0.612	0.649	0.658	***0.024***	ns
pomcb	0.895	0.991	0.928	0.994	1.022	***0.020***	ns
pomca2	0.669	0.711	0.666	0.694	0.707	***0.019***	ns
pomca2s	0.407	0.359	0.323	0.344	0.334	***0.016***	ns
agrp1	0.456^{a}	0.480^{ab}	0.449^{a}	0.510^{b}	0.488^{ab}	***0.008***	0.05
agrp2	0.562^{a}	0.600^{b}	0.607^{b}	0.616^{b}	0.596^{b}	***0.006***	0.01
npy	0.738^{ab}	0.738^{ab}	0.722^{a}	0.765^{bc}	0.777^{c}	***0.006***	0.01
Pyy	0.720^{A}	0.731^{AB}	0.726^{AB}	0.746^{AB}	0.751^{B}	***0.004***	0.1

755 Values on the same line followed by different uppercase letters are significantly different
756 (P<0.05) or there is an indication of difference (P<0.1) following Duncan test.
757 ns: non-significant
758 *same sample fish as in table 7.
759



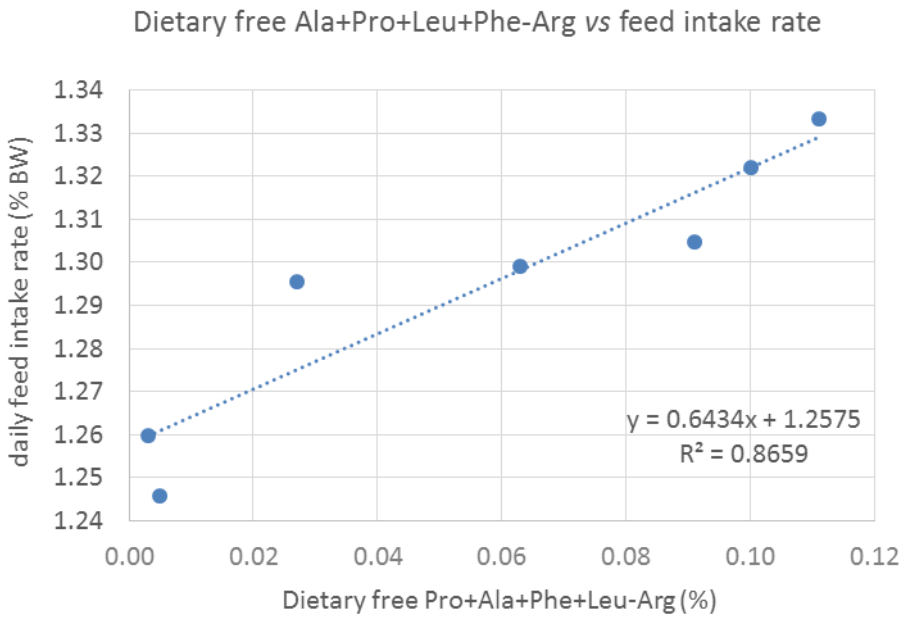
760

761 Figure 1. Calculated amounts of hypothetically released free Pro+Ala+Leu+Phe -Arg, in the

762 water volume, based on the analysed concentration values of the free form of these amino acids

763 in the diets, the water stability of the pellets and the common mean of total amounts feed fed.

