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Effects of different live prey enhancement on expression of selected immune and appetite related genes during early development of cod (*Gadus morhua* L) larvae

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Yfirlýsingar

Ég lýsi því yfir að ég einn sá um uppsetningu aðferða til mælinga á 5 af þeim 6 genum sem mæld var tjáning á, framkvæmdi allar mælingar á genatjáningu, úrvinnslu og framsetningu þeirra niðurstaða og er sá hluti verkefnisins alfarið afrakstur eigin rannsókna. Ég sá ennfremur um innsöfnun efniviðar í rannsóknina og tók virkan þátt í framsetningu niðurstaða úr öðrum hlutum verkefnisins í samstarfi við aðra höfunda greinarinnar en framkvæmd tilraunarinnar og aðrar greiningar á efnivið til rannsóknanna voru í höndum meðhöfunda og samstarfsaðila þeirra. Ég sá einnig um ritun og frágang handrits í samstarfi við meðhöfunda og er því listaður sem fyrsti höfundur greinarinnar.

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Leifur Guðni Grétarsson

Það staðfestist að verkefni þetta fullnægir að mínum dómi kröfum til prófs í námskeiðunum LOK1126 og LOK1226

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Dr. Rannveig Björnsdóttir

Formáli

Í samráði við leiðbeinanda og umsjónarmann lokaverkefna við Auðlindadeild Háskólans á Akureyri er lokaverkefni nemanda til B.S. gráðu í líftækni í formi vísindagreinar sem send hefur verið viðurkenndu fræðiriti til birtingar. Efnið er því skrifað á enskri tungu og í þeirri uppsetningu sem fræðiritið gerir kröfu um. Þessu til vitnis fylgir staðfesting á innsendingu greinarinnar.

Abstract

High mortalities in early stages of Atlantic cod (*Gadus morhua* L) larvae produced in intensive aquaculture are commonly experienced and have been linked to mainly feeding and nutrition. The immune system of cod larvae is poorly developed at hatch, and innate immune parameters therefore of importance for defence against environmental microorganism. Also higher quality larvae result in improved development during later stages. In the present study, the live prey of cod larvae was additionally enhanced using a fish protein hydrolysate or marine microalgae and offered to larvae in different treatment schedules. The results indicate that the enhancement affected the expression levels of appetite and immune related genes. The observed reduction in total lipid content and DHA:EPA ratio in live prey enhanced with marine microalgae is of concern, however, there are strong indications of improved larval survival in this group and improved growth of larvae in all groups offered additionally enhanced live prey. Also, the increased gene expression observed may result in improved disease resistance, growth and overall performance during later stages. The enhancement resulted in different mineral content of the diets, but their role in growth, development and gene expression is not fully known.

Keywords: Cod, Fish Larvae, Live Feed Enrichment, Early Development, Larvae Nutrition, Genetics

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Útdráttur

Léleg afkom lirfa Atlantshafs þorsks (*Gadus morhua* L) er þekkt vandamál í fiskeldi og hefur aðallega verið tengd fóðrun og fæðu. Ónæmiskerfi þorsklirfa er óþroskað við klak og því er ósérhæfð ónæmissvörun mikilvæg vörn gegn örverum úr umhverfinu. Einnig hafa aukin gæði þorsklirfa skilað sér í betri vexti á seinni þroskastigum. Í þessari rannsókn voru fæðudýr þorsklirfa viðbótarauðguð með ýmist ufsa prótein hýdrólýsati eða blöndu tveggja tegunda kaldsjávarþörunga og lirfurnar fóðraðar með bættu fæðudýrunum misoft og í mislangan tíma. Niðurstöðurnar sýna að hægt er að hafa áhrif á tjáningu lystar- og ónæmistengdra gena með viðbótarauðguninni. Lækkun á heildarfituinnihaldi og hlutfalli DHA:EPA í fæðudýrum auðguðum með kaldsjávarþörungum er áhyggjuefni, en engu að síður leiddi þessi meðhöndlun til betri lifunar. Sterkar vísbendingar voru um aukinn vöxt lirfa með viðbótarauðgun fæðudýra. Í ljós komu breytingar á innihalda valinna steinefna í fæðudýrunum í kjölfar auðgunarinnar, en hlutverk þeirra með tilliti til vaxtar, þroska og genatjáningar er ekki þekkt.

Lykilorð: Þorsklirfur, auðgun fæðudýra, fystu þroskastig, næring, genatjáning.

Efnisyfirlit

Myndaskrá

Töfluskrá

1 Introduction

Atlantic cod (*Gadus morhua*) is one of the most economically important species for fisheries in the North Atlantic and decrease of catches over the last decades has led to increased interest in intensive farming of Atlantic cod (Brown, Minkoff & Puvanendran 2003). However, high mortalities are commonly experienced in early stages of cod aquaculture, especially around the beginning of exogenous feeding (Lanes et al. 2012, Rosenlund, Halldórsson 2007). Significant losses are caused by diseases and growth rates are generally low, leading to high production costs and stagnation of further development of the industry in recent years (Paisley et al. 2010). High mortalities are also observed in nature, but in cod farming the losses are commonly attributed to problems in feeding, infections or pathogens in association with density and handling causing increased stress on the larvae (Bergh, Nilsen & Samuelsen 2001, Magnadóttir et al. 2004a).

