Tail Development in Different Morphs of Arctic Charr

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Tail development in different morphs of arctic aharr
Arctic charr tail development
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Abstract

There are four morphs of Arctic charr living in Thingvallvatn: two benthic Small and Large Benthivorous charr (SB and LB) and two limnetic Planctivorous (PL) and Piscivorous (PI). They differ extensively in morphology, behavior and life history characteristics. Families of PL, SB and LB as well as a domesticated aquaculture morph (AQ) from Holar aquaculture station, were created. The progeny of these morphs have been sampled at different points of development. In this study I stained 15 embryos per stage per morph using an acid-free double stain solution (Walker & Kimmel, et al. 2006). On a morphological level I found that tails start to ossify from the middle part and extent to the two sides gradually. Fin rays appear to ossify in a similar manner. In addition, heads appear to start ossifying earlier than tails for all studied morphs. From the comparison of the different morphs, I found differences in the SB shape compared to the other 3 morphs: SB tails have the two sides of the tail skin connected at the edges, but open in the middle. Furthermore at earlier stages of development SB tails have an L-shape, whereas this same bone is straighter in the other 3 morphs. Tails of AC start to ossify later than in the other morphs whereas LB fin rays develop the fastest. Subsequently I studied the gene expression of 14 genes found to be involved in bone development in other species. I tested the expression of these genes at 2 points of development in two morphs (PL and AC) using qPCR. I found 2 genes (col11a1, ihh) to be differentially expressed.

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Abbreviations

PL, Planctivorous charr from lake Thingvallavatn

AC, Aquaculture charr from Hólar aquaculture station

SB, Small Benthic charr from lake Thingvallavatn

LB, Large Benthic charr from lake Thingvallavatn

qRT-PCR, quantitative Real Time Polymerase Chain Reaction

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1 Introduction

Thingvallavatn is a freshwater system. It is also the largest lake in Iceland, 83 km2 and 114 m maximum depth. The lake was created by volcanism and rifting about 10,000 years ago. The main part of the bottom of the lake is formed by postglacial lava-flows. Water in Thingvallavatn comes from glacial runoff, the temperature of which in summer is always under 11℃ (Jonasson et al. 1992). Most of the fish in the lake are arctic charr and four morphs of arctic charr living there LB, SB, PL and PI (Sandlund. et al.1992) have been described.

Large Benthivorous (LB) and Small Benthivorous (SB) charr belong to the benthic morph type which means they have overshot mouths or short lower jaw, blunt snouts and long fins (Skulason et al. 1989). Planktiotous (PL) and Piscivorous (PI) charr have terminal mouths or relatively equal jaw lengths, pointed snouts and short fins are classified to pelagic morph type (Skulason et al. 1989). The average adult body length of the four morphs at maturity are 553 mm (LB), 133 mm (SB), 205 mm (PL) and 302 mm (PI) (Sundland et al. 1992).

The young of the year of four morphs live in the stony litoral area of Thingvallavatn and they all feed on hironomid larvae (Sundland et al. 1992). The adult of LB feeds on snails Lymnaea peregra, living epibenthically, in the littoral zone (Sandlund. Et al.1992). To some extent, SB is similar to LB, feeding on the same snails and living in the shallow littoral zone (Sundland et al. 1992).   One difference between them is that the only habitat of SB is interstitial spaces of the stony substrate (Sundland et al. 1992). On the opposite, there is a large number of PL charr in all habitats and depths of the lake (Sundland et al. 1992). PL feeds on crustacean zooplankton and chironomid pupae. The other pelagic morph, PI, which feeds on sticklebacks, Gasterosteus aculeatus, has much lower population size (Sundland et al. 1992). PI lives in the epibenthic and benthic zone, but also can be found in pelagic areas (Sundland et al. 1992).
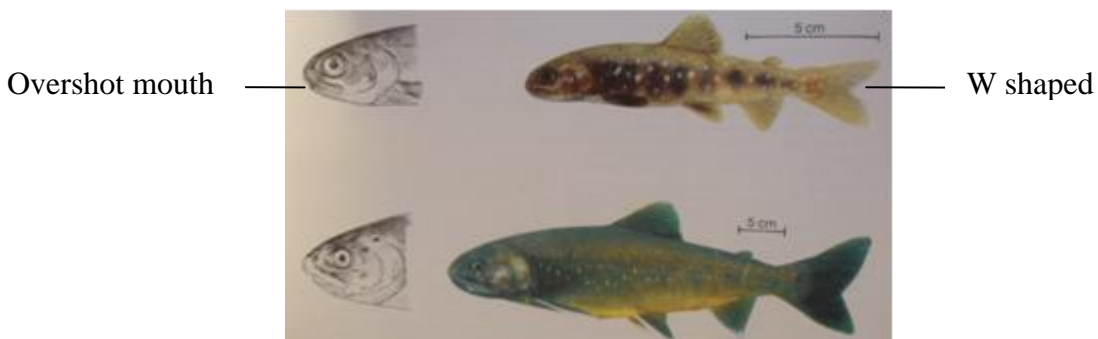
Overshot mouth