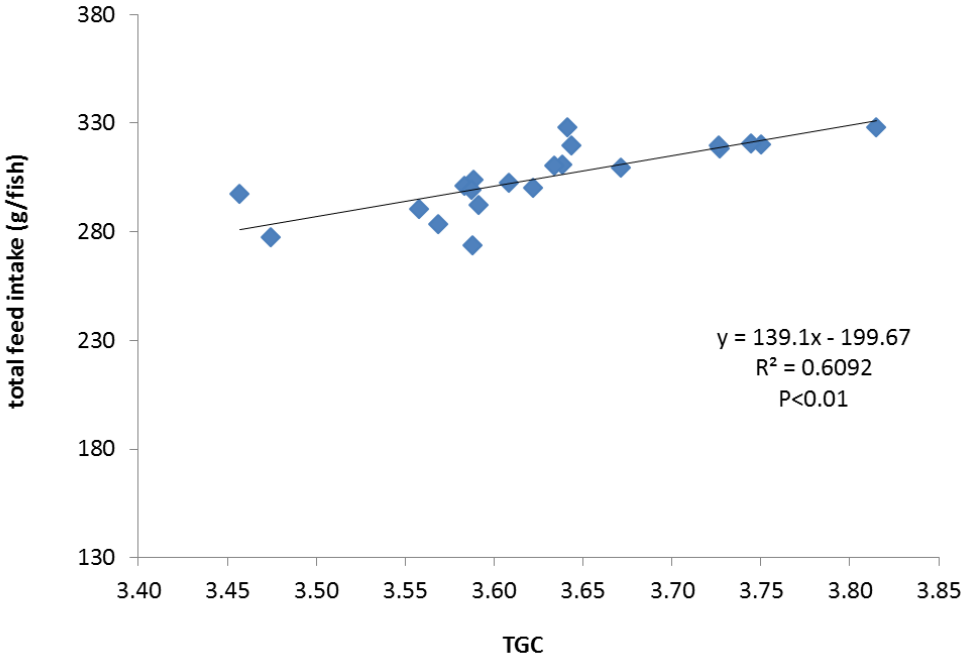
764



765

766 Figure 2. Total dietary free Pro+Ala+Leu+Phe –Arg *vs* mean daily feed intake rates as % of

767 fish body weight.

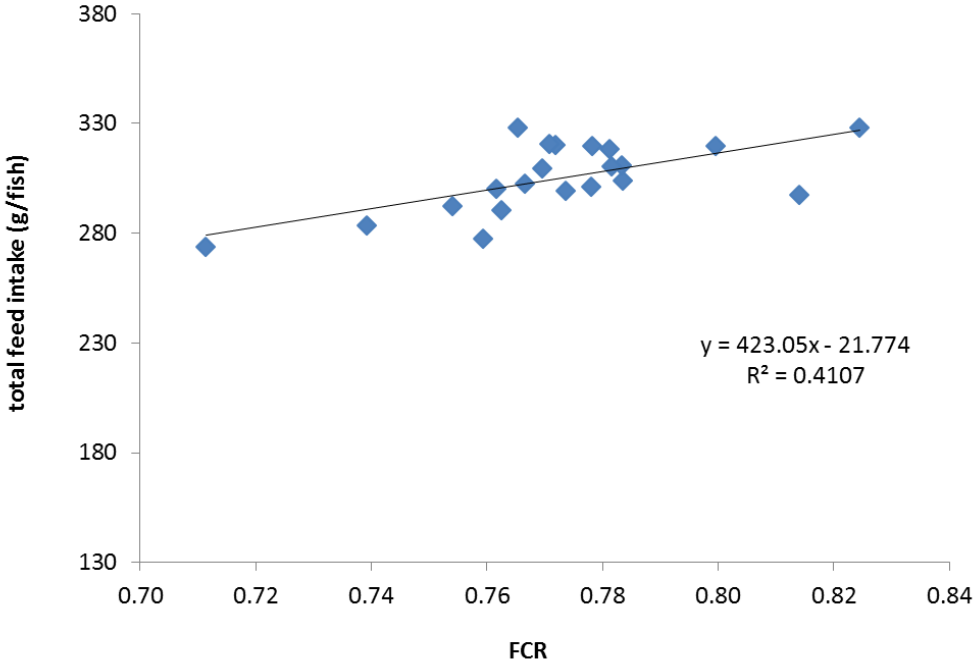


768

769 Figure 3. Correlation between individual tank mean fish TGC and total fish feed intake per tank

770 per fish (n=21 tanks).