Feeding fish larvae is a challenging step as microencapsulated or pelleted diets are unsuitable due to their small size and immature digestive system (Navarro et al. 1993). In the wild, zooplankton is the natural food source for fish larvae and larvae fed zooplankton until metamorphosis have shown clear advantages in growth compared to larvae fed rotifers(Shields et al. 1999, Mæhre, Hamre & Elvevoll 2013). However, complications such as limited availability on a year-round basis and the risk of transferring pathogens to larvae may restrict the usage of zooplankton in cod farming (Imsland et al. 2006). Nutritional requirements of cod larvae have not been defined but rotifers and *Artemia*, commonly used in the production have been found lacking or low in several essential nutrients. Hence, different enrichment procedures are used in order to improve the biochemical composition and to make the live prey more suitable for feeding (Mæhre, Hamre & Elvevoll 2013). Previous experiments with the use of fish protein hydrolysates (FPH) for additional enhancement of the live prey have indicated positive effects on morphological quality and growth of marine larvae (Johannsdottir et al. 2013). However, the mechanistic basis of this improved growth and performance is not known. Also, marine microalgae are a rich nutrient source, their protein quality is high and many species are rich sources of

antioxidant compounds, vitamins, colorants and other valuable biomolecules (Spolaore et al. 2006). Marine microalgae furthermore contain marine lipids that play an important role in providing essential polyunsaturated fatty acids for normal cell membrane function and energy for normal development (Shields et al. 1999). Marine microalgae are in general rich in essential polyunsaturated fatty acids and an abundant source of ascorbic acid and riboflavin, two key vitamins in larval development (Brown et al. 1997).

The development of the immune system is not complete at the embryonic and early larval stages. The immune competence is therefore assumed to be limited in Atlantic cod larvae (Seppola et al. 2009), making them more susceptible to infections and diseases. Previous studies have shown that innate immune responses of marine fish larvae can be stimulated (Johannsdottir et al. 2013, Bjornsdottir et al. 2009, Ruangsri et al. 2013). Furthermore, larvae with early onset of growth tend to maintain the advantage until metamorphosis and also seem to have better survival rate and viability (Izquierdo et al. 2000). Previous studies also indicate that growth advantage during early larval development persists into the growth phase (Imsland et al. 2011). Hence, it is important to stimulate natural immune defence mechanisms, growth and normal development during early production stages.

The main objective of the present experiment was to study the effects of additional enhancement of the live prey of intensively produced cod larvae using a pollock protein hydrolysate or marine microalgae. The effects were evaluated by measuring the expression of selected immune and growth related genes, as well as early larval growth and survival and the nutritional and mineral composition of larvae and their live prey.

2 Materials and methods

2.1 Live feed treatment and larval rearing

The rearing trial was performed at the Icelandic Marine Research Institute hatchery in Grindavík, Iceland in 2012, using standard hatchery practices. The standard hatchery protocol includes offering enriched rotifers (*Brachionus plicatilis*) and *Artemia* (*Artemia franciscana*) to cod larvae from hatching onwards. The rotifers were enriched with an equal mix of Algamac 3050 and Algamac Enhance (BIO-MARINE Aquafauna, California, USA) for 8-12 hours. The *Artemia* metanauplii were enriched with A1 DHA Selco (INVE Aquaculture NV, Dendermonde, Belgium) for 20 hours. Additional bioencapsulation using freeze-dried Pollock protein hydrolysate (hydrolysate product No. R-12-005, produced by Iceprotein ehf., Iceland, using procedures previously described by the group (Johannsdottir et al. 2013) was carried out for 30 minutes (100 ppm concentration) in fresh seawater following completed nutrient enrichment of the live prey. The bioencapsulation was carried out in prey densities of 1 and 0.5 million prey L^{-1}, for rotifers and *Artemia* cultures, respectively. The daily ration of live prey cultures was enhanced using the protein hydrolysate in the morning and then kept at 4°C for later feedings throughout the day. Algal enhancement was carried out in similar prey densities for 1 and 8 hours for the morning- and afternoon rations, respectively. Dense bag cultures of *Chlorella* sp. (5.000 cells mL^{-1}) and *Nannochloris* sp. (12.000 cells mL^{-1}), isolated from the sea off the North coast of Iceland, were harvested, with 1 L of each culture mixed each morning and added to the prey cultures. The enhanced prey were then collected on mesh nets and rinsed with fresh seawater prior to offering to the larvae.

Cod eggs were disinfected at 12 and 1 days prior to hatch, using 0.1 g L^{-1} of Pyceze (Novartis Aqua, Surrey, UK). Upon completed hatch the larvae were transferred to the rearing units and reared in darkness until feeding started on 2 dph and the initial light intensity was set at 200 lx at the water surface. Newly hatched yolk-sac larvae were stocked into black, polyethylene silos (150 L) in a random order, at a stocking density of 10,000 larvae $silo^{-1}$ (67 larvae L^{-1}). The experiment was carried out in triplicates, with four treatment groups fed hydrolysate- or algae-enhanced prey in

both daily feedings every day or every second day for one to six weeks from 2 dph, and standard enriched prey in other feedings, while control groups were fed standard enriched prey only. Enriched or enhanced prey were offered to the larvae in two daily rations at 9 AM and 16 PM. Three groups were fed protein hydrolysate enhanced prey (groups P1, P2 and P3), one group was fed algal enhanced prey (group A) and the control group received standard enriched prey only (group C). Groups P1 (6 week treatment) and P2 (3 week treatment) received hydrolysate enhanced prey on Mondays, Wednesdays and Fridays but regular prey on other weekdays. Group P3 (1 week treatment) received hydrolysate enhanced prey on consecutive weekdays for a whole week during 2-8 dph. Group A (6 week treatment) received algal enhanced prey on all weekdays. The larvae were fed rotifers from 2-28 dph, *Artemia* nauplii from 15-25 dph and *Artemia* metanauplii from 22 dph. Algae paste (Nannochloropsis, Reed Mariculture, USA) was used for shading the rearing water during 2-28 dph. The rearing water had a stable 32-33 g $^{L-1}$ salinity and the temperature was kept constant at 8°C during the silo stage. At 35 dph the remaining survivors were harvested and counted by sub-sampling (10 aliquots) and subsequently restocked into separate 3,000 L larval tanks (10,000-15,000 early juveniles tank^{-1}). Following transfer to the tanks the temperature was gradually raised to 10-11°C during 4-5 days. No size-grading was performed.

