

Heilbrigður þorskur
Greining á náttúrulegum varnarefnum í þorski

Lokaskýrsla til AVS vegna smáverkefnis

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Verkfræði- og náttúruvísindasvið

Líf- og umhverfisvísindadeild

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Title:

Íslensku: Heilbrigður þorskur – Greining á náttúrulegum varnarefnum í þorski

Ensku: Healthy cod – Characterisation of endogenous antibiotics in cod.

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Skýrsluágríp:

Cathelicidin eru bakteríudrepanði peptíð sem gegna lykilhlutverk í ónæmiskerfi spendýra. Cathelicidins hafa fundist í nokkrum tegundum fiska, en lítið er vitað um hlutverk þeirra þar. Í þessu verkefni var tjáning cathelicidins athugað með raun-tíma PCR. Sýkingar þorsks með bakteríunni *Aeromonas salmonicida* syndi aukna tjáningu cathelicidins í öllum vefum. Þessar niðurstöður benda til að peptíðin gegni mikilvægu hlutverki hjá þorski sem vörn á móti sjúkdómum. Við athuguðum einnig tjáningu cathelicidins í þorskun þorsks. Cathelicidin magn var breytilegt fyrstu 68 daga eftir klak. Tvær hópar af lirlfum sem gefið var mismunandi fóður voru borin saman. Cathelicidin tjáning var mismunandi milli hópanna og var meðal annars stjórnað af fóðurtegund. Miklar breytingar á cathelicidin magni í lirlfum gefa til kynna að meðfædda ónæmiskerfi þorsksins er virkt snemma í þorskun fisksins og svara örvun með aukinni tjáningu á bakteríudrepanði peptíðum.

Abstract:

Cathelicidin antimicrobial peptides are multifunctional peptides, important in the innate immune system of mammals. Cathelicidins have been identified in several fish species, but little is known about their function in fish. In this study we have examined cathelicidin expression in Atlantic cod using real-time PCR. Infection of the fish with the pathogenic bacteria *Aeromonas salmonicida* increased cathelicidin expression in all tissues. This suggest a role of cathelicidin in the defence system of Atlantic cod. We also examined the expression of cathelicidin during cod larvae development. Cathelicidin expression varied in the course of the first 68 days post hatching of the larvae. Two groups of larvae with a different food regime were compared. Cathelicidin expression was found to differ between the two groups and this was partly linked to the food input of the larvae. The rapid changes of cathelicidin expression in the larvae indicate that the innate immune system of cod is active from early on

in development and can respond to external stimuli by the production of antimicrobial peptides.

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1. INTRODUCTION

Antimicrobial peptides are confirmed as an important defence mechanism in the innate immune system of mammals (Zasloff, 2002). These peptides are found at the epithelia and form a defence front against bacteria (Lai and Gallo, 2009). In fish little is known about these peptides. The adaptive immune system, which in mammals forms an important pillar in the defence against pathogens, is less well developed in fish (Whyte, 2007). It has a limited antibody repertoire and is slow and temperature dependent and it is generally accepted that fish rely heavily on their innate immune system for defence against pathogens (Magnadottir, 2006). One group of antimicrobial peptides present in mammals as well as birds (Lynn et al., 2004), reptiles (Zhao et al., 2008) and fish (Chang et al., 2005; Chang et al., 2006; Maier et al., 2008), are the cathelicidins. We have identified seven novel cathelicidins in different fish species, one of them being Atlantic cod (*Gadus morhua*) (Maier et al., 2008) (supported by AVS grant S 002-7). Cod cathelicidins form a novel group of antimicrobial peptides rich in the amino acids arginine, glycine and serine and are very cationic suggesting a strong antibacterial activity of the mature peptides.

In this study we have compared the expression of cod cathelicidin in healthy and infected fish and found that bacterial infection increase cathelicidin expression significantly. Further we have examined cathelicidin transcription in cod larvae raised with different feeding regimes. Our data supports that cathelicidin has a role in the immune system of Atlantic cod and is already active during the development of cod.

These results could have implications for aquaculture as it has been shown that the early larval stages are very susceptible to infections with around 20% survival during larval development. Understanding therefore the role of defence molecules such as the antimicrobial peptide cathelicidin could provide methods to strengthen the immune system of fish in aquaculture and forms an applied angle to these studies.

