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TOPCOD - OPTILAR

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Vinnsla, virðisaukning og eldi

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Report summary

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Titill / Title	**TOPCOD, OPTILAR** / Lengi býr að fyrstu gerð. Kjöreldisferlar í lirfu- og seiðaeldi á þorski		
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Ágrip á íslensku:

Nýlegar rannsóknir hafa leitt í ljós að bestu aðstæður við framleiðslu lirfa gefi seiði af betri gæðum og að vaxtarforskot á fyrstu stigum eldisins skili sér að einhverju leiti á seinni vaxtarstigum. Meginmarkmið þessa verkefnis er að skilgreina bestu aðstæður við eldi þorsklirfa á Íslandi og nýta í því markmiði margvíslegar aðferðir við lausn helstu vandamála sem eru tengd framleiðslu þorsklirfa í dag. Þessi skýrsla fjallar um þá verkþætti sem Matís ohf. tók þátt í sem m.a. var að rannsaka áhrif auðgunar fóðurdýra með bætibakteríum og próteinmeltu á vöxt, þroska, ónæmisörvun og meltingarflóru lirfa svo og rannsóknir á áhrifum mismunandi frumfóðrunar á vöðvavöxt sem unnið var í samvinnu við Hafrannsóknastofnunina. Niðurstöður gefa vísbendingar um að byrjun þurrfóðurgjafar seint eða um 50 dph gefi ekki lirfum vaxtarforskot og að það sé nægilegt að fóðra með *Artemiu* þar til 40 dph. Þurrfóðurgjöf frá 30 dph leiddi til minni vaxtar og aukinnar tíðni byggingargalla. Auðgun fóðurdýra með frostþurrkaðri blöndu tveggja bætibakteríustofna hafði ekki áhrif á samsetningu bakteríuflóru lirfa og stofnar náðu ekki fótfestu í meðhöndluðum lirfum. Hinsvegar má gera ráð fyrir að léleg hrognagæði hafi haft áhrif á niðurstöður meðhöndlunar. Niðurstöður tilrauna staðfesta fyrri niðurstöður um jákvæð áhrif auðgunar fóðurdýra með próteinmeltu á afkomu og þroskun lirfa.

Lykilorð á íslensku: *þorsklirfur, frumfóðrun, vöðvavöxtur, bætibakteríur, próteinmelta*

Summary in English:

Recent research has demonstrated that production optimization during the larval and juvenile phase will to some extent be reflected in the performance of the fish during the ongrowing phase. The objectives of the project are to optimise the larval production of Atlantic cod in Iceland by applying a multidisciplinary approach to solve central bottlenecks related to larval production. This report presents tasks where Matis ohf. was involved, including analyzes of the effects of live prey enrichment using putative probionts and a fish protein hydrolysate on larval survival quality immune stimulation and intestinal bacterial community of larvae. The study also involved an analysis of the effect of startfeeding protocols on muscle growth in collaboration with MRI. The results indicate that late weaning around 50 dph may be excessive and produce no significant advantage. An intermediate weaning strategy, with artemia feeding until 40 dph, appears to be sufficient to convey important advantages in terms of growth and anatomy. Early weaning on 30 dph produced slow-growing juveniles and a higher deformity ratio. Using the freeze dried preparates of the probionts did not affect the bacterial community structure of larvae and the probionts were not found to be established within the bacterial community of treated larvae. Poor quality egg may, however, partly explain the lack of effects as a result of treatment. The present study confirms the results of previous studies where live prey enrichment using a fish peptide hydrolysate significantly improved larval survival and development.

English keywords: *cod larvae, first feeding, muscle growth, probiotics, protein hydrolysate*

Contents

1. INTRODUCTION

Over the last years, significant effort has been put into the process of developing a sustainable cod aquaculture industry in Iceland, and the focus has been on the difficult issues concerning egg and larval development. The most limiting factor in cod aquaculture up to now has been the production of sufficient amounts of high quality juveniles. The objectives of the project are to optimise the larval production of Atlantic cod in Iceland by applying a multidisciplinary approach to solve central bottlenecks related to larval production.

An effective exploitation of intensive land-based production facilities calls for detailed knowledge on the impact of key rearing factors on fundamental production characteristics. Recent research (Imsland *et al.*, 2007a; Imsland *et al.*, 2007b) has demonstrated that production optimization during the larval and juvenile phase will to some extent be reflected in the performance of the fish during the ongrowing phase. This opens for the possibility of "tailor made" cod production where improvement during the larval and juvenile phase will be carried all the way through the ongrowing stage. In particular, the economic constraints of the on-growers will require the use of offspring with desirable traits in terms of high growth and survival potential and absence of maturation prior to market-demanded harvest size.

Environmental (temperature, light, salinity) and nutritional (DHA, EPA, ArA, vitamin A, phospholipids, iodine) factors during larval rearing largely dictate the successful transformation of larvae to juveniles during metamorphosis which, in turn, determines juvenile quality (Koven, 2003). Studies on Atlantic halibut, turbot and Japanese flounder report higher metamorphic success, in terms of pigmentation, eye migration and general development when copepods were fed to the larvae. DHA, an abundant PUFA in copepods, is also vital to vision as it provides the membrane fluidity necessary for rhodopsin to function when stimulated by light. These highly potent metabolites are thought to regulate the mechanisms involved in the release of melanophore stimulating hormone and pigmentation. Differences in physiological and anatomical properties of fish juveniles produced with different first feeding methods have been indicated in Atlantic halibut (Hamre *et al.*, 2002; Næss *et al.*, 1995) and more recently in Atlantic cod (Imsland *et al.*, 2006b). Imsland et al. (op cit) reported that groups of juvenile cod fed rotifers during the larval stage had a higher incidence of deformities (23%) than fish fed zooplankton (8%). These results are in line with studies on Atlantic halibut where developmental errors have been attributed to different nutritional value startfeeding diets (Hamre *et al.*, 2002; Næss *et al.*, 1995).

Results on the size of cod larvae 100 days post hatch has been reduced in the last years and is now about 2.5 g but has been as high as 6.2 g in previous years (Agnar Steinarsson, presentation at AVS Cod Conference, 29-30 November 2007). Given that growth is an indicator of well-being of the fish, questions can be raised whether the development in feeding regimes in "commercial" production of cod larvae in Iceland is on the right track?

Optimized live prey quality is of key importance for success during early larval development. Bacterial growth in live feed cultures represents a problem and high bacterial numbers have been found to affect the overall quality of the live prey and may furthermore negatively affect larvae during early developmental stages (Bjornsdottir *et al.*, 2009). Bacterial growth

furthermore increases the risk for transfer of opportunistic and pathogenic bacteria to larvae. High temperatures and high loads of organic nutrients stimulate bacterial growth, and measures for decreasing and controlling the bacterial flora of live prey organisms are therefore of high importance. Changing and stabilising the microflora can be achieved by algal incubation (Olsen *et al.*, 2000) or exchange of the detrimental flora with probiotic bacteria (Lauzon *et al.*, 2010; Makridis *et al.*, 2000). Microbiota control, higher larval survival, increased tolerance, growth and development as well as stimulative immunological effects and probiont establishment in rearing systems are the main criteria for the selection of probiotic techniques. At the moment there are no well documented probiotic bacteria for use in first feeding of cod. However, bacterial strains have been isolated from Icelandic cod and halibut aquaculture environments and applied by various treatment schedules, and have been found to be promising (Bjornsdottir *et al.*, 2010b; Lauzon *et al.*, 2010). The effects of these bacteria have to be tested in order to validate e.g. the possible growth promoting effects. Algal cleaning has been developed for *Artemia* and preliminary studies have shown that it can be adjusted to apply for rotifers as well. More research is, however, needed before this technology can be implemented in commercial hatcheries.

The innate immune system represents the first line of defence and many of its components are well developed in fish. Stimulation of innate immune parameters is therefore considered a promising approach for improved survival and overall larval quality. Results from a previous study where the live prey was enriched using a protein hydrolysate derived from Pollock indicated significant improvements in growth and development during early larval stages of cod (Bjornsdottir *et al.*, 2010a).

Growth rate improvements can be achieved through the adjustment of parameters such as temperature, photoperiod, salinity, food quality/ration, stocking densities, etc. Each of these parameters has to be adjusted accordingly to the species and development stage. Of all environmental factors that influence growth in fish, temperature is the single most dominant (Brett, 1979). Temperature is a rate-controlling factor for all chemical processes in poikilotherms. However, the temperature effect varies within the growth temperature range of the species. In juvenile cod, growth rate is significantly influenced by temperature, following a pattern typical of most fish species. This pattern is characterized by a rapid increase in growth rate as temperature increases, a peak signalling the optimum temperature for growth ($T_{opt}G$), and frequently, a precipitous decline as higher temperatures become adverse beyond $T_{opt}G$. The findings of different temperature optima for different size classes together with the downward trend of the $T_{opt}G$ and $T_{opt}FCE$ with size can be summarized in the “stepwise-temperature-hypothesis”. Instead of using constant rearing temperatures one utilizes specific “temperature-steps” where the fish are reared at optimum temperatures defined for each size class. The benefit of “temperature-steps” throughout the whole larval and juvenile cycle and under realistic production scale has not yet been verified in Atlantic cod although there are now clear indications that this rearing method may yield substantial growth benefit (Björnsson *et al.*, 2007; Imsland *et al.*, 2007a; Imsland *et al.*, 2006a; Imsland *et al.*, 2005). Studies in Canada and Norway (Koedijk *et al.*, 2008; Lambert *et al.*, 1994) have demonstrated considerable growth improvement at intermediate (15‰) salinity.

The larval part of the TOPCOD project and the whole OPTILAR research project is structured according to two main tasks; *Optimal larval culture of cod* and *Development of an optimal rearing protocol for larval and juvenile cod.* The projects were funded by the AVS fund (OPTILAR) and the Technology Development Fund (TOPCOD) in Iceland. This report presents tasks where Matis ohf. was involved. These tasks include analyzing the effects of live prey enrichment using putative probionts and a fish protein hydrolysate in Task 1.2 and collaboration with Hafro in analysing the effect of startfeeding protocols on muscle growth in Task 1.1.