2.2 Larval sampling and measurements

All samplings were performed immediately prior to the morning feeding. For measurements of larval growth, pooled samples of ~40 larvae from each tank were collected at 10, 26 and 35 dph. The standard length of ~10 larvae from each tank was determined and the mean weight of ~40 larvae tank^{-1} measured on pre-weighted nets following drying at 70°C for 48 h (dry weight). For quantitative real-time PCR (RT qPCR), pooled samples of 10 larvae from each tank were collected at 10, 26 and 44 dph, using fine mesh dip net. The larvae were euthanized using tricainemethanesulphonate (MS-222), subsequently rinsed in UV treated sea water and 10 larvae transferred into 1.5 mL RNAse free Eppendorf tubes (Sarstedt AG & Co.), one larva tube^{-1}. Excess water was removed using RNAse-free pipette tips

(Ambion®), the samples snap frozen in liquid nitrogen and stored at -80°C until further processing. All equipment and working surface was cleaned using RNase*Zap*® (Ambion®).

Samples of larvae and their live prey were subjected to analysis of nutritional and mineral content. For the nutritional analysis, the protein and lipid composition was measured in freeze-dried samples using near-infrared resonance spectroscopy technique (NIR), with reflectance measurements performed over the wavelength range of 800-2500nm using a Bruker Multi Purpose Analyzer (MPA) system with a fiber probe (Bruker Optics, Rheinstetten, Germany) and fish meal used as reference. The relative content of selected minerals was measured in freeze-dried samples using inductively coupled plasma mass spectrometry (ICP-MS). Lipid and fatty acid analysis was carried out by Dr. Chris Parrish and his group at Memorial University of Newfoundland, Canada.

2.3 Quantitative real-time PCR

Total RNA was purified from whole larvae. Samples were then homogenized in TRIzol® reagent (Invitrogen, California, USA) according to the manufacturer‘s protocol and three larvae per tank pooled prior to phase separation. The quality and quantity of RNA was evaluated through electrophoresis on 0,8% agarose gels and spectrophotometry using Qubit® Fluorometer (Invitrogen, California, USA) and Quant-iT™ RNA Assay Kit (Invitrogen, California, USA) as described by the manufacturer. Complimentary DNA (cDNA) was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA) according to the manufacturer’s protocol. Real-time PCR (qPCR) was performed using a StepOne Real-Time PCR System (Applied Biosystems, California, USA) with SYBR Green chemistry (Power SYBR Green PCR Master Mix, Applied Biosystems, California, USA). No-Template and minus reverse transcriptase controls were included for each primer pair. The thermal profile for qPCR was 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 56-60°C for 60 sec (depending on the optimal annealing temperature for the primers used; see Table 1)

Table 1. Primer pair sequences, annealing temperature conditions and GenBank accession numbers for genes used for RT-qPCR.

Primer	Sequence (5'-3')	T (°C)	Accession number
mIgM_F [a]	AAGGAATGAAGTGGTTCTGTGAGG	60	AJ87128
mIgM_R [a]	TTCAGTCAGGACAAGAAACGCAT	60	X58871
Ubi_F [b]	GAGGTCGAGCCCAGTGACA	60	EX735613
Ubi_R [b]	GCTTGCCAGCGAAGATCAG	60	EX735613
Def3_F [c]	TTTTGTTGAGAATGAGGCAGC	58	JF733714
Def3_R [c]	ATGAGACACACAGCACTGGAAT	58	JF733714
Pis1.1_F [d]	GTATCTGAAAGGATGAGGTATATTG	58	FJ917596
Pis1.1_R [d]	CTTGAAACATGCGAACTGC	58	FJ917596
RTpis2_F [d]	CCGTGCAGACAAAGCTGAGGA	58	HQ184322
RTpis2_R [d]	GCCGATTTATTTATCACCAACA	58	HQ184322
RTpis2b_F [d]	TGACTGTTGTGTGTTTTTAGG	56	HQ184323
RTpis2b_R [d]	TAAAATACCTTCACACCAATG	56	HQ184323
CART_F [e]	AATTTGGCCAAGTACCAACG	60	DQ167209
CART_R [e]	AGGGCAGTCACACATCTTCC	60	DQ167209

T=annealing temperature
[a] (Seppola et al. 2009)
[b] (Thorarinsdottir 2010)
[c] (Ruangsri et al. 2013)
[d] (Ruangsri et al. 2012)
[e] (Kortner et al. 2011b)

The specificity of PCR amplifications was determined by melting curve analysis during RT qPCR assays. Quantification of the six genes of interest in cDNA samples was carried out in duplicate using ubiquitin (*ubi*) as reference gene and data normalized against ubiquitin using the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen 2001)

2.4 Statistical analysis

Statistical analysis was performed with SigmaPlot® release 11.0 (Systat Software, Inc. CA 94804-2028, USA). After confirming that data had equal variance and were normally distributed, the effects of different feed on gene expression and growth were analysed by one-way ANOVA followed by Tukey's Multiple Comparison Test. The level of statistical significance was set at $p<0.05$.