771



772

773 Figure 4. Correlation between tank FCR and total feed intake per fish per tank (n=21 tanks).

774

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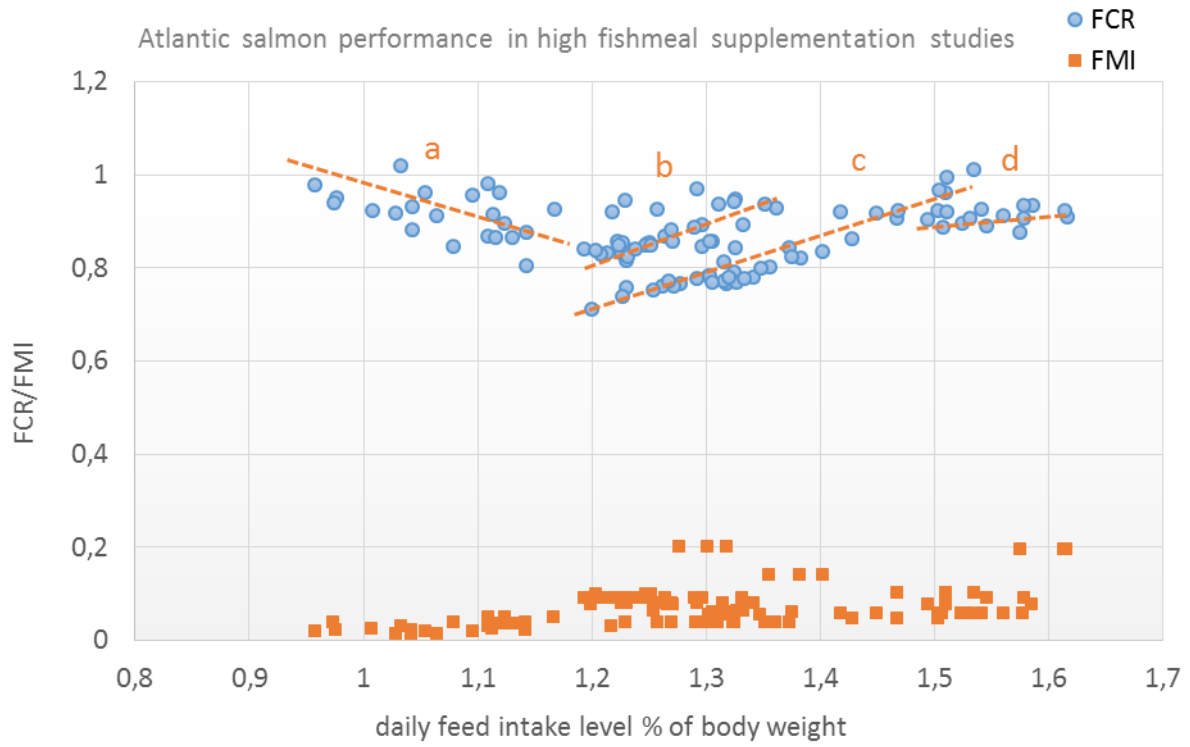
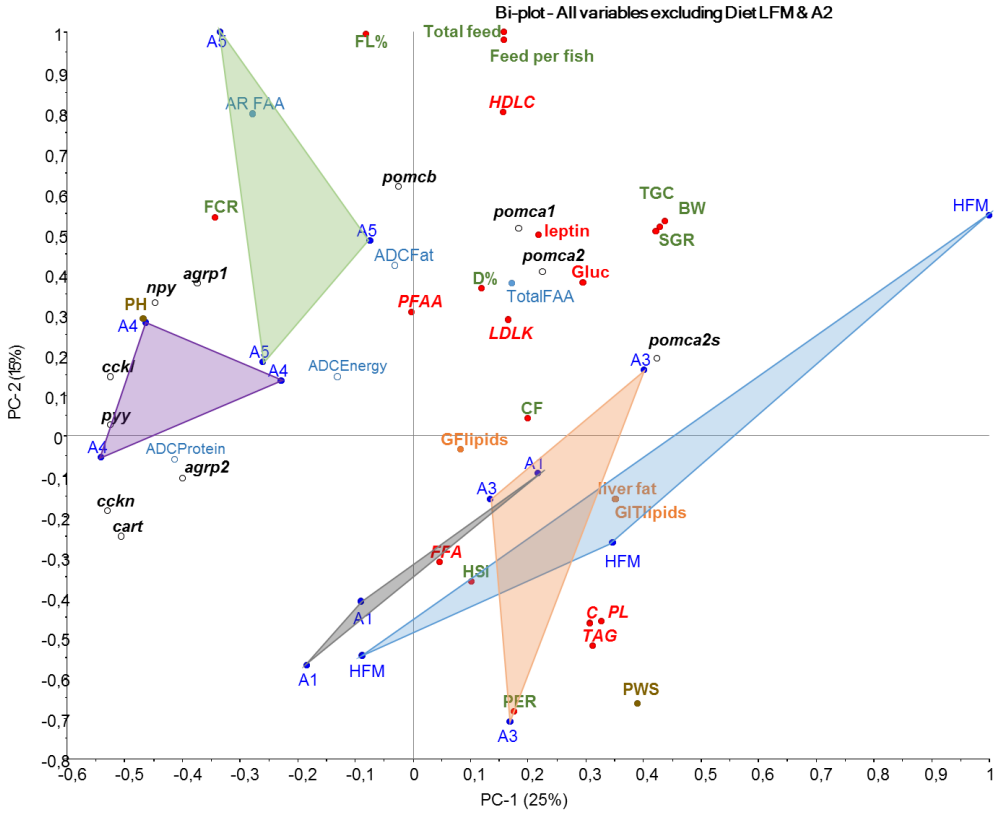
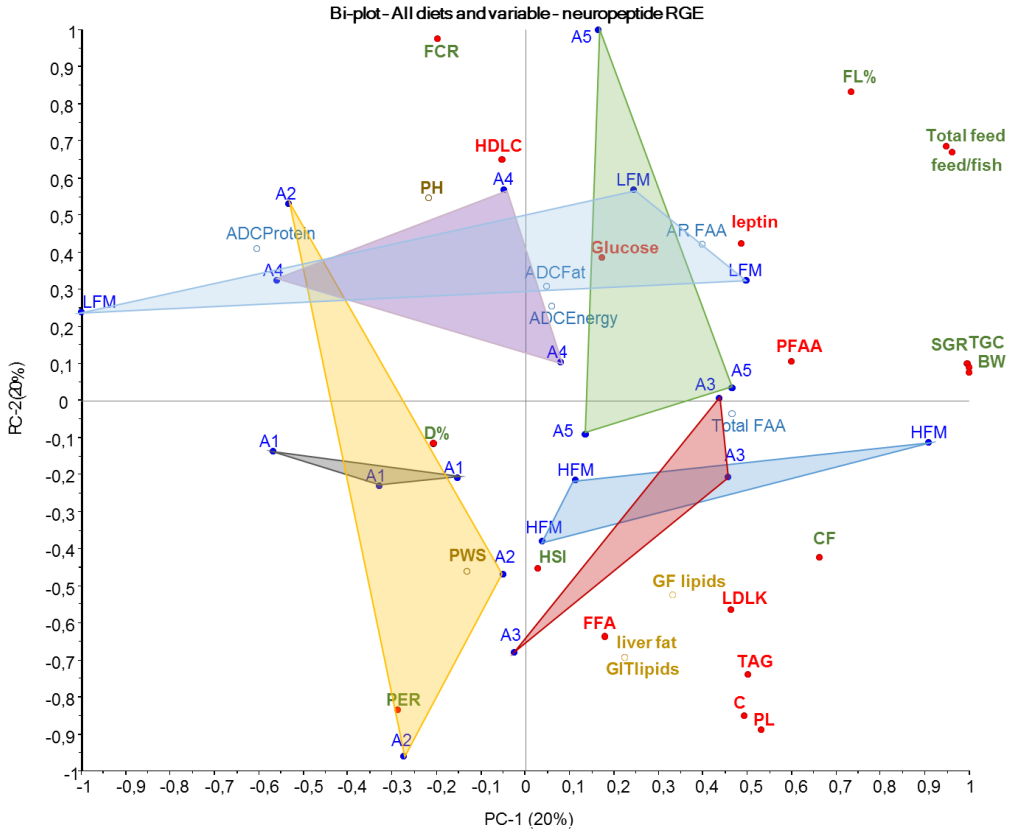