W shaped

Figure 1(a): Two pelagic morphs of Arctic charr from Thingvallavatn adapted from Sandlund et al. 1992

Terminal mouth — ... — V shaped

Figure 1(b). Two benthic morphs of Arctic charr from Thingvallavatn adapted from Sandlund et al. 1992

All the morphs spawn in the littoral area but the spawning times of the morphotypes are quite different. LB spawns from July to August and SB spawns from August to December. While PL and PI spawn from September to November (Skulason et al. 1989).

The four morphs show clear differences in habitats, size, spawning, diet and development process. Eiriksson et al 1999 show that in later stages of embryos, pure progeny of SB has a higher number of fin rays than pure progeny of PL, while in early stages, the skeletal development of PL proceeds faster than SB. Moreover in early stages of development SB are found to grow slower than PL. This trend continues between 1and 2 years of age. However in this period of time, PL and PI show a similar growth speed (Snorrason et al. 1993)

Some early research has also proven that the differences in mophology and development are to some extent gene determined (Skulason et al. 1989, Eirikson et al. 1999). It is believed that the morphological diversity in Thingvallavatn is a result of adaption to different habitats and diets. The benthic has a short lower jaw so that it is able to eat snails. At the same time, the pelagic has a terminal mouth and it feeds on zooplankton.

For the gene expression investigation in the caudal fin compartment of Arctic charr embryos, as a part of my study, I have selected 14 genes with a broad range of different and sometimes overlapping functions during embryonic development and morphogenesis. The primary selection criteria was a list of genes differentially expressed in the RNA-transcriptome data produced by the Arctic charr group in university of Iceland (unpublished data). The list of genes exceeds thousands and the transcriptome data is based on two far distinct morph types (natural occurring SB from Thingvalavatn and the Hólar AC strain with pelagic characteristics). The aim of this preliminary expression profiling was mostly an effort; (I) to detect the possible caudal fin specific expression of a handful of candidate genes with already shown whole embryo expression in RNA transcriptome, and (II) to investigate the potential expression differences between two more close pelagic morph types (planktivorous and aquaculture strain). One important reason for the second part is the more available pelagic eggs due to the unique biological and environmental characteristics of Icelandic Arctic charr which makes the task of benthic egg collection more difficult than pelagic counterparts. Later the interesting results might be used or directly integrated into the caudal tail expression study of distinct benthic morphs as well.