3 Results

The results indicate improved larval growth, measured by dry weight, following additional enhancement of the live prey, however, no significant differences in larval weight (p=0.357-1.000) or length (p=0.058-1.000) were observed between the groups when measured at 12, 20 or 35 dph (Table 2). The standard length of larvae at 35 dph was highest in the P3-group but the survival was also lowest in this group and density effects on growth rate therefore cannot be excluded. The growth was lowest in the P1-group which may indicate that a six week treatment was excessive in duration as compared with the three week treatment.

Table 2. Larval growth and survival in response to different enhancement of live feed. Larval growth was measured by the dry weight (DW) and standard length (SL) of ~40 larvae tank-1 (~120 larvae treatment-1) collected at 12, 20 and 35 days post hatch (dph). Shown are mean values ± S.D. Larval survival was evaluated at 35 dph by counting surviving larvae from each tank. The larvae were fed control diets of enriched rotifers (C) substituted by rotifers additionally enhanced using a fish protein hydrolysate (P) or a mixture of two marine microalgae species (A). The hydrolysate enhanced prey was offered in both daily feedings every second day three times a week for six weeks (P1), for three weeks (P2) or in both daily feedings every day for one week (P3). The microalgae enhanced prey was offered once every day for six weeks (A).

	12 dph		**20 dph**		**35 dph**		
	SL (mm)	DW (mg)	SL (mm)	DW (mg)	SL (mm)	DW (mg)	Survival (%)
C	5,72 ± 0,44	0,16 ± 0,04	6,57 ± 0,66	0,31 ± 0,02	8,03 ± 1,03	0,36 ± 0,01	47,0 ± 6
P1	5,80 ± 0,50	0,17 ± 0,02	6,61 ± 0,74	0,32 ± 0,03	7,67 ± 1,03	0,43 ± 0,05	42,0 ± 5
P2	5,70 ± 0,45	0,18 ± 0,02	6,72 ± 0,75	0,38 ± 0,08	8,31 ± 1,05	0,41 ± 0,08	45,3 ± 9
P3	5,65 ± 0,48	0,18 ± 0,03	7,00 ± 0,52	0,37 ± 0,06	8,42 ± 1,11	0,44 ± 0,11	35,3 ± 8
A	5,70 ± 0,66	0,16 ± 0,02	6,98 ± 0,82	0,33 ± 0,03	8,08 ± 1,07	0,50 ± 0,29	50,0 ± 10

Larval survival was excellent in all groups, but there was a tendency towards a lower survival in the peptide-treated groups (groups P1, P2 and P3) when evaluated at 35 dph, while algal enhancement (A-group) had positive effects on larval survival (50%) as compared to the P3 group (35%) (p=0.211-0.999) (Table 2).

Gene expression was found to vary considerably between individual larvae within each treatment group, but the overall results indicate that the hydrolysate and algae enhancement of the live prey may have affected the expression of the selected genes. A significant up-regulation of *Pis2b* and *Cart* was observed at 10 dph in the P3-group as compared to the control group (Figure 1). *Cart* was also up-regulated in the P1-group and *IgM* was up-

regulated in larvae fed hydrolysate enhanced prey every second day for three weeks (P2-group).