2. MATERIALS & METHODS

The hatchery production of marine fish species is still entirely dependent upon the culturing and harvesting of live prey items for the voracious fish larvae. Rotifers and *Artemia* are easily mass produced and thus provide means of replacing the natural zooplankton diet in marine hatcheries around the world. However, studies indicate that hatchery produced juveniles often exhibit diminished growth capacity as well as deviations in anatomical development, which can be directly traced to an incomplete start-feeding diet (Imsland *et al.*, 2006b). Another negative aspect of cultured prey is the associated bacterial flora, which provides a vector for harmful bacteria into the larvae, potentially leading to reduced survival of the larval population. The aim of this task is to challenge these problems and seek ways to improve the performance of the cod hatchery on all levels. The task is sub-divided into the three sub-tasks but this report will discuss the following two tasks, *Improved startfeeding protocols for larval cod* and *Probiotics for larval and juvenile cod*. First experiment using probiotics did not show positive effects on larval survival and it was therefore decided to treat larvae with fish protein hydrolysate which had shown promising results in previous experiments.

All rearing experiments were carried out at the MRI hatchery in Grindavík and samples transported to the Matis laboratories at Akureyri.

2.1. Improved startfeeding protocols

This task was aimed at testing long term effects of early weaning and devising an optimal start-feeding protocol that favours growth potential and juvenile quality. A long term rearing experiment was launched in May 2009 at the MRI hatchery in Grindavík. The larval rearing was conducted in six hatchery tanks (3400 L) stocked with 150.000 larvae per tank. Fertilized eggs were obtained from Icecod. Three different protocols were tested in duplicate, i.e. weaning by 50, 40 and 30 days post hatch (groups A (tanks E13-14), B (tanks E15-16) and C (tanks E17-18), respectively). All groups were fed rotifers and dry feed (*Gemma Micro Diamond*) but the early weaning group (group C) was not fed any *Artemia*. Larval samples for length, dry weight and anatomy were collected weekly and survival calculated at transfer to nursery tanks (49 dph). All groups were graded to prevent cannibalism and a sub-population of 200 juveniles from each weaning group reared and monitored in three rearing tanks at the MRI. Deformities were checked and classified visually at 135 dph.

2.1.1. Muscle growth

Matis role in this task was to test the effects of early weaning on muscle growth measured by hypertrophy/atrophy. Muscle growth occurs through hyperplasia (an increase in fibre number) and hypertrophy (increase in fibre size). Following grading of larvae from all groups (larger and smaller larvae) larval samples (5-10 larvae) of both size groups were collected from each group at 59 and 90 dph for analysis of the muscle growth using image analysis. Samples from each treatment group were collected following grading of larvae, fixed in buffered formalin and sent to the Matis laboratory in Akureyri. Three larvae from each group were measured for standard length (SL, measuring from the tip of snout to end of notochord) and myotome height (MH) and then cross-sectioned at the anus level (Fig. 1). The front part of larvae was then post-fixed and processed prior to embedding in a paraffin blend (Tissue Embedding Medium, Surgipath) followed by sectioning in 3µm transverse sections that were stained with Hematoxylin and Eosin (HE) for image analysis. Initial analytical approach using freezing in liquid nitrogen following fixing in buffered formalin and subsequent cryosectioning and staining (toluidine blue or hematoxylin/eosin) gave poor results, with irregular muscle fibres that could not me measured accurately for size. Post-fixing and embedding in liquid paraffin blend was therefore selected for analysis of all samples. Three frames, located in the dorsal epaxial quadrant of sections cut at the level of the posterior gut, i.e. immediately posterior the anus (Fig. 1) and representing superficial and deep myotomes, were analysed using 400x magnification. Outlines of all muscle cells within each frame were digitally traced and all cells counted and measured for equivalent diameter and area, using the Leica QWin program. Because of the large variation in the SL of the sectioned larvae between the graded groups, muscle data are presented as a function of larval SL.

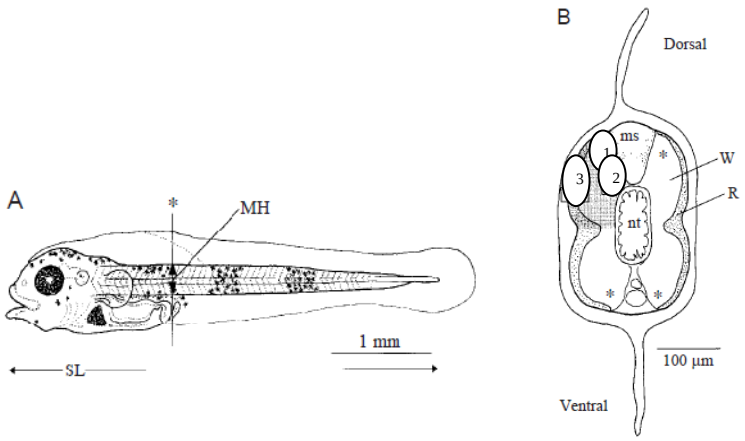

***Figure 1**. (A): Larval measurements, myotome height (MH) and standard length (SL), and where transverse sections were cut (*). (B): Localization of frames used for image analysis of muscle cells within the epaxial quadrant (shaded area). 1= dorsal germinal zone, 2= deep fibers, 3=lateral fibers (Galloway et al., 1999).*

2.2. Live prey enrichment

This task aimed at developing preventive measures for improved larval growth, development and/or survival. Putative probionts and bioactive peptides were tested through various treatments of larvae at hatch and during the first weeks post hatch. The effects of early treatment were studied in three distinct experiments, with bioencapsulation of the live prey using a mixture of two putative probiotic bacterial strains in two distinct experiments and one experiment where the live prey of larvae were bioencapsulated using a fish protein hydrolysate.

The bacterial community structure of larvae and their live prey was studied and the effects of treatment on early larval development and survival were evaluated. The expression of selected immune related genes was furthermore studied in a parallel project, using larvae from the probiotic experiments and the distribution as well as intensity of selected immunological parameters was studied in the peptide experiment. Gene expression was quantified in pooled samples of 5 larvae at each sampling dates, and the analysis carried out using three biological replicate samples. The methods are further described in Thorarinsdottir (2010). The distribution and intensities of immunological parameters were evaluated using specific antibodies and immunohistochemistry.

2.2.1. Experiments 1 & 2

The effects of probiotic treatment of larvae at hatch and during the first two weeks post hatch were studied. Two bacterial strains (*Arthrobacter bergerei*, GeneBank code AJ609631 and *Enterococcus thailandicus* FP48-3, GeneBank code EF197994), isolated from the cod farming environment in a previous study (Lauzon *et al.*, 2008), were cultured in the laboratory and growth inhibition activity of fresh and freeze dried preparations against selected fish pathogens (*Aeromonas salmonicida salmonicida*, *Vibrio salmonicida* and *Vibrio anguillarum*) tested *in vitro,* using four different methods; the pour plate method, the paper disc method, the well diffusion assay and the agar spot method. Briefly, MA plates were sawn with each pathogenic strain and 10µl of fresh MB-cultures (Lauzon *et al.*, 2008) or various dilutions of freeze dried preparation of the strains, placed on the surface of the agar, either by placing the volume on a paper disc, directly on the agar surface or in a well prepared in the agar surface (Hermannsdóttir, 2008). Following incubation (15 °C, 7 days), the inhibitory activity of the isolates towards the pathogenic strains was evaluated by measuring the diameter (mm) of the clear zones appearing around the colonies. Partial inhibition was also registered, characterized by reduced density of bacterial growth surrounding the colonies.

Freeze-dried preparations were then prepared for probiotic treatment of the rotifers and *Artemia* in various treatment schedules including time of treatment, rinsing and additional treatment of the live feed using UV radiation (carried out at IMR) (Matis-experimental report). The effect of treatment on growth and survival of the live feed were studied (IMR) as well as on the numbers of bacteria grown on selective nutrient media (nitrite actidione polymyxin agar (NAP pH 5.5) for isolating lactic acid bacteria and Thiosulphate Citrate Bile Salts Sucrose Agar (TCBS) for number of presumptive *Vibrio*). The bacterial community structure was also analysed through 16S rDNA characterization using PCR and the

Denaturing Gradient Gel Electrophoresis (DGGE) method, followed by sequence analysis of bands of interest. The most favourable application was then selected for probiotic treatment of the live feed offered to larvae in two separate comparative experiments that were carried out during April-July 2009:

- *Experiment 1*. Bacterial treatment (10^{10} bact/L) through two hour bathing at hatching and repeated at 2 dph. Bacteria-treated (10^{10} bact/L) live feed offered to larvae at hatching and daily during 3-6 dph, 13-14 dph and 17-18 dph. The experiments were carried out in triplicate, with three incubators used as controls. Larval samples were collected at three selected time points: at hatching, 3dph and 7dph for analysis of the bacterial community structure. Samples were furthermore collected at 3 and 7dph for analysing genetic expression of selected immunological factors (IgM and Lysozyme) in a parallel project.
- *Experiment II*. Bacterial treatment (10^{10} bact/L) through one hour bathing at hatching and repeated at 2 dph. Bacteria-treated (10^{10} bact/L) live feed offered to larvae at hatching and daily during 3-5 dph, 10-11 dph and 18-19 dph. The experiments were carried out in duplicate, with two incubators used as controls. Larval samples were collected at 2, 7, 13, 21, 26 and 36 dph for analysis of the bacterial community structure. Samples were furthermore collected at 2, 7, 26 and 36dph for analysing genetic expression of selected immunological factors (IgM and Lysozyme) in a parallel project.

The effects of treatment on early larval development and survival were evaluated by the staff at IMR. The bacterial community structure of larvae were studied using cultivation on selective nutrient media and as well as through PCR and the DGGE method. The gene expression was quantified using the RT-PCR method that has been set up and standardized with the appropriate housekeeping genes selected for comparison of the expression of selected immune related genes in cod larvae at various developmental stages. The gene expression was analysed in samples of 5 pooled larvae at each sampling dates, and the analysis carried out using three biological replicate samples. The methods are further described in (Þórarinsdóttir, 2010).

2.2.2. Experiment 3

The protein hydrolysate (88% protein) was manufactured from pollock fillets by Iceprotein Ltd. in Iceland, using the Hultin-Process method that is based on changes in the pH of the solution in a chilled environment (Kristinsson & Rasco, 2000). Cod larvae were fed the hydrolysate through bioencapsulation of the live prey (rotifers and *Artemia*) for 30 minutes prior to offering to larvae in both daily feedings, three times a week from 2-45dph. The treatment was carried out in quadruple (10.000 larvae $tank^{-1}$), with four tanks containing larvae of a common origin serving as control fed untreated live prey. Samples of larvae and juveniles were collected at 3, 10, 17, 31, 46, 55, 80 and 160dph. The mean weight and length of pre-juveniles was evaluated at 48 dph and the remaining survivors from each protocol then pooled and transferred to a new incubator (~1.500 post-juveniles $tank^{-1}$). Long-term effects of treatment were evaluated at 160 dph when normal development was also estimated by visually examining ~50 juveniles from each tank with respect to skeletal deformities, gaping and poorly developed swim bladder.