Figure 5. Relation between daily feed intake level expressed as % of daily body weight and feed conversion ratio (FCR) in Atlantic salmon fed low fish meal diets in four independent studies (a-d) including the present one (b) (a: Kousoulaki et al., 2013; c: Kousoulaki et al., 2009; d: Kousoulaki et al., 2012). Dietary fishmeal level indicator (FMI) of 0.2 corresponds to 30% fishmeal in the diet.



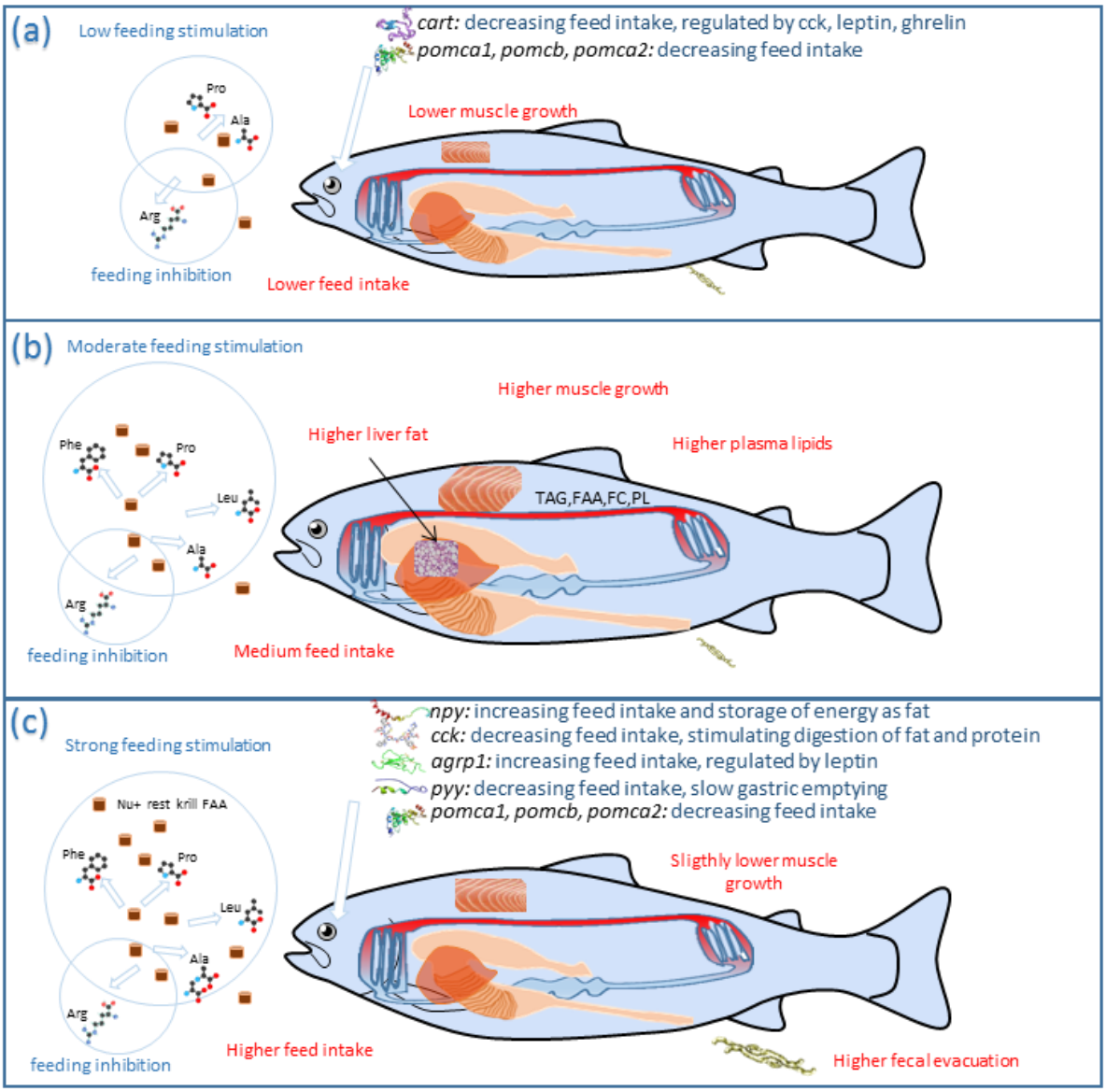
782

783 Figure 6. PCA bi-plot of all trial variables in treatments MFM, A1, A3, A4 and A5.



784

785 Figure 7. PCA bi-plot of all variables except neuropeptide relative gene expression.



786

787 Figure 8. Schematic representation of hypothesised physiological effects in farmed Atlantic

788 salmon by supplementation of feeding modulators in very low fishmeal diets.