2. MATERIAL & METHODS

2.1 Collection of cod material

For the infection study ten juvenile cod were injected intra peritoneal (i.p.) with 10^6 CFU of *Aeromonas salmonicida* ssp. *achromogenes* (Asa) and as a control further 10 fish were injected with PBS as described earlier (Maier et al., 2008). Twenty four hours after infection the fish were dissected, samples were taken from various organs and immediately snap frozen in liquid nitrogen.

Larval rearing trials were conducted at the Marine Research Institute's Experimental Station, Stadur, Grindavik, Iceland, in the spring of 2009. Two groups of larvae (groups 1 and 2) in commercial hatchery tanks were reared on different start-feeding protocols and monitored from the egg stage, through the larval stage and well into the pre-juvenile stage. Rearing conditions were identical for both groups. Eggs were incubated at 7°C and sterilized with Pyceze (Novartis Animal Vaccines Ltd.) at -8 and -2 days pre-hatch (100 ppm for 30 minutes). Upon completed hatching, two hatchery tanks (3200 Litre) were stocked with 170.000 larvae each. Antibiotics (25 ppm Lincospectin, Pfizer Ltd.) were routinely administered on 1, 4 and 7 days post hatch (dph). Lights were provided at 3 dph (150 lux at surface) and increased incrementally to 700 lux from 10 dph onwards. Photoperiod was initially fixed at a 18:6 ratio (L:D hours) but adjusted to 24:0 from 22 dph onwards. Rearing temperatures were held at 8°C until 7 dph and gradually increased to 12-13°C from 34 dph onwards. Water flow was provided at 3 dph (3.5 L/min) and increased incrementally to 13 L/min from 45 dph onwards. Concentrated *Nannochloropsis* algae (Instant algae, Reed Mariculture) were added for shading twice daily (60-80 ml per day).

The group 1 larvae were fed enriched (Algamac 3000, Aquafauna Bio-Marine) rotifers (*Brachionus plicatilis*) from 3-30 dph (26-75 millions per day), *Artemia* (*Artemia salina*) nauplii and metanauplii from 16-50 dph (0.5-60 millions per day) and dry feed (Gemma Micro Diamond, Skretting) from 20 dph onwards. The group 2 larvae received the same rotifer protocol but were not fed any *Artemia* and dry feeding commenced earlier (on 14 dph), as seen in Figure 6 B.

Larvae were collected directly into RNAlater (Ambion) at the indicated days. Larvae counts were different at various sampling timepoints, with 50 larvae for the first days post

hatching (dph), around 10 - 20 larvae until day 40 and thereafter 2-4 pre-juveniles for each timepoint.

2.2. RNA isolation, cDNA synthesis and quantitative real-time PCR

Atlantic cod larvae were homogenized with a Pellet Pestle Cordless Motor (Kimble-Kontes) and RNA was extracted using TRI reagent (Ambion). For the infection studies approximately 50 mg of frozen tissue was ground in a pestle and mortar and total RNA was extracted using TRI reagent. RNA was prepared using manufacturer's instructions and dissolved in 30 µL RNase-free water. To minimize contamination with DNA ten micrograms of RNA were digested with DNase (New England Biolabs) followed by ethanol precipitation and the resulting RNA pellet was dissolved in 20 µL RNase-free water. Quantity and quality of resulting RNA was assessed using NanoDrop ND-1000 UV/Vis-Spectrophotometer (NanoDrop Technologies) and the integrity of the RNA from half the samples was evaluated via agarose gel electrophoresis.

cDNA was prepared from 300 ng of RNA using the First Strand cDNA synthesis kit (Fermentas), according to manufacturer's protocol. The absence of genomic DNA was confirmed by preparing several samples without reverse transcriptase in the reaction (called – RT). cDNA was diluted 10 fold (infection study) and 100-fold (larvae) in water for further use in the quantitative real-time PCR.