The presence and distribution of IgM and lysozyme in cryosectioned larvae was furthermore studied through immunohistochemistry using specific antibodies (kindly donated by Dr.

Merete Bjørgan Schrøder at the Norwegian College of Fishery Science), with binding of specific antibodies visualized using horseradish peroxidase labelled secondary antibodies.

3. RESULTS

3.1. Improved startfeeding protocols

3.1.1. Larval survival and development

The startfeeding experiment was carried out at IMR and larval development and survival were evaluated by the staff at IMR (Agnar Steinarsson).

The survival from larva to juvenile was relatively low i.e. 15,3 and 7,7% in group A, 7,7 and 7,0% in group B and 6,3 and 7,0% in group C. Growth rates based on dry weight were similar (8-12%/day) in all groups during the rotifer stage (0-30 dph) but group C (no artemia) fell far behind groups A and B during the artemia stage. Growth rates in group C stagnated around 10%/day, while groups A and B rose to 15-17%/day. A sharp decrease in growth rate was observed in all groups during weaning, most notably in group B around 40 dph. Group B, however, quickly recovered with a compensatory growth spurt of 23%/day (Fig. 2).

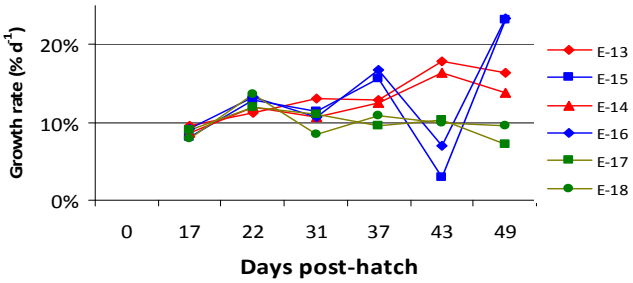

Figure 2. *Growth rates during 0-49 dph. Shown are growth rates of larvae in the different treatment groups; group A (E13-14) weaning by 50 dph, group B (E15-16) weaning by 40 dph and group C (E17-18) weaning by 30 dph.*

At the end of the hatchery stage (49 dph) the mean dry weight in groups A and B was 2-5 times higher than in group C (Fig. 3). The mean weight was highest in group A, but due to large relative differences between replicate tanks there was no significant difference between groups A and B ($P > 0.05$). Group C, however, grew significantly slower than groups A and B ($P < 0.05$). These initial differences still persist on the juvenile stage and at 135 dph the mean live weight in groups A, B and C was 16,2, 15,3 and 11,1 g, respectively. The variation in live weight is greatest in group C (CV=38,2%), reflecting a higher incidence of slow growing juveniles than in groups A and B (CV=34-35%) (results not shown).

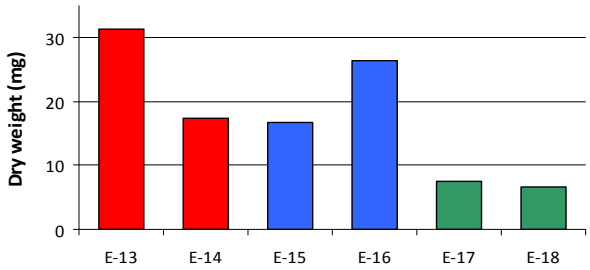

Figure 3. *Mean dry weight of larvae in diffent treatment groups at 49 dph; group A (E13-14) weaning by 50 dph, group B (E15-16) weaning by 40 dph and group C (E17-18) weaning by 30 dph.*

Deformity ratios were relatively low in all groups but deformities were manifested differently among the groups. Group C stood out with a relatively high ratio of jaw- and fin deformities i.e. 4 and 15%, respectively. Head deformities were similarly noted in all the groups (10-15% incidence) but the majority was only mild to moderate cases. Distinct head deformities had a similar incidence in all the groups i.e. around 3%. Serious intolerable deformities were assessed at 6, 5 and 11% in groups A, B and C, respectively (Fig. 4).

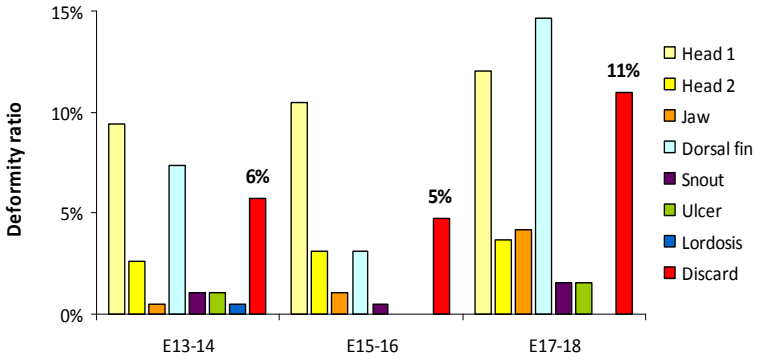

Figure 4. *Deformity ratios at 135 dph. Shown are results from diffent treatment groups, group A (E13-14) weaning by 50 dph, group B (E15-16) weaning by 40 dph and group C (E17-18) weaning by 30 dph.*

3.1.2. Muscle growth

Muscle growth is commonly measured by the recruitment of new muscle fibres and comparing the relative number of small myofibres (Martell & Kieffer, 2007). Previous studies indicate that increased number of myofibres is primarily observed within superficial muscle layers (frames 1 and 3 in Fig. 2) (Galloway *et al.*, 1999).

Figure 5 shows an example of HE stained section and Table 1 shows results from the weight and size (SL and MH) of all larva sampled.

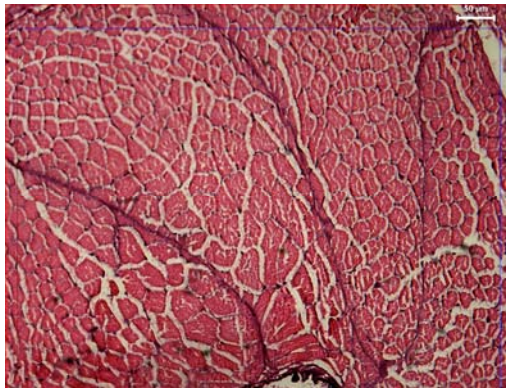

Figure 5. *HE stained, transverse section located within frame 2 in the dorsal epazial quadrant showing deep fibers.*

Table 1. *Weight, standard length (SL) and myotome height (MH) of larvae from all groups, collected at 59 and 90 dph.*

Sampling	Group	Size grading	Sampling	Sample no.	Weight (g ±SD)		SL (cm ±SD)		HM (cm ±SD)	
59 dph	50 dph	+	59 dph	*E-1-1*	0.283	0.431 ±0.20	3.0	3.4 ±0.51	0.5	0.7 ±0.15
				E-1-2	0.357		3.3		0.7	
				E-1-3	0.653		4.0		0.8	
		-		*E-2-1*	0.134	0.190 ±0.05	2.3	2.6 ±0.25	0.4	0.5 ±0.06
				E-2-2	0.240		2.8		0.5	
				E-2-3	0.195		2.6		0.5	
	30 dph	+		*E-3-1*	0.307	0.278 ±0.03	3.1	3.1 ±0.10	0.5	0.5 ±0.06
				E-3-2	0.275		3.0		0.5	
				E-3-3	0.253		3.2		0.6	
		-		*E-4-1*	0.041	0.104 ±0.07	1.8	2.2 ±0.40	0.3	0.4 ±0.10
				E-4-2	0.175		2.6		0.5	
				E-4-3	0.097		2.2		0.4	
	40 dph	+		*E-5-1*	0.650	0.563 ±0.09	3.9	3.8 ±0.15	0.7	0.7 ±0.06
				E-5-2	0.471		3.6		0.6	
				E-5-3	0.569		3.8		0.7	
		-		*E-6-1*	0.076	0.127 ±0.05	2.0	2.3 ±0.26	0.3	0.4 ±0.06
				E-6-2	0.140		2.4		0.4	
				E-6-3	0.165		2.5		0.4	
90 dph	50 dph	+	90 dph	*J-3-1*	1.780	2.690 ±1.49	5.6	6.1 ±0.92	1.0	1.1 ±0.12
				J-3-2	4.410		7.2		1.2	
				J-3-3	1.879		5.6		1.0	
		-		*J-4-1*	1.143	1.375 ±0.83	4.9	5.0 ±0.80	0.8	0.8 ±0.25
				J-4-2	0.690		4.2		0.6	
				J-4-3	2.293		5.8		1.1	
	30 dph	+		*J-5-1*	1.107	1.701 ±0.66	4.7	5.3 ±0.56	0.8	0.9 ±0.10
				J-5-2	1.582		5.4		0.9	
				J-5-3	2.414		5.8		1.0	
		-		*J-6-1*	0.497	0.589 ±0.17	3.6	3.8 ±0.38	0.6	0.6 ±0.06
				J-6-2	0.486		3.5		0.6	
				J-6-3	0.784		4.2		0.7	
	40 dph	+		*J-11-1*	1.965	2.505 ±0.51	5.5	6.0 ±0.42	1.0	1.1 ±0.10
				J-11-2	2.581		6.1		1.1	
				J-11-3	2.969		6.3		1.2	
		-		*J-12-1*	0.738	1.520 ±0.81	4.1	5.2 ±1.01	0.7	0.9 ±0.15
				J-12-2	1.460		5.3		0.9	
				J-12-3	2.362		6.1		1.0	

The results indicate an increasing number of primarily superficial myofibres observed 59 dph as well as 90 dph in larvae from both group A and group B (within frames 1 and 3 in Fig. 2) (Fig. 6-13). An increase in the number of deep myofibres (within frame 2 in Fig. 2) was furthermore observed in the smaller size group of larvae from group A. A similar ratio of the various size groups of superficial and deep fibres was observed in larger size groups of groups A and B, at 50 dph as well as 90 dph. In both size groups of group C, an increase in number of myofibres was only detected at 90dph, indicating increased growth compared with 59dph when no increase in number of myofibres was observed. Overall, higher variability in number and size of miofibres was observed in larvae from the smaller as compared with the larger size groups within each group.