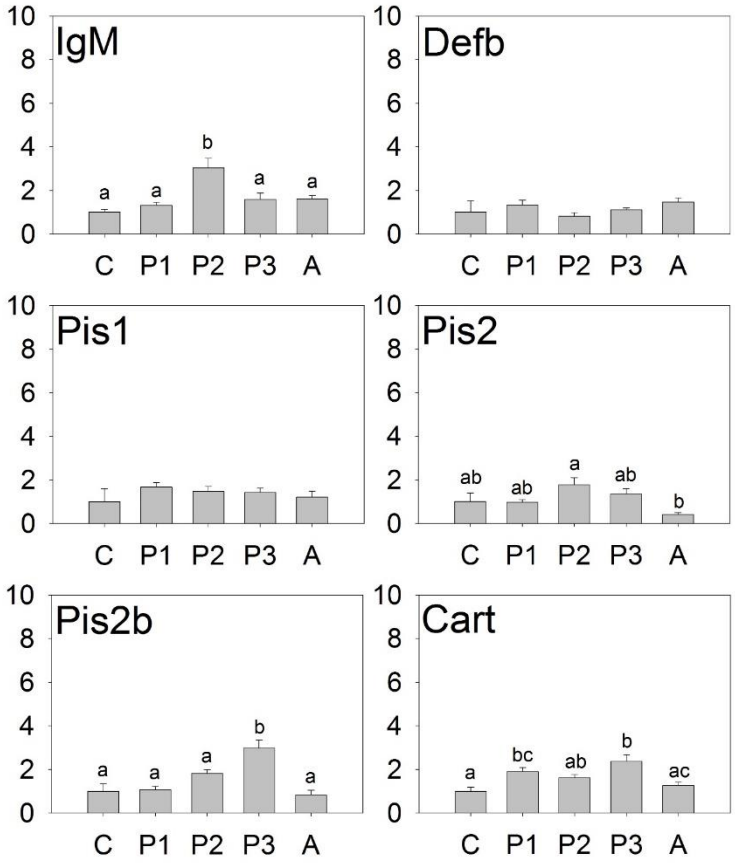

Figure 1. Relative expression of selected genes in Atlantic cod larvae in response to different enhancement of live feed measured at 10 dph. Quantification by real-time PCR of Immunoglobulin M (IgM), Beta-Defensin (Defb), Piscidin 1 (Pis1), Piscidin 2 (Pis2), Piscidin 2-β (Pis2b) and Cocaine and amphetamine-related transcript (Cart) mRNA levels, relative to Ubiquitin (Ubi) in cod larvae 10 days post hatch (dph). The larvae were fed control diets of enriched rotifers (C) substituted by rotifers additionally enhanced using a fish peptide hydrolysate (P) or a mixture of two marine microalgae species (A). The protein hydrolysate enhanced prey was offered in both daily feedings every second day three times a week for six weeks (P1), for three weeks (P2) or in both daily feedings every day for one week (P3). The microalgae enhanced prey was offered once a day for six weeks (A). Each bar represents mean (n=6) values of relative expression of larvae within each group, with error bars indicating the SEM. Different letters denote significant differences between treatment groups (p<0.05)

At 26 dph, a significant up-regulation of *IgM*, *Defb*, *Pis1* and *Pis2* was observed in the A-group and *Pis1* was also significantly up-regulated in the P2-group (Figure 2).

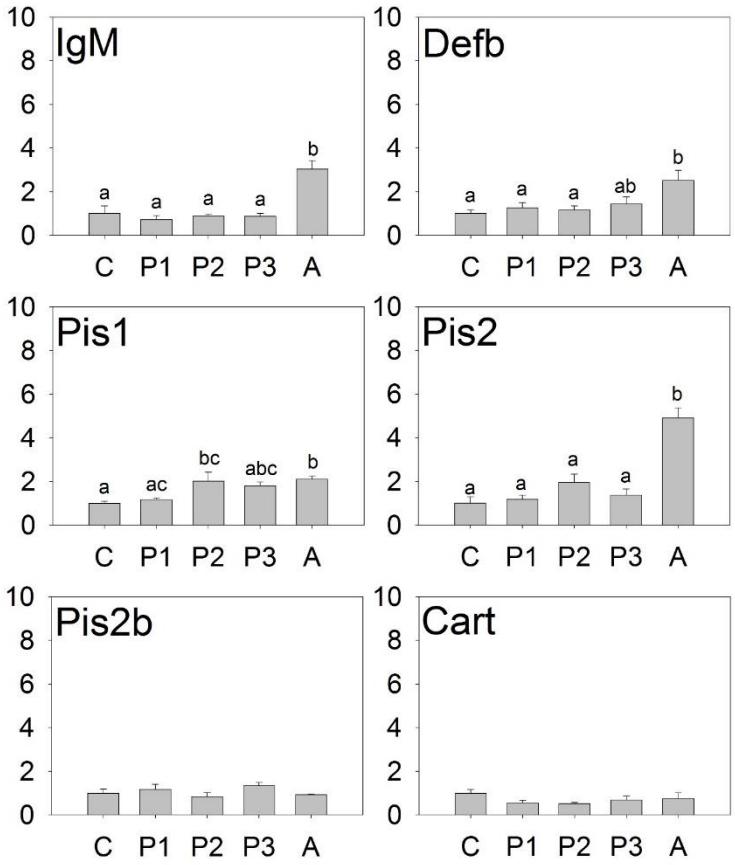

Figure 2. Relative expression of selected genes in Atlantic cod larvae in response to different enhancement of live feed measured at 26 dph. Quantification by real-time PCR of Immunoglobulin M (IgM), Beta-Defensin (Defb), Piscidin 1 (Pis1), Piscidin 2 (Pis2), Piscidin 2-β (Pis2b) and Cocaine and amphetamine-related transcript (Cart) mRNA levels, relative to Ubiquitin (Ubi) in cod larvae 26 days post hatch (dph). The larvae were fed control diets of enriched rotifers (C) substituted by rotifers additionally enhanced using a fish peptide hydrolysate (P) or a mixture of two marine microalgae species (A). The protein hydrolysate enhanced prey was offered in both daily feedings every second day three times a week for six weeks (P1), for three weeks (P2) or in both daily feedings every day for one week (P3). The microalgae enhanced prey was offered once a day for six weeks (A). Each bar represents mean (n=6) values of relative expression of larvae within each group, with error bars indicating the SEM. Different letters denote significant differences between treatment groups (p<0.05)

At 44 dph, a significant up-regulation of *IgM* was observed in the P2-group, but down-regulation of *Defb* and *Cart* as compared to the control group (Figure 3). *Defb* was also down-regulated in the A-group and the P3-grou, and *Pis1* in the A-group and the P1-group. A highly significant up-regulation of *Pis2* and *Pis2b* was observed in both the A-group (13.3-fold and 15.3-fold respectively) but also the P3-group (10.5-fold and 11.4-fold respectively), and *Cart* was up-regulated in both groups as compared to the control group.