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Based on reported functions of these genes in teleost embryonic development, we might be able to categorize them according to their most important roles concerning to cartilage and skeletal formation. For example decorin has displayed a critical activity in embryonic convergent extension and cartilage formation (Zoeller et al., 2008), whereas Foxq1b is a member of well-conserved forkhead gene family which is recently reported as an immediate Aryl hydrocarbon pathway responsive gene (Planchart et al., 2010). Aryl hydrocarbon has extensive effects on skeletogensis and cartilage formation (Xiong et al., 2008; Olifsen and Arukwe 2011) and is detected as highly active pathway in our transcriptome data. Moreover Foxq1b has shown conserved spatial expression in both pharyngeal arches and caudal tail mesenchyme in teleost fish (Wotton et al., 2008; Wotton and Shimeld 2011). Bone morphogenic proteins 2 and 4 (Bmp2/4) were selected in this study, although there were not differentially expressed in transcriptome sequencing (produced by the Arctic charr group in university of Iceland), because of their broad function and expression in different organs during development, particularly in head and tail morphogenesis (Winnier et al. 1995; Mishina et al. 1995; Wan and Cao et al., 2005). Twisted gasturaltion 1 (tgs1) is a Bmp antagonist with particular roles in dorso-ventral embryonic patterning which was already detected in the transcriptome data (Ross et al., 2001). Members of the Hedgehog signaling pathway were among the genes studied; two Hedgehog ligands (i.e. Shh and Ihh), the Hedgehog suppressor receptor (Ptch1b) and a Hedgehog downstream transcription factor (Gli), however only Ihh was already detected as differentially expressed in the transcriptome. Hedgehog signaling has a direct role in osteogenesis and embryonic patterning and interestingly the distinct expression behavior of this pathway is reported in two benthic and pelagic cichlid species closely related to each other (Roberts et al., 2011). Two collagen family members (col1a1 and col11a1) and UDP-glucuronic acid decarboxylase 1 (uxs1), were also included in the study because all had already shown high expression differences in the transcriptome, in addition to their crucial role during teleost fish embryonic development and more specifically skeletal morphogenesis (Bass et al., 2009; Eames et al., 2011). Finally, the expression level of two important transcription factors dlx5 and sox9 was investigated since the activity of them has interconnection and also sox9 is a transcriptome detected candidate (Lee et al., 2011; Yan et al, 2005). Furthermore, two major downstream genes of sox9 (i.e. Sparc and col2a1) with important role in cartilage formation were differentially expressed in the transcriptome data (Rotllant et al., 2008; Suzuki et al., 2006).

2 Methods

2.1 Sampling

All samples used for this study were from the Arctic charr development project at the University of Iceland. Briefly: Three morphs LB, PL and SB sampled in August-October 2010 from Lake Thingvallavatn and the forth morph AC were fertilized using milt from several males from the corresponding populations. Water-hardened eggs were reared at

5℃ in an EWOS hatching tray under constant water flow and in complete darkness at The Holar College Experimental facilities in Verið, Sauðárkrókur.

2.2 Processing

Embryos were staged according to the method described by (Gorodilov 1996) Samples were taken at various developmental stages and fixed in 4% PFA in the fridge overnight, washed in increasing percentage of Methanol and stored in 100% Methanol at -20C.

2.2.1 Selecting samples and staining

I wanted to gain a general idea about the development of the fish before going deep into the research. The first step I did was selecting and staining a few stages of PL and SB. For the reason that we had enough PL and AC, but not few individuals of SB, I stained 10 individuals for each stage of PL and only 3 individuals for each stage of SB. Table 1 describes the sampling procedure:

Table 1: General idea of Arctic charr tail development in degree days (DD)

PL	239	245	254	266	275	280	293	304	315	327	338	346	354	370
PL											400	419	437	453
SB	---	---	---	---	---	---	293	304	313	326	336	346	354	370
AC	---	---	---	---	---	280	293	304	315	327	336	347	360	370
AC											400	419	437	453

After analyzing the pictures of different morphs, I selected a few relevant stages for tail development. I then stained 15 individuals per stage, per morph. Table 2 shows the selected stages for 4 morphs:

Table 2: Comparison of tail development in 4 morphs of Arctic charr in degree days (DD)

PL	304	315	327	336	346	370
AC	304	315	327	336	346	370
SB	304	315	327	336	346	370

LB	304	315	327	336	---	---

I used Acid-free double stain solution as described by Walker & Kimmel, 2006 to stain the samples but adapted the protocol to my research. I found that it is better to stain the samples for one or two days. I kept the fish older than 400DD staining for two days, and the rest were stained for only one day.

At the clearing part, I left the samples in 20% glycerol+0.25% KOH for 7-11 days instead of the 30min to overnight as described in the original protocol. The specific time differed depending on the age of the fish.

2.2.2 Selecting samples and staining

2.2.2.1 Preparation

To take pictures, I immobilized the fish on an agarose plate (1% agarose) using pins and covered it with 50% glycerol+0.25% KOH.

2.2.2.2 Taking photos

All the samples were photographed with Leica microsystem CMS GmbH, D-35578 Wetzlar. The type is DFC10 FX (11547002).