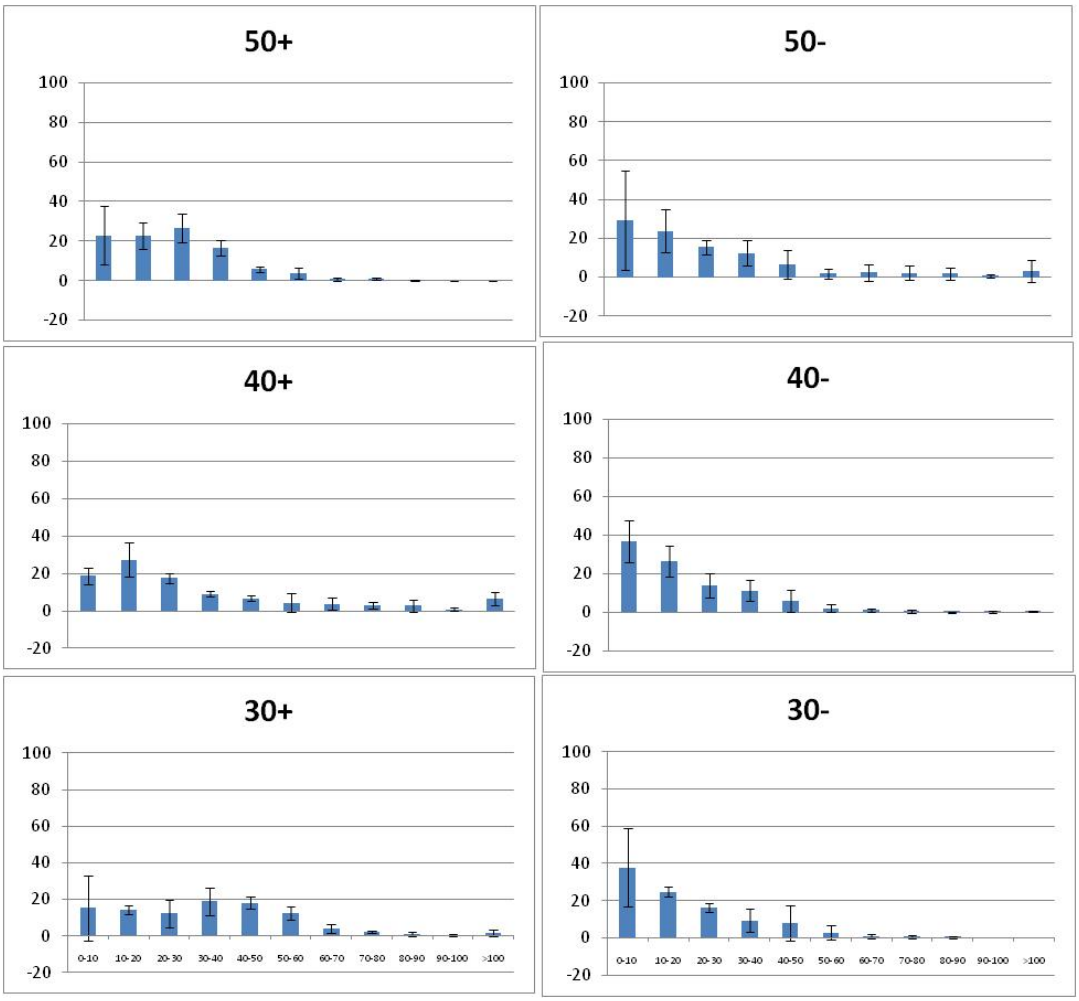

***Figure 6**. Dorsal germinal zone. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) at 59 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups, group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

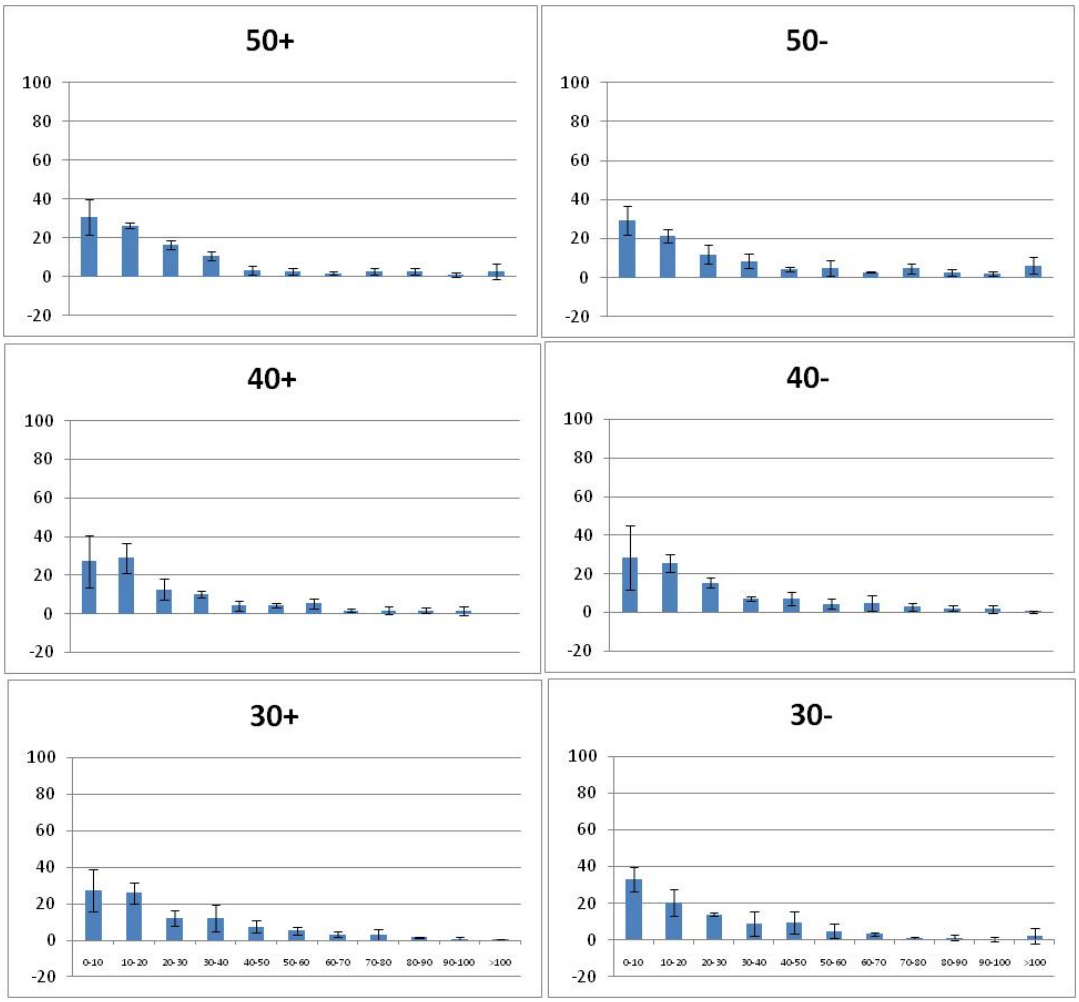

Figure 7. *Dorsal germinal zone. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) at 90 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

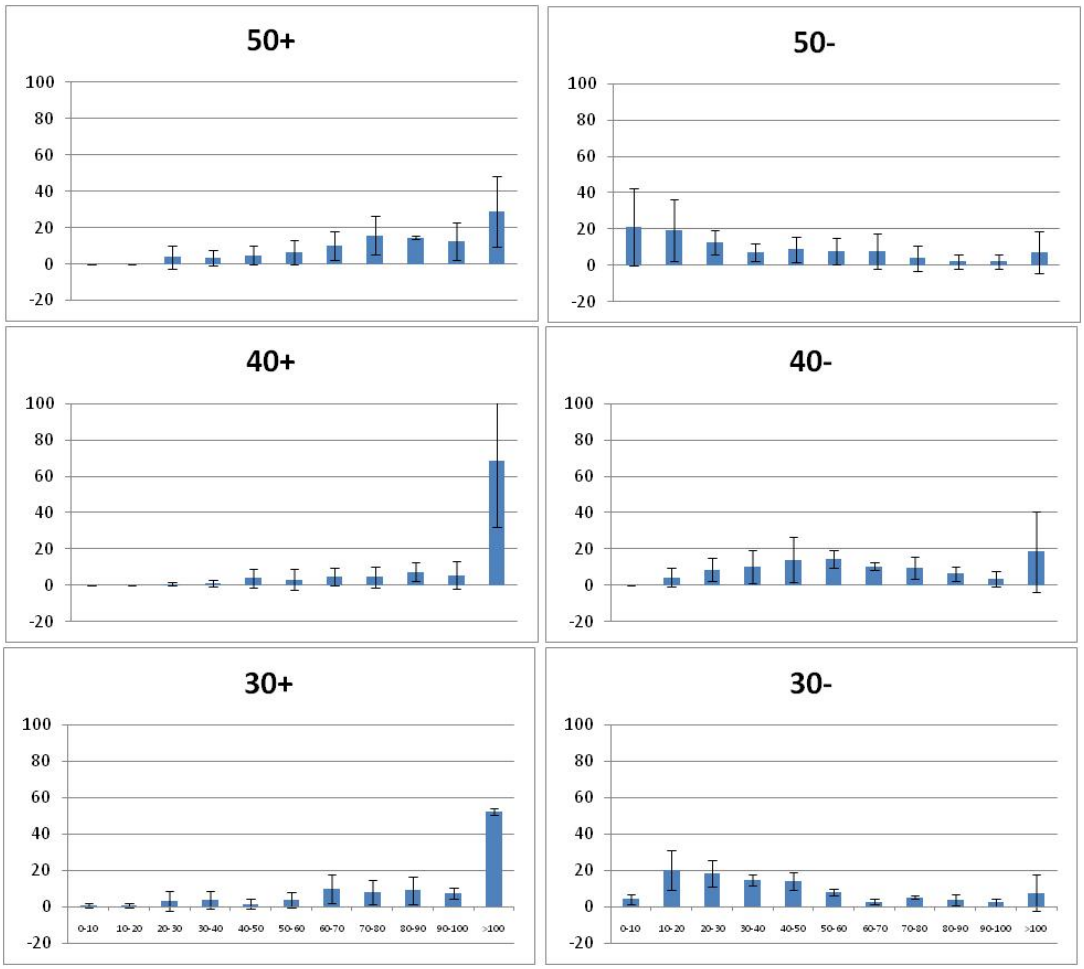

***Figure 8**. Deep muscle fibers. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) at 59 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