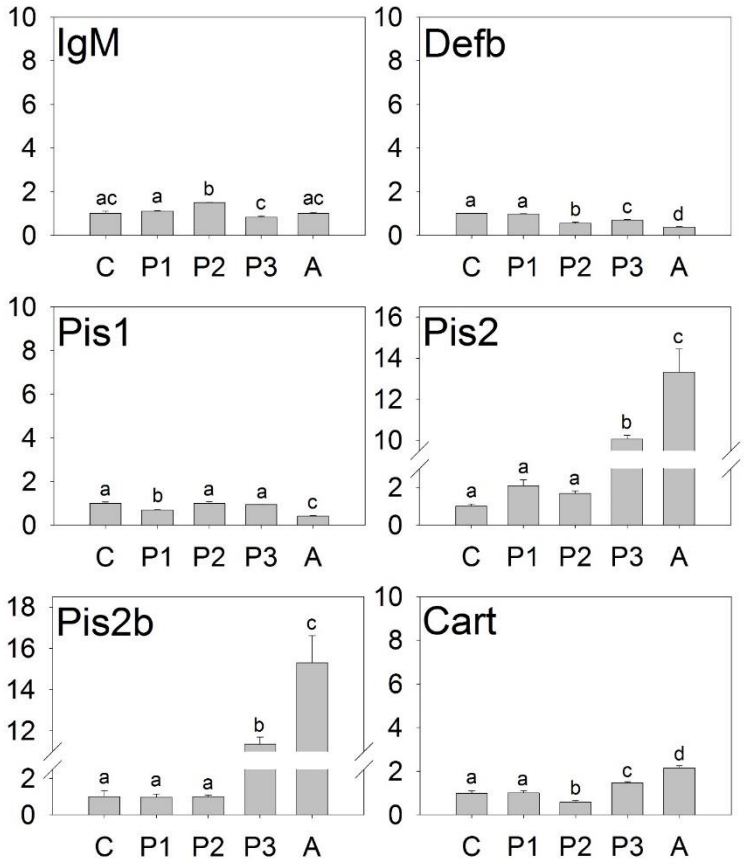

Figure 3. Relative expression of selected genes in Atlantic cod larvae in response to different enhancement of live feed measured at 44 dph. Quantification by real-time PCR of Immunoglobulin M (IgM), Beta-Defensin (Defb), Piscidin 1 (Pis1), Piscidin 2 (Pis2), Piscidin 2-β (Pis2b) and Cocaine and amphetamine-related transcript (Cart) mRNA levels, relative to Ubiquitin (Ubi) in cod larvae 44 days post hatch (dph). The larvae were fed control diets of enriched rotifers (C) substituted by rotifers additionally enhanced using a fish peptide hydrolysate (P) or a mixture of two marine microalgae species (A). The protein hydrolysate enhanced prey was offered in both daily feedings every second day three times a week for six weeks (P1), for three weeks (P2) or in both daily feedings every day for one week (P3). The microalgae enhanced prey was offered once a day for six weeks (A). Each bar represents mean (n=6) values of relative expression of larvae within each group, with error bars indicating the SEM. Different letters denote significant differences between treatment groups (p<0.05)

The protein hydrolysate contained ~81% protein and ~16% lipid. Analysis of the nutrient content revealed no significant effects of the hydrolysate enhancement on the protein and lipid composition of the live prey items (Table 3). There are, however, indications of a reduction in the protein and lipid content of the live prey as a result of the microalgae enhancement, but the difference between the groups was not significant and is not reflected in the nutritional composition of larvae at 20 dph.

Table 3. The protein and lipid content (% of dry weight) of the protein hydrolysate, live prey items and larvae collected at 20 days post hatch (dph). Shown is the nutritional content of the peptide hydrolysate used for live prey enrichment and of untreated rotifers (Control) as compared with rotifers following enhancement using 100ppm of the protein hydrolysate (Peptide) or marine microalgae (Algae). Also shown is the nutritional content of untreated larvae (C) as compared with larvae offered live prey items enriched with marine microalgae (A) or a fish protein hydrolysate in different treatment protocols (P1-P3). Different letters denote significant differences in ion content between the treatment groups of rotifers or larvae collected at 20 dph.

		Protein (%)	Lipid (%)
Protein hydrolysate		80.79	16.05
Rotifers	Control	61.86 ± 0.0^{a}	36.28 ± 0.1^{a}
	Peptide	62.68 ± 0.8^{a}	35.92 ± 1.5^{a}
	Algae	47.87 ± 14.3^{a}	28.78 ± 5.6^{a}
Larvae 20 dph	C	82.37 ± 1.7^{a}	17.40 ± 1.5^{a}
	P1	83.68 ± 0.7^{ab}	17.10 ± 0.4^{ab}
	P2	83.38 ± 1.1^{a}	18.56 ± 0.5^{ac}
	P3	77.20 ± 12.3^{a}	17.01 ± 0.3^{ab}
	A	82.16 ± 0.6^{ac}	18.09 ± 0.7^{ac}

There was a tendency towards increased percentage of EPA, measured as % of total fatty acids identified, in both hydrolysate and microalgae enhanced rotifers, but the groups did not differ significantly from the control group (p=0.11-0.14). However, a significant reduction in the percentage of DHA was observed as a result of additional prey enhancement using both the protein hydrolysate and marine microalgae (p=0.03) and a significantly lower percentage of ω-3 fatty acids, measured as % of total fatty acids identified, was observed following additional enhancement of the live prey using marine microalgae as compared to the control group (p=0.05) (Figure 4).