All the photos were taken at 2 times magnification. I used the following camera settings: Exposure time 6.21ms, Saturation 1.20, Gamma 0.71, and Gain 1.0x.

All heads were placed to face to the left. Figure 2 describes how the embryos were placed when photos were taken.
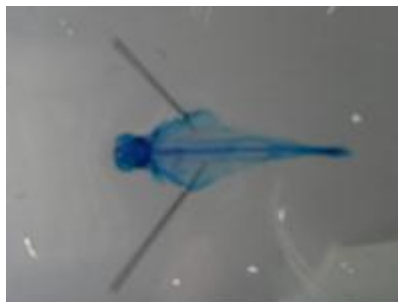

Figure 2: Shows how I placed the embryos when taking the photos

Taking photos. All heads should be forward to the left and the tail is to the right.
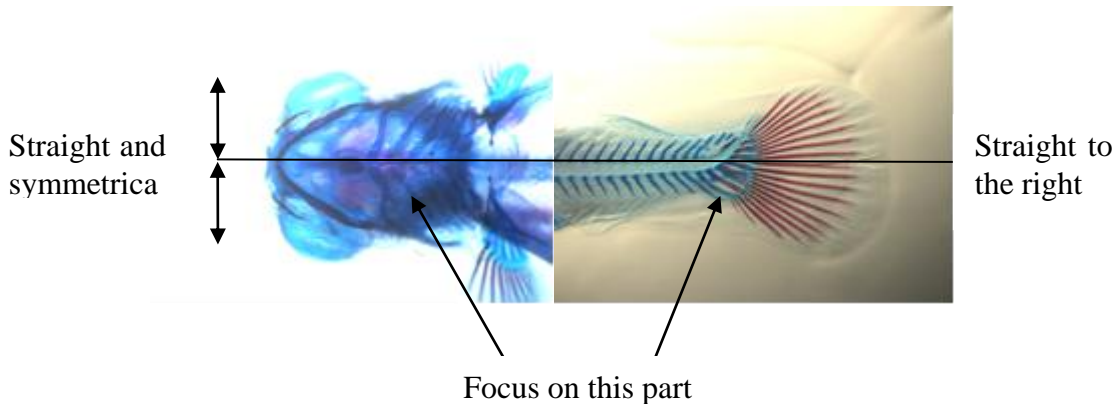
Straight and symmetrica

Straight to the right

Focus on this part

Figure 3: Shows how the samples look like when taking photos

2.3 Molecular work

After analyzing the morphological development of Arctic charr tails, I looked into some 14candidates which have been described to be involved in bone development in other species. I selected two morphs PL and AC and two developmental stages: 383DD and 327DD.

2.3.1 RNA extraction

For each stage and morph I extracted RNA from 6 individuals. First I cut the fish following the line shown in Figure 4, and put the tail part in 350ul TRI-reagent (Sigma). After homogenizing the sample, I added another 650ul of TRI. Then samples were centrifuged directly without staying at room temperature for a few minutes, as suggested by the protocol. Next, I transferred 3x160µl of the uppermost phase to a new eppendorf. Next steps were exactly the same as described in the lab protocol for RNA extraction dissolved the pellet in 50ul cold MillQ water and then measured the RNA concentration using NanoDrop.
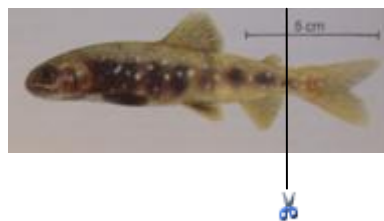

Figure 4: cutting the tails in this line

2.3.2 DNA digestion

To get rid of contaminating genomic DNA, I performed a DNA digestion step.

I did the DNA digestion in a total reaction volume of 25 ml (Table 3).

Table 3 DNA digestion solutions

RNA(µg)	Buffer(µl)	DNase(µl)	H_2O (µl)
5	2.5	1	to 25 µl

When doing Nanodrop after the digestion, MilliQ water was added to make RNA solution to around 100µg/µl samples were then stored at -80°C.