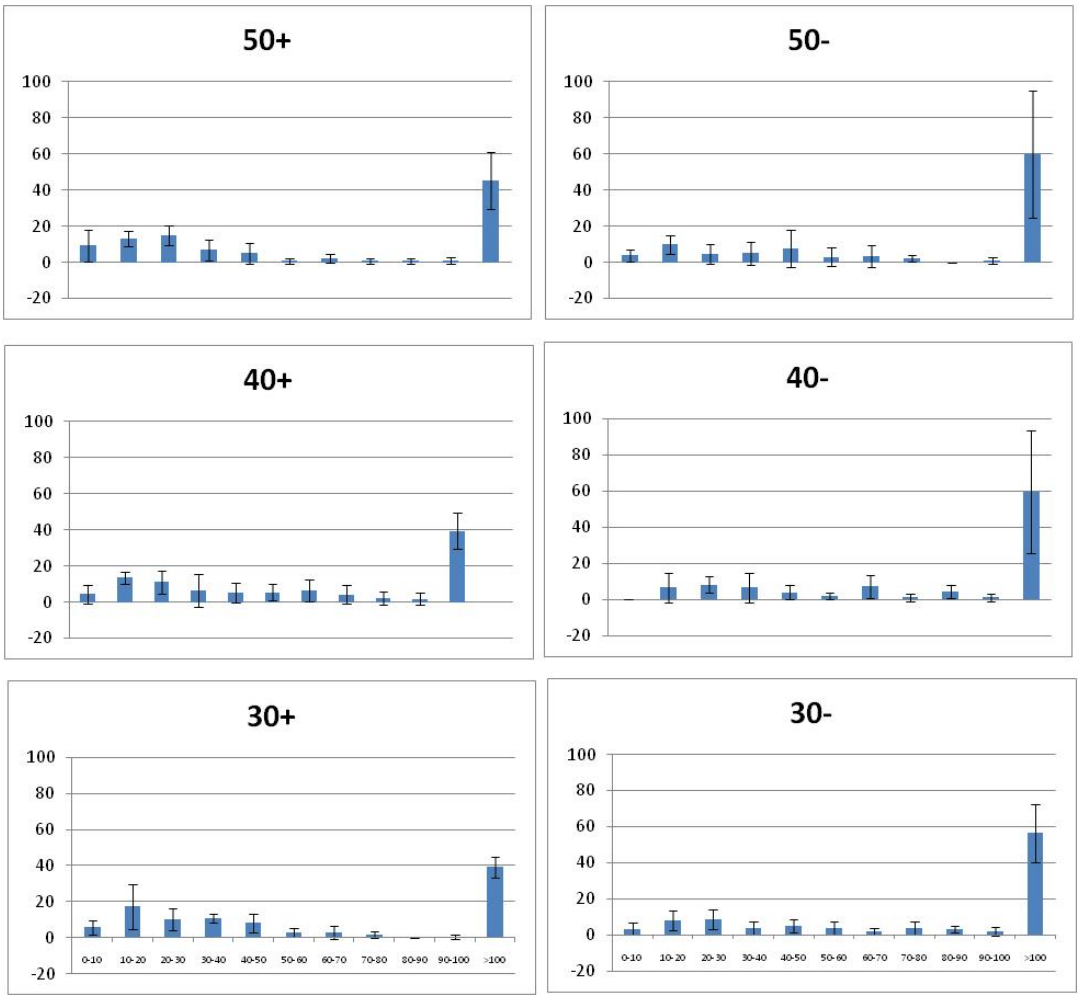

Figure 9. *Deep muscle fibers. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) at 90 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

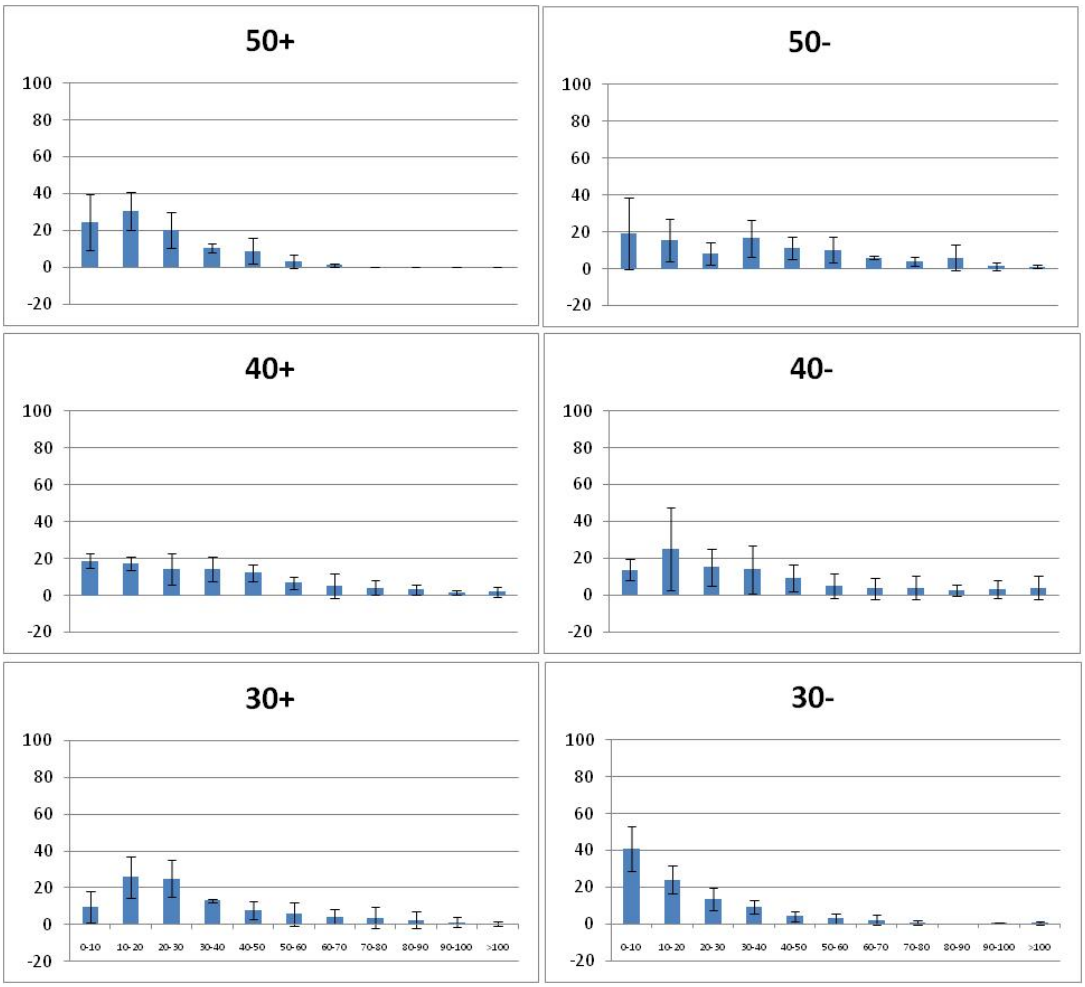

Figure 10. *Lateral fibers. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) at 59 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups, group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

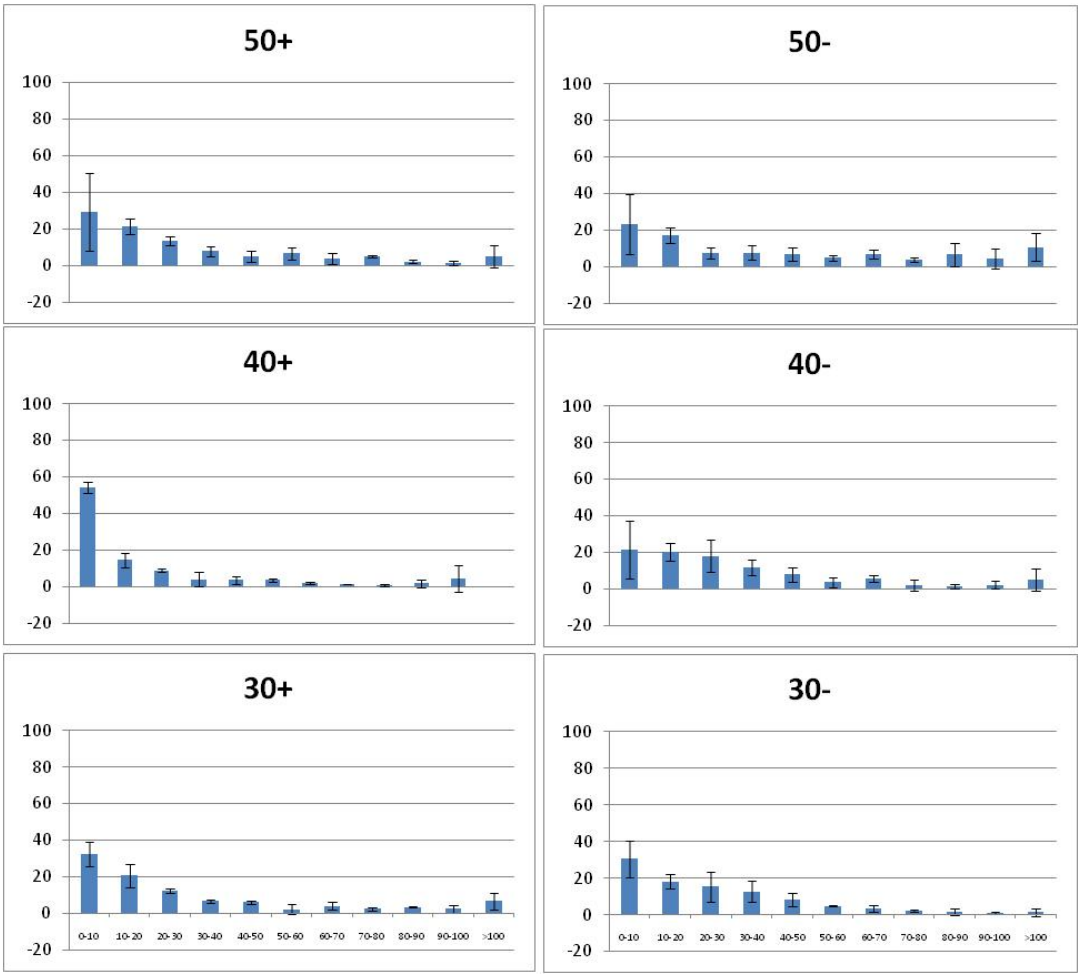

Figure 11. *Lateral fibers. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) at 90 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