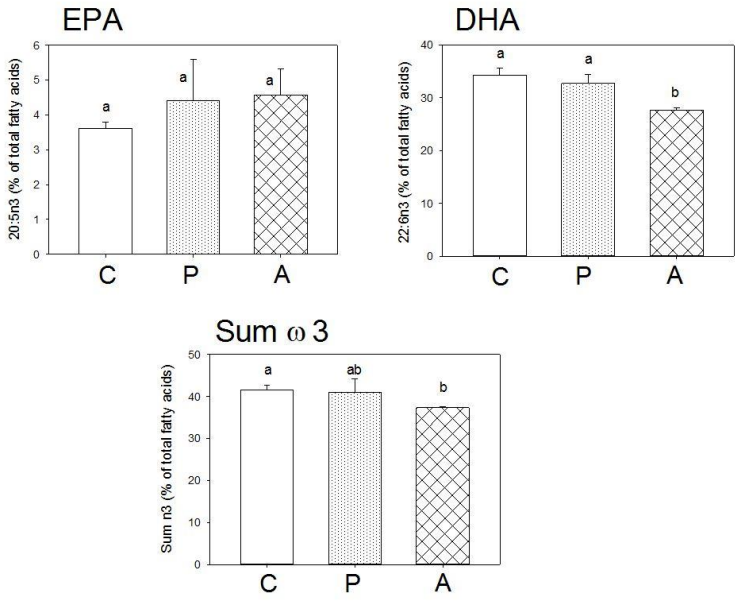

Figure 4. Percentage of total fatty acids identified in rotifers subjected to different enhancement protocols. Shown are mean values ±S.D in the rotifers following standard enrichment (white columns) and additional enhancement using a fish protein hydrolysate (dotted columns) or marine microalgae (striped columns). Dissimilar letters indicate statistical differences between treatment groups ($p<0.05$).

The composition of selected elements in the peptide hydrolysate, enhanced live prey items and larvae collected at 20 dph is shown in Table 4. Significantly higher concentrations of Fe ($p=0.026$) and Cu ($p=0.009$) were measured in rotifers enhanced with the protein hydrolysate as compared with control prey cultures. Similarly, significantly higher concentrations of Fe ($p=0.007$) were measured in rotifers enhanced with the microalgae as compared with the control group. However, significantly lower concentrations of Cu ($p=0.02$) were observed in rotifers enhanced with the microalgae as compared with rotifers enriched with the protein hydrolysate. These differences are partly reflected in the larvae at 20 dph, where there is a tendency towards elevated concentrations of Fe in larvae offered both the hydrolysate and microalgae enhanced live prey.

Table 4. Element composition of the protein hydrolysate, rotifers and larvae collected at 20 days post hatch (dph). Shown is the concentration (mg/kg dry weight) of selected elements in untreated rotifers (Control) as compared with rotifers following enhancement using 100ppm of the protein hydrolysate (Peptide) or marine microalgae (Algae). Also shown is the element composition of untreated larvae (C) as compared with larvae offered live prey items enriched with marine microalgae (A) or a fish protein hydrolysate in different treatment protocols (P1-P3). Different letters denote significant differences in ion content between the treatment groups of rotifers or larvae collected at 20 dph.

		Iron (Fe)	Cupper (Cu)	Sinc (Zn)	Selen (Se)
Protein hydrolysate		19.11	6.40	24.75	1.78
Rotifers	Control	63.25 ± 1.29^{a}	14.06 ± 3.01^{a}	80.69 ± 19.44^{a}	0.16 ± 0.01^{a}
	Peptide	161.22 ± 12.21^{b}	78.46 ± 6.38^{b}	78.51 ± 4.64^{a}	0.19 ± 0.00^{a}
	Algae	148.48 ± 4.48^{b}	6.91 ± 0.33^{c}	64.91 ± 3.57^{a}	0.19 ± 0.01^{a}
Larvae 20dph	C	87.32 ± 7.06^{a}	24.33 ± 17.00^{a}	154.33 ± 46.52^{a}	0.98 ± 0.17^{a}
	P1	86.71 ± 7.29^{a}	13.14 ± 4.30^{a}	143.80 ± 56.46^{a}	0.93 ± 0.14^{a}
	P2	86.89 ± 12.36^{a}	13.28 ± 3.89^{a}	145.58 ± 29.09^{a}	0.88 ± 0.13^{a}
	P3	93.35 ± 7.49^{a}	9.59 ± 0.78^{a}	146.76 ± 11.67^{a}	0.91 ± 0.15^{a}
	A	99.63 ± 4.30^{a}	11.43 ± 1.47^{a}	192.44 ± 27.91^{a}	1.07 ± 0.10^{a}

4 Discussion

Optimal dietary and feeding protocols for cod larvae are not yet fully known and current rearing techniques used in fish farming of other species have therefore been adapted for cod larval rearing (Garcia, Parrish & Brown 2008). The general lack of knowledge on the molecular level of the digestive capability and endocrine function of the larvae has kept back advancement in this field (Kortner et al. 2011a). The results of the present study, comparing different feeding protocols of additionally enhanced live prey items, reveal different mRNA expression levels of appetite and antimicrobial and other immune related genes following additional enhancement of the live prey using a fish protein hydrolysate or marine microalgae.

FPH has been shown to have high nutritional value (Šližytė et al. 2005) and contain various bioactivity (Bøgwald et al. 1996). Previous studies on the effects of FPH on development of cod larvae have reported stimulation of innate immune parameters and improved development of Atlantic cod larvae (Johannsdottir et al. 2013) . However, the different protocols for additional enhancement of live prey items following traditional enrichment did not significantly affect the lipid or protein content of the rotifers. A significantly decreased percentage of DHA in algae enhanced prey items indicates DHA-reduction by the marine microalgae enhancement while DHA and particularly high DHA:EPA ratios have been found to promote growth and stress resistance (Kraul 1993, Sorgeloos, Dhert & Candreva 2001, Park et al. 2006). The microalgae species used, *Chlorella* sp. and *Nannochloris* sp. have been found to contain high levels of polyunsaturated fatty acids (Brown et al. 1997) and the present results indicate that these valuable components may be partially transferred to the live prey items through a short-time enhancement following the traditional enrichment. The minor effects of nutrient enhancement of the live prey on the nutrient content and the reduced DHA:EPA ratio observed as a result of the microalgae enhancement of the live prey therefore indicate that the enhancement protocols need further improvement, possibly through starvation of the microalgae prior to harvesting. Also, further optimization of the enhancement protocols are needed.