2.3.3 cDNA synthesis

cDNA synthesis was done as shown in Table 4:

Table 4: cDNA synthesis

RNA(µl)	Hexamers(µl)	NTP(µl)	Ddwater(µl)	Buffer(µl)	Enyzme(µl)	Total(µl)
10.0	2.0	0.8	4.2	2.0	1.0	10.0

2.3.4 qPCR

I tested 14 genes which were found to be involved in bone formation in other species for their involvement into the development of the tail in Arctic charr.

Genes tested by qPCR were: deco, foxq1b,gli, shh, ihh, ptch1, tgs1, col11a1, col1a1, uxsl, bmp2, bmp4, dlx5, sox9. We used ACTB, ub213 and if5al as reference genes.

Before doing the qPCR, I further diluted the cDNA into 30µl ddwater.

Table 5: qPCR solutions

SYBR Green Mix(µl)	forward primer(µM)	reverse primer(µM)	Ddwater(µl)	cDNA(µl)	Total(µl)

5.0	5	5	3.0	1.0	10.0

2.4 Data processing

2.4.1 Morphology

Fin ray numbers of different stages of four morphs were observed in the taken pictures. I looked at the appeance of important morphological structures in the tail in time, compared the shape of these features at different points of development and looked at the ossification pattern and timing in four different morphs of Arctic charr.

2.4.2 Gene expression

Relative expression ratios were calculated taking primer efficiencies (E) into account. For this the earlier AC sample Cq (327DD) in each primer pair was set to one and the other sampling points were calculated in relation to that time point, according to E ΔCt (early AC Cq − Cq sample).

I calculated qPCR result according to the protocol by Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data used real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-8.

3 Results

3.1 Head and tail development of Arctic charr

Heads start to ossify at 245DD, which is earlier than the tails. Tails start to ossify from 360DD (Figure 5, N, arrow c). Ossification is almost complete by 417DD (Figure 5, T, arrow d). The ossification begins from the middle and extends to the two sides gradually (Figure 5, N, a).The fin starts to ossify from the middle fin rays, before 280DD. From 400DD, we can also observe the last feature within the time frame of the study starting to ossify (Figure 5, R, arrow e). During development not only the size of the tail increases but the fin ray number increase as well (Table 6). From 315DD, the first little fin ray appears (Figure 5, J, arrow a), and then the second one becomes visible at 346DD (Figure 5, L, arrow b).

The head morphology differs among morphs in the developmental stages covered by this report: SB and LB have larger heads, more open lower jaw and pronounced teeth, whereas PL and AC have more narrow heads, sharper lower jaw and long maxilla.
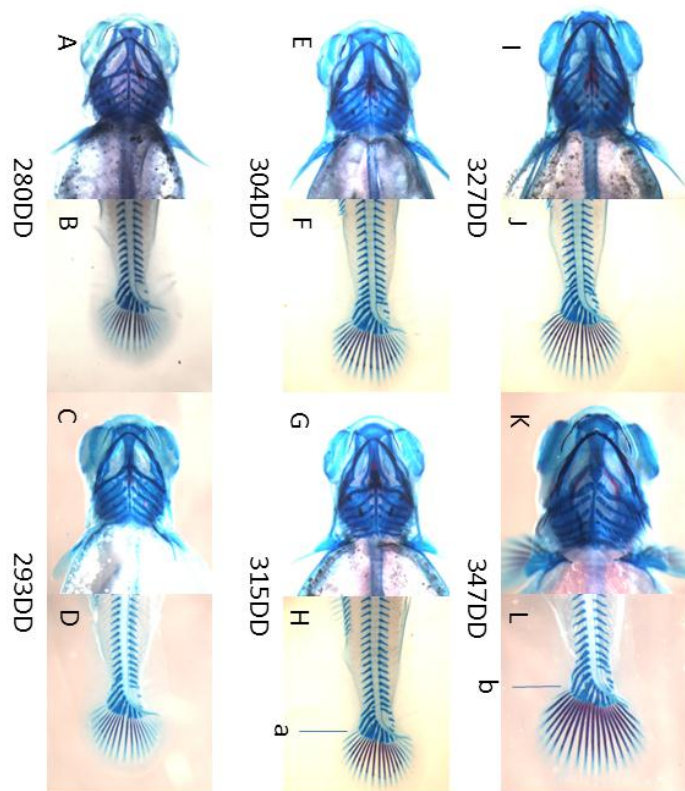
Figure 5 (a): Development of the head and the tail in AC 280, 293, 304, 315, 327 and 347 DD
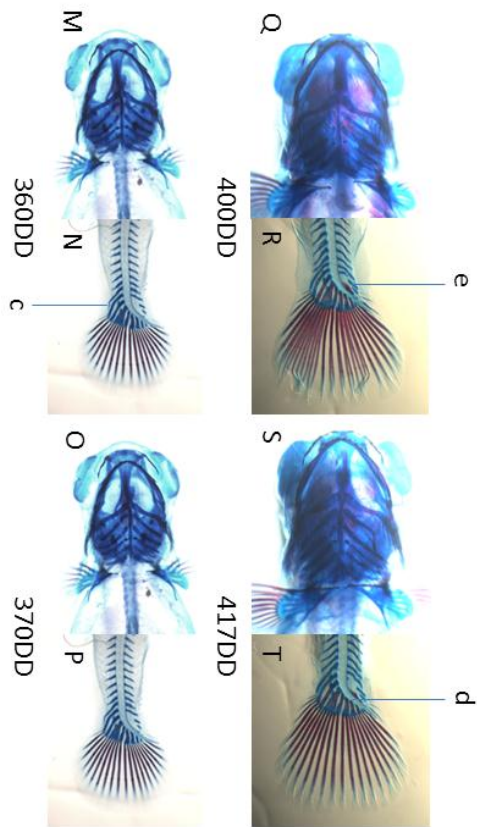
Figure 5 (b): Development of the head and the tail in AC 360, 370, 400 and 417 DD