789

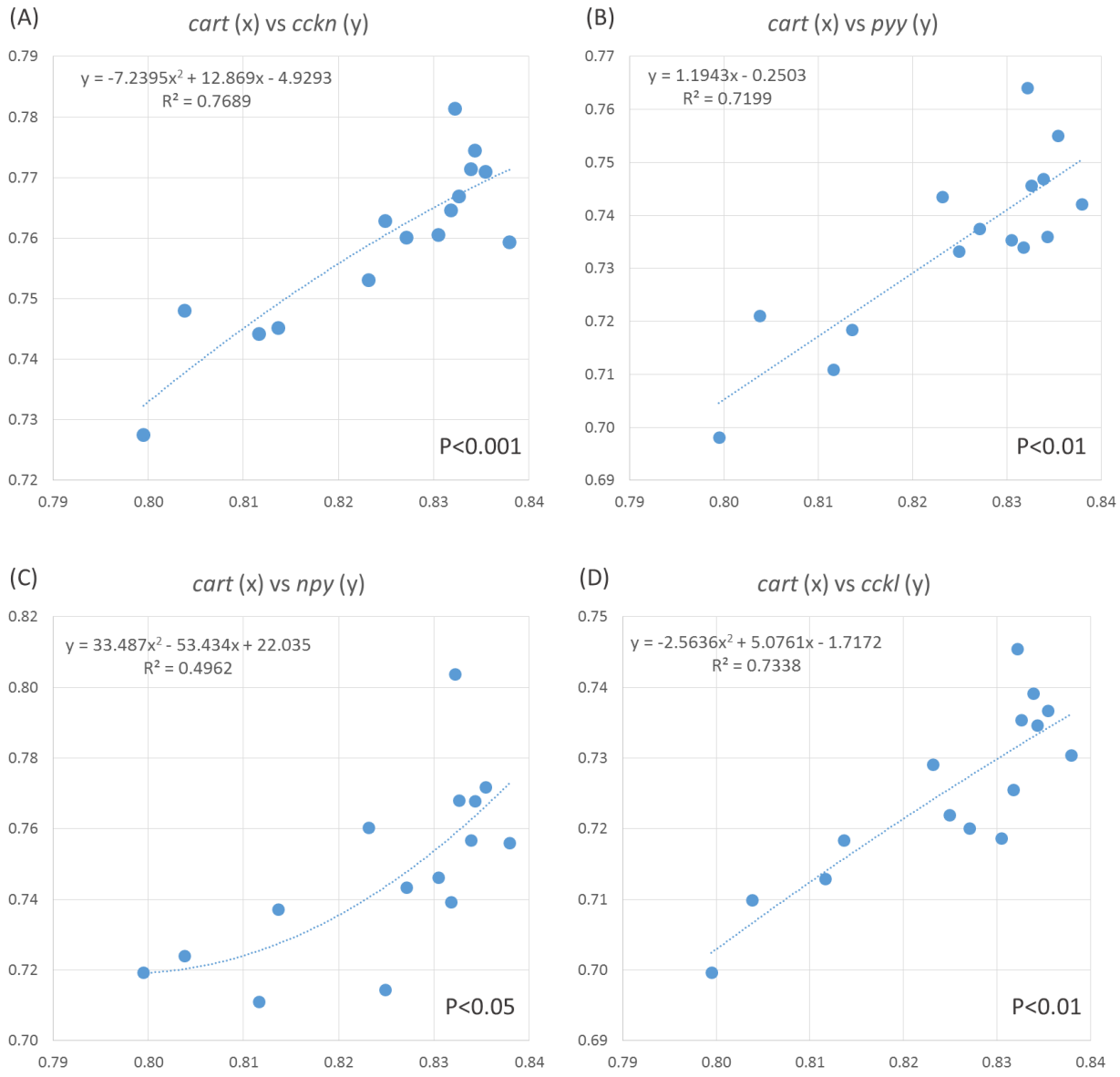


Figure 9. Correlations among relative expression levels of different appetite regulating neuropeptides in Atlantic salmon fed diets supplemented with different levels of feeding modulators in very low fishmeal diets.