The different enhancement protocols of the live prey resulted in significant changes of the mineral content of the diets. However, these differences were only partly reflected in the mineral content of larvae at 20 dph. The increased concentrations of Fe and Cu as a result

of some of the enhancement protocols is of concern, whereas these metal ions are known pro-oxidants and may therefore cause rancidity ravage the ω-3 fatty acids necessary for normal larval development and growth (Blazer 1992, WAAGBØ 1994). The increased levels of these ions were however only partly reflected in larvae offered the enhanced prey and the significance of these findings therefore needs further analysis in long-term growth studies for determining the effect of mineral concentrations in early diets on growth during on-growing.

The standard enrichment and hatchery protocol produced extremely high survival rates in all groups and can thus be considered highly efficient with respect to larval survival, not least due to the low temperatures applied in this study. However, these high survival rates were achieved at the cost of greatly reduced growth rates. The strong negative correlation between growth and survival is not necessarily a desirable trade-off as improved growth during early production stages has been found to persist during the on-growing phase (Imsland et al. 2011). On the other hand, improved larval development and survival may be expected to result in improved disease resistance, growth and overall performance during later stages (Brown, Minkoff & Puvanendran 2003, Kortner et al. 2011a). The results suggest a slightly positive effect of the microalgae enhancement on survival but a tendency towards a lower survival was seen in the peptide-treated groups, especially in the 6-week treatment group, where the enhancement may have been excessive and produced adverse effects on survival. Based on mean dry weights at the end of the feeding trial (35 dph), all the treatments produced a positive effect on growth rates as compared to the control group, with the highest mean weight being reached in the microalgae group. The combination of high survival and improved growth from the microalgae enhancement was a potentially interesting result and suggest that microalgae enhancement may indeed be a promising protocol for improving the quality of hatchery produced cod juveniles.

Immune related parameters can either be maternally inherited and transferred to the offspring or transcriptionally active, and these early defence mechanism has broad specificity against pathogens (Mulero et al. 2007, Magnadóttir 2006). Immunoglobulins are believed to increase larval survival (Swain et al. 2006). In cod, maternal *IgM* was not observed in the eggs or early larvae suggesting that *IgM* is not maternally inherited but appears later in the development process (Magnadóttir et al. 2004b). The present result support previous findings of the group, whereas additional hydrolysate enhancement of the

live prey resulted in increased expression of *IgM* (Johannsdottir et al. 2013). However, in the present study an increased expression of *IgM* was observed in larvae fed hydrolysate enhanced prey every second day for three weeks but not for six weeks as in the previous study. Feeding hydrolysate enhanced prey in all feedings for one week (P3) did not affect the expression of *IgM*, but significant increase in the expression of genes related to antiparasitic activity were found at 44 dph. Daily feeding of algal enhanced prey for six weeks (A) caused a significant up-regulation of *IgM* and other immune related genes at 26 and 44 dph. An increased expression of antimicrobial genes (*Pis*) has been observed when injecting Atlantic cod with attenuated bacterial pathogens (Ruangsri et al. 2012). An increased expression of *Pis* genes and tendency towards improved larval survival observed in the present study may therefore indicate that additional enhancement of the live prey using the marine algae stimulates antimicrobial defence mechanisms and may result in more robust larvae.

Fish primarily detect food in the natural aquatic environment through olfaction (smell) and sight, however, appearance, feel, and taste of the diet are key factors in determining whether food will be swallowed or rejected by fish held in captivity (Lall, Tibbetts 2009). Cocaine- and amphetamine-regulated transcript (*Cart*) peptides was first identified in mammals as an mRNA induced by administration of cocaine and amphetamine as well as other psychomotor stimulants (Hunter et al. 2004). *Cart* has been shown to affect the intake of food in fish as central injections of *Cart* decrease appetite and food consumption. Interactions between *Cart* and other appetite-related peptides furthermore occurs as *Cart* inhibits neuropeptide Y (NPY)- and orexin (OX) induced hyperphagia (Volkoff, Peter 2000, Volkoff 2006).

The marine microalgae cultures used for the enhancement, were harvested daily from continuous algae cultures, and thus maintaining favourable cultural conditions, while unfavourable environmental conditions are commonly applied for increased accumulation of lipids and reduced water content prior to marine algae harvesting. This may explain the significantly lower lipid content of the algae-enhanced live prey as compared with the control and peptide-enhanced diets. The observed up-regulation of *Cart* as a result of algal enhancement of the live prey therefore indicates a decrease in appetite and reduced feed intake. However, the tendency towards improved larval growth as compared to the control group may indicate that additional enhancement using the marine algae may better fulfil

the nutritional requirements of the larvae as compared to the other diets tested or favourable composition of minerals. The expression pattern of *Cart* over time in the present study is in line with previous findings suggesting that *Cart*, NPY and OX may be affected by feeding at the first stages of larval development (Kortner et al. 2011b)

Overall, the present results indicate promising effects of additional enhancement of the live prey of cod larvae using a fish protein hydrolysate or a mixture of two cold water marine microalgae species. However, further experiments are required for the development of best practice protocols.

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Viðauki I – Staðfesting frá Aquaculture Nutrition

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Effects of different live prey enhancement on the expression of selected immune and appetite related genes during early development of cod larvae (Gadus morhua L)

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