3.2 Comparison of head and tail development among 4 morphs of Arctic charr

As shown in Figure 6, the tails of AC and PL are similar in shape, but they start ossifying at different times.

The fin rays of SB appear to be quite different from the other three morphs. They appear to be connected at the edges and open in the middle, whereas the other 3 morphs do not show this feature. Another anatomical feature distinguishing SB tails is the presence of an L shaped bone in the tail (Figure 6, f) which is present in the majority of SB analyzed. This feature is also present in PL and LB but to a lesser extent and in a minority of the fish I looked at, whereas in AC this bone appears to be straighter. Later in development the L-shaped bone tends to become straighter.

Table 6 shows the development speed of LB is the highest, while SB is the lowest. PL and AC appear to have similar development. By 370DD, there are 2 small fin rays at the most. The first small fin ray of PL appears at 315DD, the second is at 370DD, while the second fin ray of AC becomes visible at 346DD. The first small fin ray of LB has already appeared at 304DD, and the second appears at 327. Compared to the other three morphs, this is quite early. We can see the first small fin ray of SB at 327DD, but the second one is still not visible by 370DD. This is in accordance with previous studies by Snorrason et al. 1993: at early stages PL allocates more to development of bones than SB.

Table 6: Fin ray numbers in different developmental time points of AC, PL, SB and LB. The first number stands for number of the larger fin rays, the second number comes after plus stands for the small fin rays.

	304 DD	315 DD	327 DD	336 DD	346 DD	370 DD
PL	18	19+1	19+1	19+1	19+1	19+2
AC	19	19+1	19+1	19+1	19+2	19+2
SB	18	19	19+1	19+1	19+1	19+1
LB	19+1	19+1	19+2	19+2	---	---