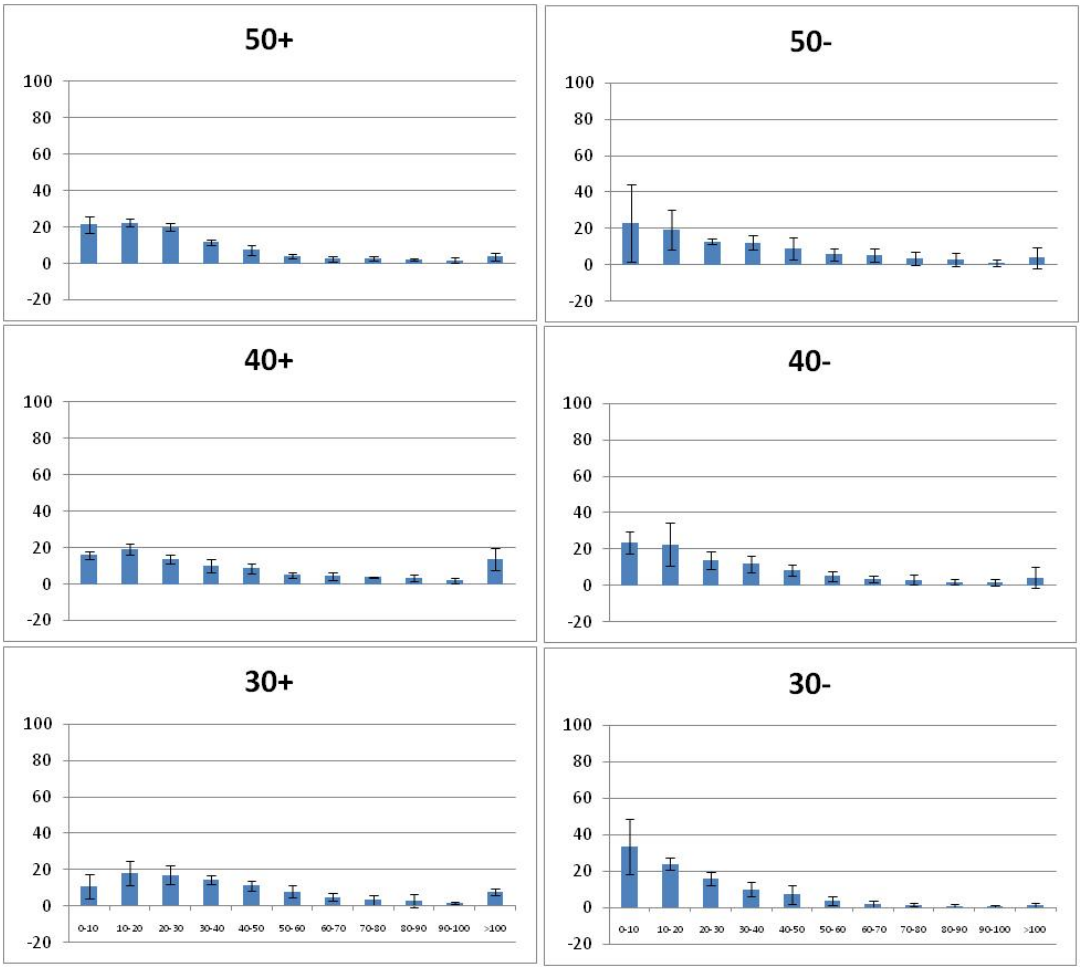

***Figure 12**. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) within the epaxial quadrant (see Fig. 2) of larvae (at 59 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups, group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

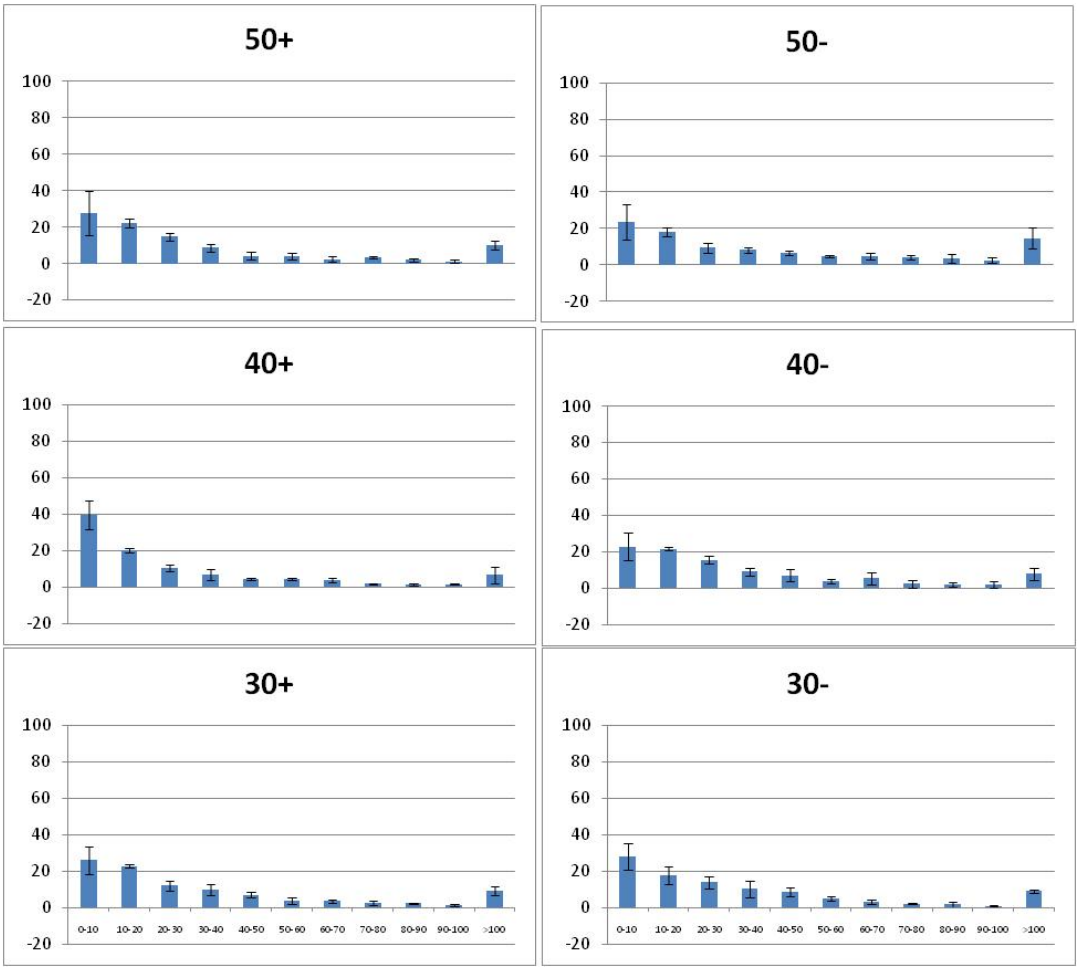

***Figure 13**. The ratio (Y-axis) of myofibers of various size groups (area) (X-axis) within the epaxial quadrant (see Fig. 2) of larvae (at 90 days post hatch. The analysis was carried out on three larvae from two size groups (+ and -) within each of the three experimental groups, group A weaning by 50 dph, group B weaning by 40 dph and group C weaning by 30 dph.*

3.2. Live prey enrichment using putative probionts

3.2.1. *In vitro* growth inhibition of selected pathogens

Previous results indicate that the rotifer cultures are not negatively affected by the bacterial treatment and treatment using freeze-dried preparations of the two probiotic strains even resulted in improved harvesting of the live feed (Jóhannsdóttir *et al.*, 2009). The inhibitory activity was best determined using the paper disc or agar spot method. The probiotic bacterial isolate 04-279, (*Arthrobacter bergerei*) showed inhibitory activity towards all three of the four pathogens included in the study (14-16 mm diameter clear zone), with no inhibitory activity observed against *Vibrio salmonicida*. Freeze drying of the bacteria did not affect the inhibitory activity of this isolate. Similar inhibitory activity was observed for freeze dried preparation of 04-394 (*Enterococcus thailandicus*) and increased growth inhibition activity may have been observed for fresh cultures of this isolate. Fresh cultures of *Enterococcus thailandicus* furthermore showed inhibitory activity against *V. salmonicida*

while freeze dried preparation showed no inhibitory activity against this pathogen. Similar inhibitory activity was observed in undiluted and diluted cultures of the probiotic isolates. *To conclude, the results indicate inhibitory activity of both probiotic isolates towards the selected fish pathogens. Freeze drying did not affect the inhibitory activity of* Arthrobacter bergerei *against the fish pathogens tested, but reduced inhibitory activity of* Enterococcus thailandicus *against some of the pathogens tested was observed* (Matis-experimental report).

3.2.2. Larval performance

Two separate experiments were carried out analysing the effects of probiotic treatment on larval survival, growth and development. During experiment I the water flow decreased after 9 days, resulting in increased water temperatures (up to 9-10°C) and elevated larval mortalities. Subsequently the experiment was terminated.

In experiment II, larval survival at 40 dph was estimated about 6% in both groups. Whereas no grading was performed, the poor survival rate may be at least partly explained by cannibalism. Survival from larvae to juvenile was only 0.9% and 0.5% in control and treated group, respectively, which is extremely poor in comparison with the 15-20% average survival at the MRI hatchery (Steinarsson, 2004). Poor egg quality has been correlated with low survival rates and it has been observed that fish larvae hatching from poor quality egg groups show lower viability and a lower degree of normal development up to the juvenile stage, as compared to larvae that hatch from good quality egg groups (Kjørsvik *et al.*, 2003). Larval growth was generally good, with a mean wet weight of 75.5 g and 77.7 g measured in the control and bacteria treated group, respectively, at the end of the hatchery stage (49 dph). Head deformities and ratio of discarded larvae was relatively high in both groups (Fig. 14). The ratio of deformed larvae was higher in the bacteria-treated as compared with the untreated group, but the types of deformities observed were similar for both groups.