Real-time PCR was performed on 96well-PCR plates on a ABI 7500 real-time PCR System (Applied Biosystems) using Power SYBR green PCR Master Mix as recommended by the manufacturer (Applied Biosystems) with the exception of using 10 µl final reaction volume. Reactions were run in duplicates. Real-time primers for cathelicidin were designed in PerlPrimer (Marshall, 2004) and the sequence for the forward primer used is:

5'GGTTGAAACTGTCTATCCAGAGG 3' and reverse primer

5'AACTCTTGTGCAGGGAATGTC 3'. The relative expression of cathelicidins was

measured against one or two reference genes, Ubiquitin and ribosomal protein S 9 (RPS 9) as these had to be shown to be stable in both adult cod (Olsvik et al., 2008) as well as larvae (Saele et al., 2009) and the primers were taken from these studies. Efficiencies for all primers and tissues were calculated and shown to lie within the 90 – 110 % required in order to use the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) for the calculation of the fold differences in

expression. For the infection study skin from healthy fish were set as value 1 and all other tissues were compared to this, one representative experiment is shown. In the larvae analysis ubiquitin was used as reference gene and the hatching day (0 day post hatching) was set as value 1 and the later measurements were compared to this. Statistical differences between tissues and treatments were assessed from three fish for each condition, with a one-tailed t-test unequal variance. Values below 0.05 were considered significant.

3. RESULTS

3.1. Expression of Atlantic cod cathelicidin

Cathelicidin expression was examined in seven organs using real time PCR. Injection of cod with *A. salmonicida* ssp. *achromogenes* and incubation of the fish for 24 h, increased cathelicidin expression in all tissues in comparison to fish injected with PBS alone (Fig. 1, due to the high individual variation a representative experiment is shown). For the statistical analysis three healthy fish and three infected fish were compared. A one-tailed t-test with unequal variance showed a statistically significant increase in expression in all tissues ($p < 0.05$), except gills, when comparing infected to healthy fish. Constitutive expression was found to be most pronounced in kidney and spleen. When compared to all other tissues the high basal expression of cathelicidin in kidney or spleen was found to be statistically significant ($p < 0.01$ for kidney and $p < 0.05$ for spleen). Due to the relative high expression of cathelicidin in uninfected kidney and due to the availability and size of the organ, this tissue was used in the purification of the mature cathelicidin peptide – not described here.

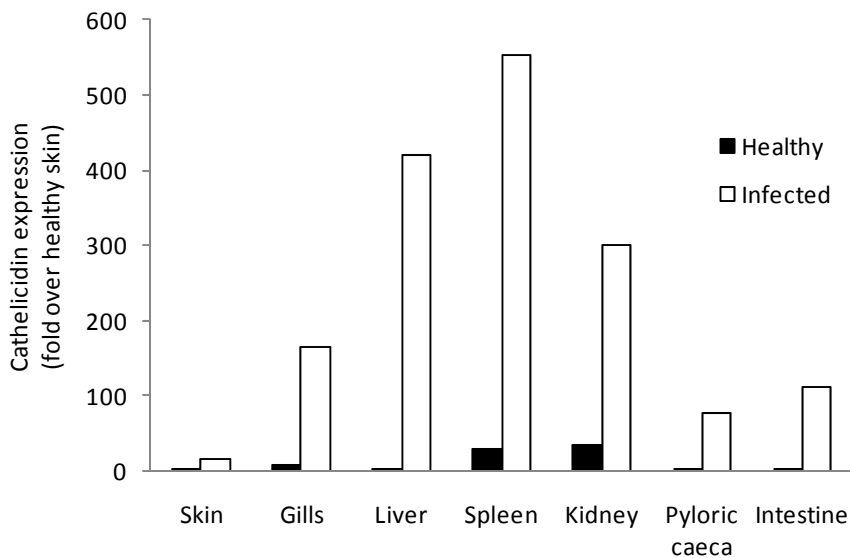
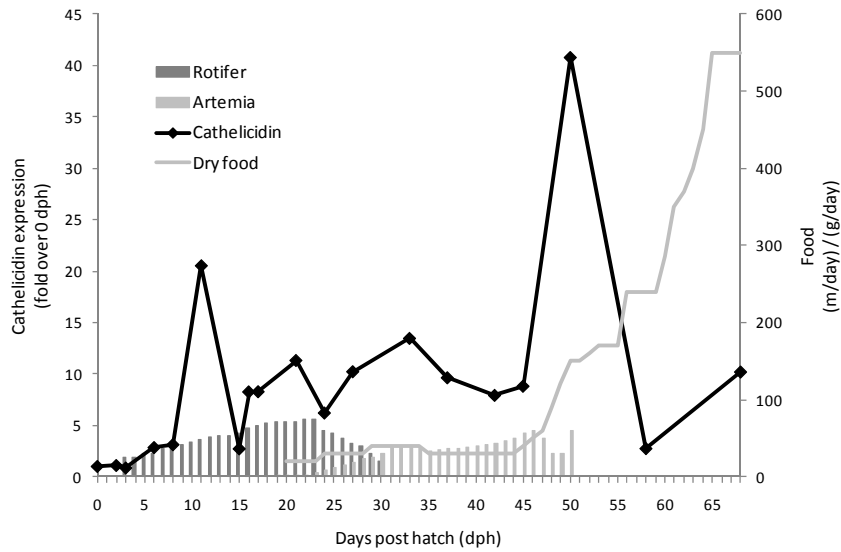