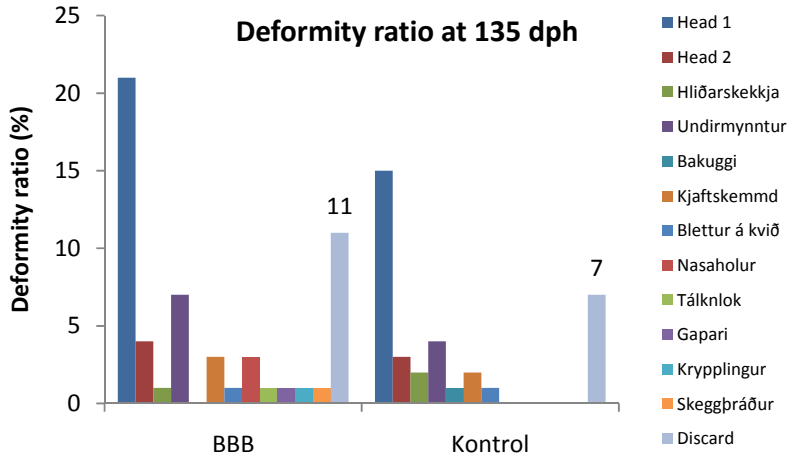

Figure 14. *Larval deformities at 135 dph. Shown is the ratio of deformed larvae fed bacteria-enriched (BBB) and untreated (kontrol) live prey.*

3.2.3. Cultivable bacteria

A total of 22 samples of rotifers, 1 sample of eggs and 35 samples of larvae and their culture water have been analysed. Bacterial treatment of rotifers resulted in higher numbers of CFU observed in samples of treated compared to untreated rotifers. A sudden increase in bacterial numbers of surface sterilized larvae was observed during the first week in feeding, with 3-6 log-unit increase observed in total viable counts, 1-2 log units increase in the numbers of lactic acid bacteria and 3-4 log units increase in the numbers of presumptive *Vibrio* bacteria. *Overall, the results indicate that probiotic treatment during hatching and offering probiotic-treated live feed during the first days in feeding, resulted in increased numbers of cultivable bacteria in the gut of larvae, with 1-3 log unit higher numbers observed in treated as compared to untreated larvae.* The lactic acid bacterial isolate used for treatment was found to be successfully recovered using a selective media for isolation (NAP). The sudden and decisive increase in bacterial numbers observed during the first week in feeding opens for the option to and emphasizes the importance of controlling the bacterial environment of larvae during the first week following hatching.

3.2.4. Bacterial community structure

DNA was extracted from a total of 93 samples of larvae, their life prey and their rearing water. The samples were collected at various time points throughout the experiments.

Live prey:
Results from the DGGE analysis indicate the dominance of 4-5 bacterial groups. Very little variability was observed between the different samples analysed. DNA sequencing of the groups revealed the overall dominance of *Vibrio* sp. *The results furthermore indicate that UV radiation of the culture water did not eliminate the bacterial groups observed nor did it affect the bacterial composition of the live prey.*
Grazing on freeze dried preparations of the probionts resulted in changes in the bacterial community structure of the rotifers. Both probionts were observed only in samples of treated rotifers and *Arthrobacter bergerei* was still detected following rinsing of the rotifers. Both probionts were furthermore observed in the rearing water of treated rotifers.

Larval treatment - Experiment I: Analysis of the bacterial community using DGGE resulted in the identification of both probionts at 3 dph, following bathing treatment using a mixture of the isolates. However, neither probiont was detected in treated larvae or their rearing water at 7 dph, i.e. a few days following treatment.

Larval treatment – Experiment II: The effect of bacterial treatment on the bacterial community of larvae and their rearing water were studied at various time points. The main results are the following:

- *Enterococcus thailandicus*, was identified in treated as well as untreated larvae at 2 dph, as well as in the rearing water from treated larvae (Fig. 15). This isolate was detected in both treatment groups until 36 dph, indicating that the strain belongs to the "normal" bacterial community of cod larvae at early developmental stages.
- *Arthrobacter bergerei* was only identified in treated larvae in samples collected 21 dph and 26 dph, and in no samples of control larvae (Fig. 16).

- Prior to offering live prey to larvae at 2 dph, the bacterial community of larvae consisted of 5-7 groups. The bacterial community structure changed after the onset of exogenous feeding, but continued to be dominated by 5-7 bacterial groups throughout the sampling period (7-36 dph). Higher diversity of bacterial groups was observed in the control group and *Arthrobacter bergerei* was only observed in treated larvae.

Overall, treatments using the putative probionts did not affect the bacterial community structure of larvae and the putative probionts were not found to establish within the bacterial community of treated larvae.

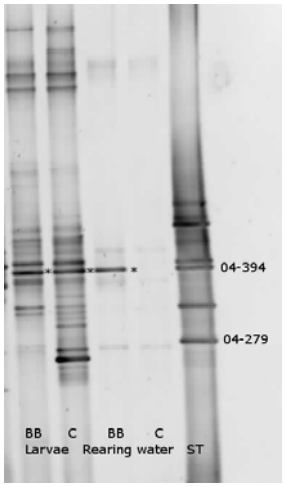

Figure 15. *DGGE profiles of surfice sterilized larvae and rearing water 2days post hatch. Shown are profiles of probiotic treated larvae and the rearing water (BB) and respective controls (C). Asterisk indicate bands excised for sequencing and subsequently identified as 04-394. Included in the relative mobility standard (ST) are both probiotic strains (04-394 and 04-279).*

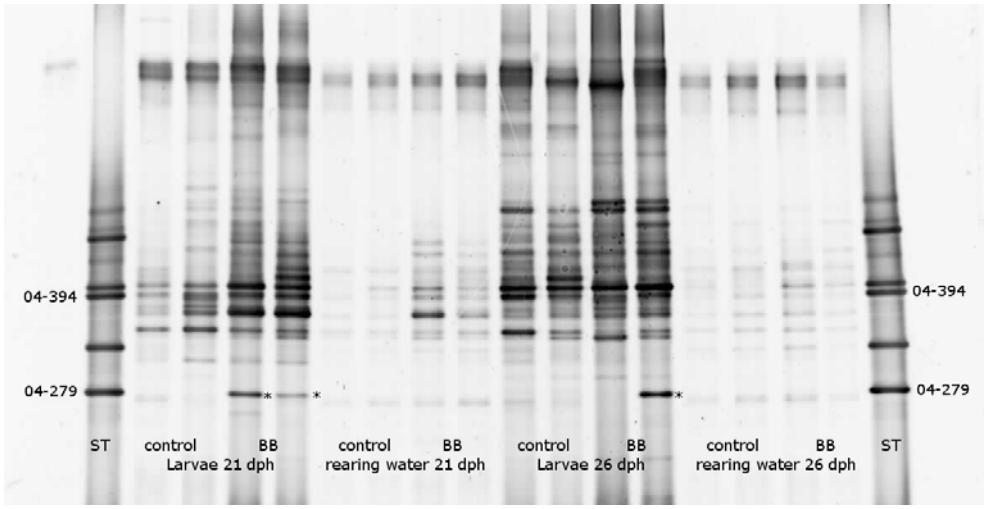

Figure 16. *DGGE profiles of surface sterilized larvae and rearing water 21 and 26 days post hatch (dph). Shown are profiles of probiotic treated larvae and the rearing water (BB) and respective controls (control). Asterisk indicates bands excised for sequencing and subsequently identified as 04-279. Included in the relative mobility standard (ST) are both probiotic strains (04-394 and 04-279).*

3.2.5. Expression of selected immunological genes

The expression of selected immune related genes (IgM, Lysozyme and hepsidin) was analysed in a parallel project ("Relative expression of selected immune related genes in larvae of Atlantic cod (*Gadus morhua* L.)" MSc Thesis Þórarinsdóttir 2010).

Normalised expression levels of membrane bound IgM (mIgM) revealed no significant difference between the treatment group's expression levels, neither prior to the onset of exogenous feeding of larvae at 2 dph nor at 7 dph (approximately four days after the onset of exogenous feeding). A significant difference in expression levels was, however, observed between the untreated control and probiotic treated larvae at the onset of metamorphosis at 26 dph (p= 0.028) as well as during metamorphosis at 36 dph (p=0.009) (Fig.17). A significant increase (p= 0.035) in the expression levels of mIgM was observed in treated larvae between 7 and 26 dph, while the expression levels in untreated larvae were at approximately the same level as at 2 and 7 dph.

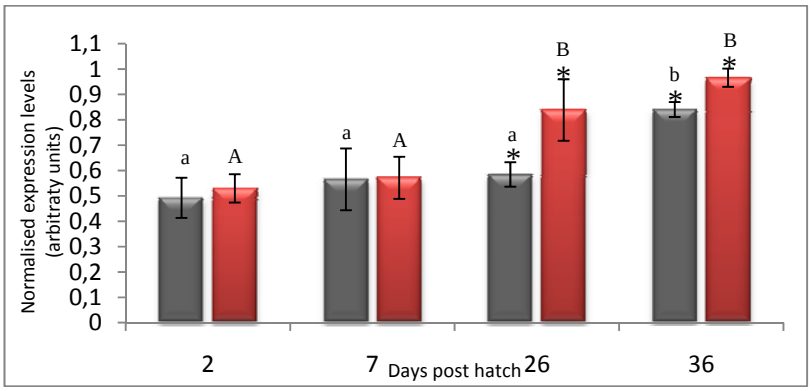

Figure 17. *Levels of mIgM on selected days post hatch. Grey columns represent samples of control larvae and red columns probiotic treated larvae. The expression levels were normalised to RPL4 and EF1-α and calibrated with respect to the sample showing the highest expression. Bars marked with an asterisk (*) indicate statistical difference (p<0.05) between the groups on that particular day post hatch. Bars marked with "ab" indicate statistical difference (p<0.05) between sampling days in the control group and "AB" between sampling days in the probiotic group.*

Similar expression levels of g-type lysozyme were observed in larvae from the two groups prior to the onset of exogenous feeding at 2 dph (p = 0.698). A significant increase in the expression levels occurred between 7 and 26 dph, corresponding to early metamorphosis, but no significant differences in the expression of g-type lysozyme were observed between the two groups at any sampling date (Fig. 18) (Þórarinsdóttir, 2010).