Figure 1 Cathelicidin expression is increased during infection. Cod were injected with PBS (Healthy) or *Aeromonas salmonicidae* ssp. *achromogenes* (Infected) and incubated for 24 hours. Tissues were sampled as indicated. Relative cathelicidin expression was analysed using quantitative real time PCR and the fold induction was compared to healthy skin. Ubiquitin and RPS9 were used as internal controls and a representative experiment is shown.

3.2. Expression profile of cathelicidin during cod larvae development

In order to study cathelicidin expression during Atlantic cod development, cod eggs were examined and cathelicidin mRNA was found in both immature and mature eggs (data not shown). Subsequently cathelicidin expression was followed using quantitative real-time PCR for 68 days post hatching (dph) of the larvae. Cathelicidin mRNA was found to be present at low levels in newly hatched larvae and its expression increased and decreased over the 68 days examined (Figure 2). Previous results from a pilot study on larvae raised in 2008 had indicated that the increase in cathelicidin expression was partly linked to the food of the larvae (data not shown). We therefore compared in the present study the effect of food on cathelicidin expression by following an experiment where one group of larvae was fed on mainly rotifers (small zooplankton), brine shrimp (*Artemia*) and finally dry feed (group 1), while the other group (group 2) did not get any *Artemia*. The length growth rate for the larvae following the normal regime was found to be around 3.5% per day, with a mean length of pre-juveniles at 49 dph of 26.3 mm. The larvae fed without *Artemia* (group 2) also started off with a length growth rate of 3.5% but following premature dry feed weaning the rate decreased to only 2.5% per day and the mean length of the pre-juveniles at 49 dph was only 15.6 mm. Similarly the survival of the larvae in group 1 was 14% (50 days post hatching (dph)) while the survival for group 2 was 6% (55 dph). Due to lower survival in group 2 than in group 1 (10.000 versus 23.000 pre-juveniles, respectively) the amount of dry feed given to group 2 was lower than given to group 1 (Fig 2). Comparison of the cathelicidin expression between these two groups showed a different expression profile. Both groups had an about 15-20 fold increase of cathelicidin expression around day 11 post hatch (Fig. 2) and this increase was similar in the 2008 study (data not shown). Larvae raised on the normal food regime had about 40-fold increase in cathelicidin expression at day 50, while a similar increase was seen in group 2 at day 37 post hatch. This increase in cathelicidin expression coincided with dry feed weaning in both groups i.e. with the end of feeding with live feed and sole dependency on dry feed.

Group 1



Group 2

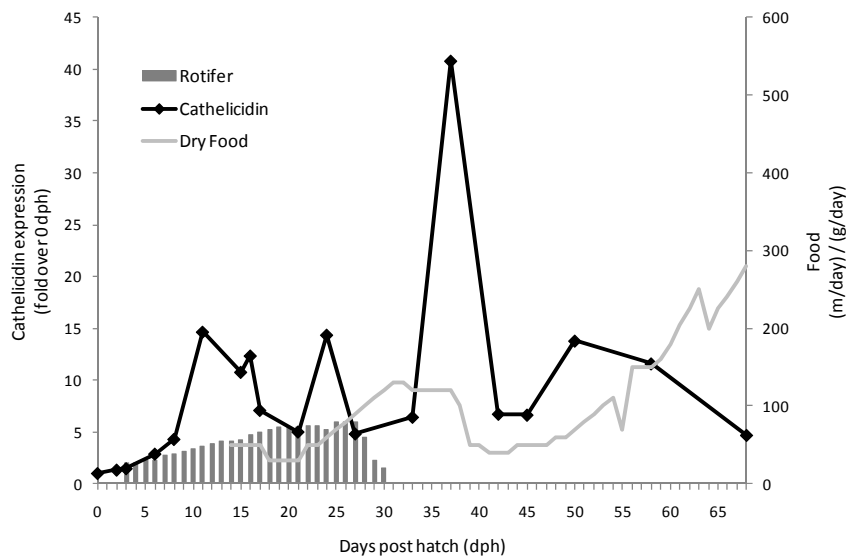


Figure 2 Cathelicidin expression during Atlantic cod larval development. Samples were taken from 0 to 68 days post hatch (dph) and analysed by real-time PCR. The relative cathelicidin expression was normalized to the expression on the hatching day and calculated using ubiquitin as endogenous control. (A) Larvae were either raised on a normal diet of rotifers (million/day), *Artemia* (million/day) and dry food pellets (gram/day) (Group 1) or (B) raised without *Artemia* (Group 2).