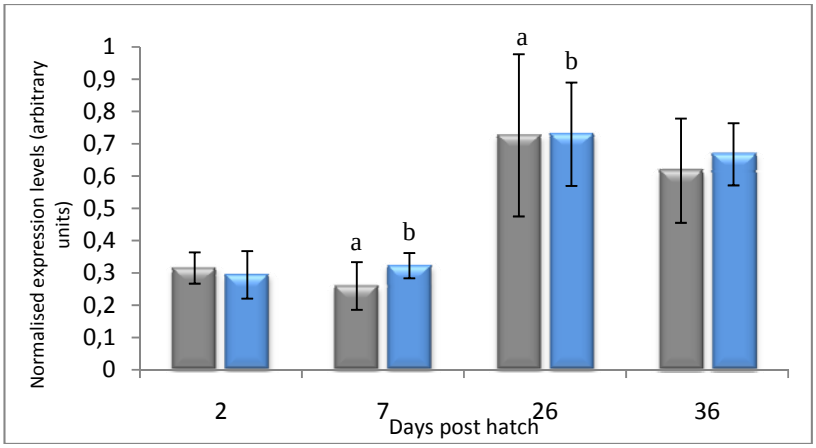

Figure 18. *Expression levels of g-type lysozyme on selected days post hatch. Grey columns represent samples of control larvae and blue columns probiotic treated larvae. The expression levels were normalised to RPL4 and EF1-α and calibrated with respect to the sample showing the highest expression. Bars marked with "a" indicate statistical difference (p<0.05) between sampling days in the control group and "b" between sampling days in the probiotic group.*

No significant difference was detected in the normalised expression levels of hepcidin between the groups at any sampling date, nor between sampling days (p>0.05). The highest expression levels observed in both groups were at 7 dph, but decreased expression was observed in both groups at the onset of and during metamorphosis at 26 and 36 dph. Results from the expression studies are further described in Thorarinsdottir (2010).

3.3. Live prey enrichment using a fish protein hydrolysate

3.3.1. Larval performance

The overall survival from larvae to juvenile was relatively low, but feeding the peptide enriched live prey to cod larvae positively affected larval survival at 48 dph, with 7.0% and 4.3% survival observed in treated and untreated group, respectively (Table 2). Larval growth was generally successful, with a mean wet weight of larvae at 48 dph measured 78.1g and 71.3g in the treated and untreated group, respectively. A general observation at the hatchery indicates that a lower mean weight of larvae reflects improved larval survival, with a mean weight of 10-50 mg generally observed at 46 dph.

Table 2. *Survival (%) and weight of larvae (mg wet weight) at 48 and 160 dph. Shown is the mean weight ±S.D. of ~20 larvae from each tank (~80 larvae treatment^{-1}).*

	48 dph				160 dph	
	Survival (%)		Mean weight (mg)		Survival (%)	Mean weight (mg)
Treated	5,9 14,2 3,7 4,4	**7,1 ± 4,9**	81,2 37,2 110,6 83,4	78,1 ± 30,4	36	33,9 ± 10,4
Control	2,9 5,7 4,0 4,7	4,3 ± 1,2	66,4 67,2 97,2 54,2	71,3 ± 18,3	35	35,6 ± 10,1

Offering peptide enriched live prey to larvae resulted in significantly improved larval development (Fig. 19).

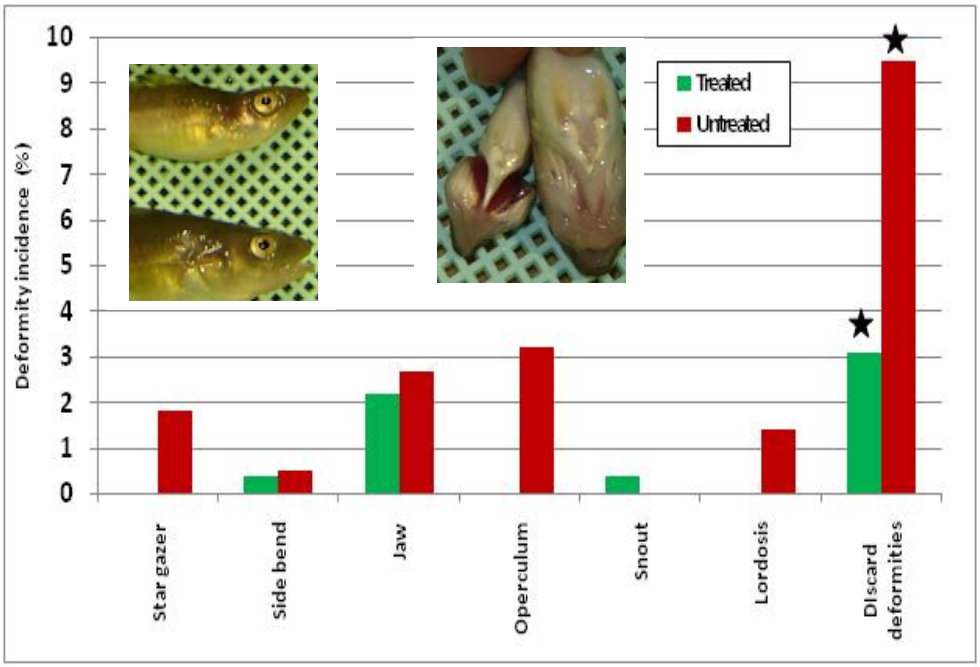

Figure 19. *Incidence (%) of deformities observed at 160 dph (225 treated and 222 untreated larvae). Asterisks indicate the ratio of larvae discarded due to severe deformities in each treatment group.*

3.3.2. Distribution and intensities of immunological products

Larval samples collected at 46, 55 and 80 dph were analysed for the presence and distribution of IgM and lysozyme using specific antibodies and immunohistochemistry. The overall results show significantly improved larval development. Highly variable development was furthermore observed within the control group as compared with the treated group, where some of the larvae analysed had well distinguished organ structure while others were poorly developed at early developmental stages.

The main results furthermore include:

- ✓ detection of IgM in larvae already at 28dph (14-17mm larvae), mainly in the foregut and the epithelial lining of the digestive tract as well as in the epidermal mucus of the skin. IgM and lysozyme were more widespread and detected in higher intensities in the gut wall of treated as compared to control larvae at this early stage of the development.
- ✓ detection of lysozyme and IgM in high intensities in the gut wall and skin mucus of treated larvae at 46dph, while high variability in the intensity and distribution of IgM and lysozyme was observed amongst individuals in the untreated group.

Overall, the results indicate that early treatment using peptide-enriched live prey may promote growth and normal development during early developmental stages of cod larvae.

4. DISCUSSION & CONCLUSIONS

4.1. The influence of startfeeding protocols on muscle growth

The survival from larva to juvenile (6-15%) was generally disappointing, compared to the routine survival of 15-20% in the MRI hatchery (Steinarsson, 2004). The average survival in Icecod's own hatchery was only 5% at the same time, which may point to poor egg quality as a likely cause. The growth rates in groups A and B were however excellent and average dry weight around 50 dph was 3-4 times greater than is common in most commercial cod hatcheries (Steinarsson, 2004). Higher variability in the number and size of miofibres was observed in juveniles from the smaller as compared to the larger size groups of juveniles from all experimental groups. The insignificant differences between groups A and B support previous observations that late weaning around 50 dph may be excessive and produce no significant advantage. An intermediate weaning strategy, with artemia feeding until 40 dph (group B), appears to be sufficient to convey important advantages in terms of growth and anatomy. Early weaning on 30 dph produced slow-growing juveniles, with greater size variation and a higher deformity ratio. No increase in the number of myofibres was observed in larvae from group C at 59dph, indicating a lack of growth at that time point. Increased numbers of muscle fibres was, however, observed at 90 dph, indicating that compensatory growth may have occurred in this group as a result of slow early growth rate. Even with a 20-30% dismissal due to deformity and/or slow growth, the early weaned juveniles will be out-performed by the later-weaned juveniles.
The results suggest that the early growth advantage established during the pre-juvenile stage will manifest itself in the post-juveniles, leading to a significant difference in long-term growth potential. Continued monitoring of tagged individuals from all weaning groups will gradually cast a greater light on this important matter.

4.2. Probiotic bacteria

Present study showed that freeze drying of the bacterial strains did not affect the inhibitory activity and the use of dried pellets is believed to be the most practical method for farm stations compared to applying fresh bacterial suspensions. Previous results indicate that the rotifer cultures are not negatively affected by the bacterial treatment and treatment using freeze-dried preparations of the two probiotic strains even resulted in improved harvesting of the live feed (Jóhannsdóttir *et al.*, 2009). Freeze-dried preparations of bacteria have been considered an efficient mode of delivery of probiotic bacteria in aquaculture (Panigrahi *et al.*, 2005). The present results, however, suggest that the use of fresh bacterial suspensions represent a better form for delivery of the respective bacterial isolates compared to the freeze-dried form as Lauzon *et al.,* (2010) observed increased larval survival in treated groups. The present study indicated that using the freeze dried putative probionts did not affect the bacterial community structure of larvae and the putative probionts were not found to establish within the bacterial community of treated larvae. In the present study survival from larvae to juvenile was very low compared with the average survival at the MRI

hatchery and deformities were relatively high in both treatment groups. This could be caused by poor egg quality, that has been correlated with low survival rates and it has been observed that fish larvae hatching from poor quality egg groups show lower viability and a lower degree of normal development up to the juvenile stage, as compared to larvae that hatch from good quality egg groups (Kjørsvik *et al.*, 2003). Poor larval survival and quality may therefore partly explain the lack of effects as a result of treatment.
A significant increase in expression levels of IgM was, however, observed at the onset and during metamorphosis as a result of the probiotic treatment using freeze dried preparations of the bacterial isolates. Probiotic treatment may furthermore have accelerated the expression of mIgM, indicating the proliferation of B cells in the larvae, as has been suggested by (Nayak & Mengi, 2010) and references therein. Overall, the results on the expression profile of both mIgM and lysozyme are in agreement with previous studies by (Seppola *et al.*, 2009).

4.3. Bioactive products

Previous studies have indicated better results from feeding larvae with peptide enriched live prey compared with adding the products to the culture environment of larvae. The overall survival was however relatively low in the present study showing positive affects on larval survival and quality when feeding with enriched live prey. The present study confirms the results from a previous experiment when feeding the peptide hydrolysate to cod larvae resulted in significantly improved larval development already at 28 dph and a lower ratio of larvae developing curved notochord and other deformities observed in treated larvae at transfer to weaning at 160 dph. The results are furthermore, in agreement with previous studies demonstrating various bioactivity in proteins and peptides isolated from fish (Kristinsson & Rasco, 2000). Small peptides may furthermore provide nutritional benefits in addition to improved bacterial resistance and immunostimulating effects observed in fish larvae in this and other studies (Hákonardóttir & Hrólfsdóttir, 2008; Olafsen, 2001; Pedersen *et al.*, 2004).

5. ACKNOWLEDGEMENTS

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