4. DISCUSSION & CONCLUSIONS

Cod cathelicidin was constitutively expressed in gills, spleen and kidney and infection with bacteria increased the expression in all tissues. Interestingly cod skin showed the lowest cathelicidin expression of all the tissues studied. This is unexpected as cathelicidins in mammals have been found at the forefront of innate defences, such as mucosal membranes and epithelia (Lai and Gallo, 2009). Further studies on the activity and localisation of the cathelicidin mature peptide are currently performed in our lab in order to understand its function in the innate immunity of fish.

Another aim of this study was to follow the cathelicidin expression during the development of cod larvae. Our data confirms that cathelicidin is present from very early on in development, indeed the eggs might be already protected, as we have seen cathelicidin transcription in the unfertilised eggs which is likely due to the transfer of maternal RNA. Transfer of maternal proteins (Magnadottir et al., 2004) or mRNA (Seppola et al., 2009) has been seen in several studies on cod eggs and larvae. Cathelicidin expression increases after hatching and first feeding with rotifers. In our pilot study with larvae from 2008 a large increase in cathelicidin transcription after feeding with *Artemia* was also observed. The zooplankton given as live feed are grown in elevated temperatures and are very susceptible to bacterial contamination (Olafsen, 2001). Interestingly in the 2009 larvae, examined in this study, a different antimicrobial compound was used to wash the *Artemia* before feeding the cod larvae. This change in rearing practice coincided with a smaller cathelicidin transcription around 30 dph than seen in the year before and seen by a Norwegian group studying the transcription of several immune genes during ontogeny (Seppola et al., 2009). Further studies examining the bacterial load of the feed would be needed in order to confirm that the change in cathelicidin expression is due to these changes in *Artemia* rearing. Comparison of two different rearing methods showed remarkable differences in cathelicidin expression during days 30 – 60 post hatch. Larvae fed on the normal diet had a large increase in cathelicidin expression around day 50 post hatch and this was similar in the larvae studied a year earlier. Larvae fed without *Artemia* also had an about 40 fold increase in cathelicidin expression, but this increase was much earlier in the development at around day 37 post hatch. Both these increases in cathelicidin expression coincided with the end of live feeding and the increase in dry feeding. Cod larvae will preferably feed on live feed if supplied (Agnar Steinarsson, personal observation) and therefore the termination of live feeding is a significant step for the

developing pre-juveniles. Group 1 pre-juveniles were moved from one tank to another at day 49 post hatch, therefore an excess of *Artemia* were given at this timepoint in order to settle the larvae (Fig. 6 A, 50 dph), but this is thought to have a small effect on the overall food consumption of the pre-juveniles (Agnar Steinarsson, personal observation). The increase in cathelicidin expression at 50 dph, could therefore also be due to the stress during movement, but this is unlikely due to the fact that the group 2 pre-juveniles were moved at 55 dph and did not show an increase in cathelicidin expression at this timepoint. The cathelicidin increase observed in both groups could either be due to the stress during food exchange or alternatively be due to components in the dry food. In conclusion our results suggest that the innate immune system in cod larvae in regards to antimicrobial peptides is already active and responds to external stimuli. This is confirmed by the study of Seppola et al who also examined the antimicrobial peptide hepcidin in cod larvae and saw a similar increase in transcription as seen for cathelicidin. Other immune factors such as pentraxin and lysozyme seem to be at very low levels during the first weeks after hatching and have a gradual increase in transcription during development (Seppola et al., 2009). Our studies also stress the point that the larvae are very susceptible to changes in food composition. Probiotic bacteria have been shown to increase fish growth and reduce mortality (as reviewed in (Olafsen, 2001)). We speculate here that beneficial effects seen by treatment of the larvae with probiotics might be due to strengthening of the innate immune system of the larvae, including the increase in production of the antimicrobial peptide cathelicidin.

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