AC PL SB LB

304DD

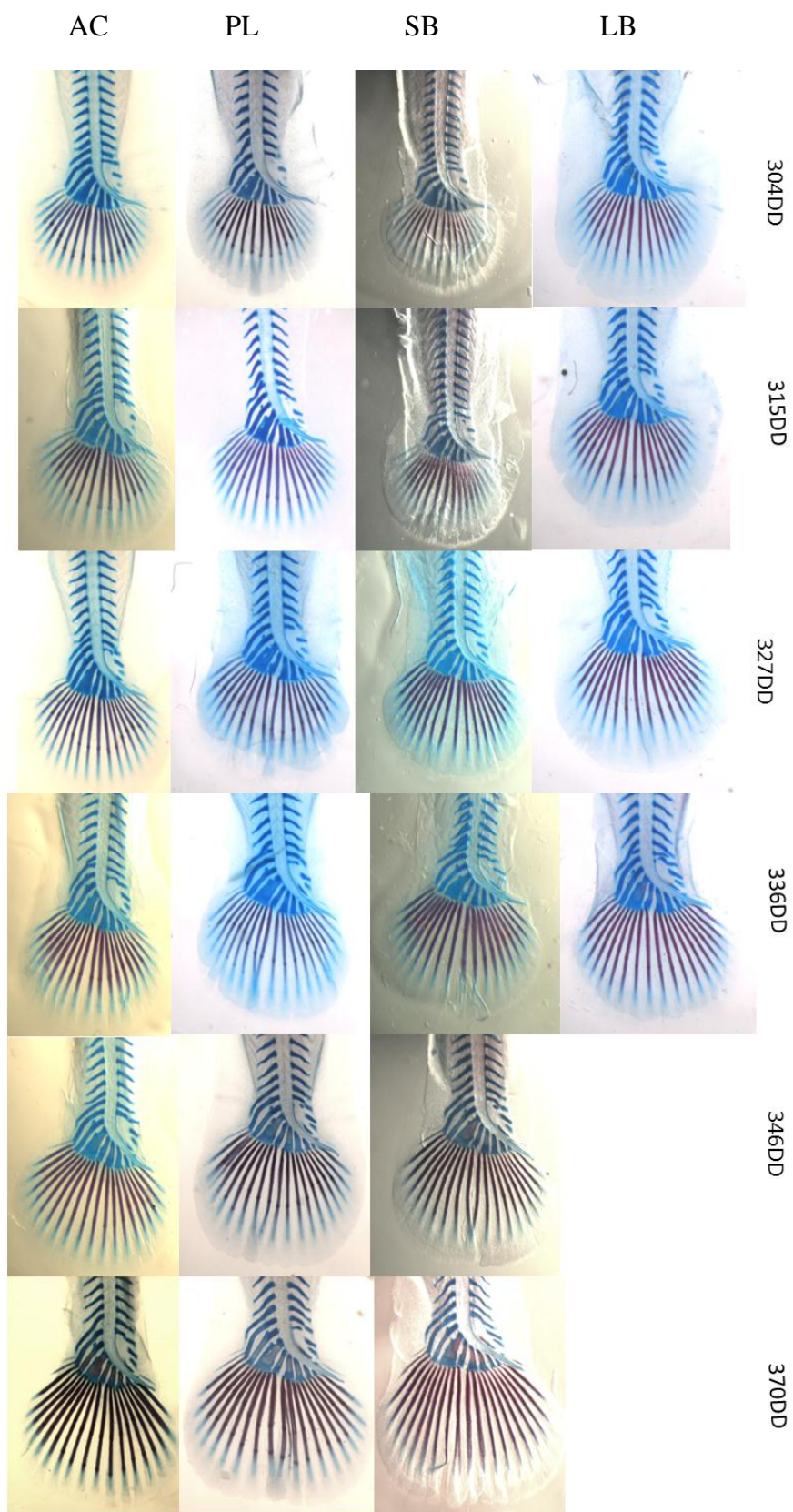315DD

327DD

336DD

346DD

370DD

Figure 6: Comparison of tail development PL, AC, SB and LB

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3.3 Gene expression analysis

I studied the gene expression of 14 candidate genes in two stages and two morphs using 3 reference genes to normalize my data. Among these 14 genes, six appear to be differentially expressed between different morphs. Foxq1b, tgs1 show higher expression in AC at both stages. Pitch1b, gli and col11a1 express more in PL than AC at two stages. Expression of Bmp2 in different morphs are almost equal.

Expression of foxq1b in tails and heads are both higher in AC than PL. This is related to its activity pattern. Gli shows less difference at 273DD, but the different expression becomes obvious at 383DD. Tgs1 expresses more in AC, but the distance is not clear. Col11a1 expresses more in the later stage and in PL.

We can also see that all these six genes except foxq1b express more in the later stage. But the regular patterns of other genes are not clear.

The light green stands for 327PL, gray stands for 327AC, dark green represents 383PL and black is 383AC.
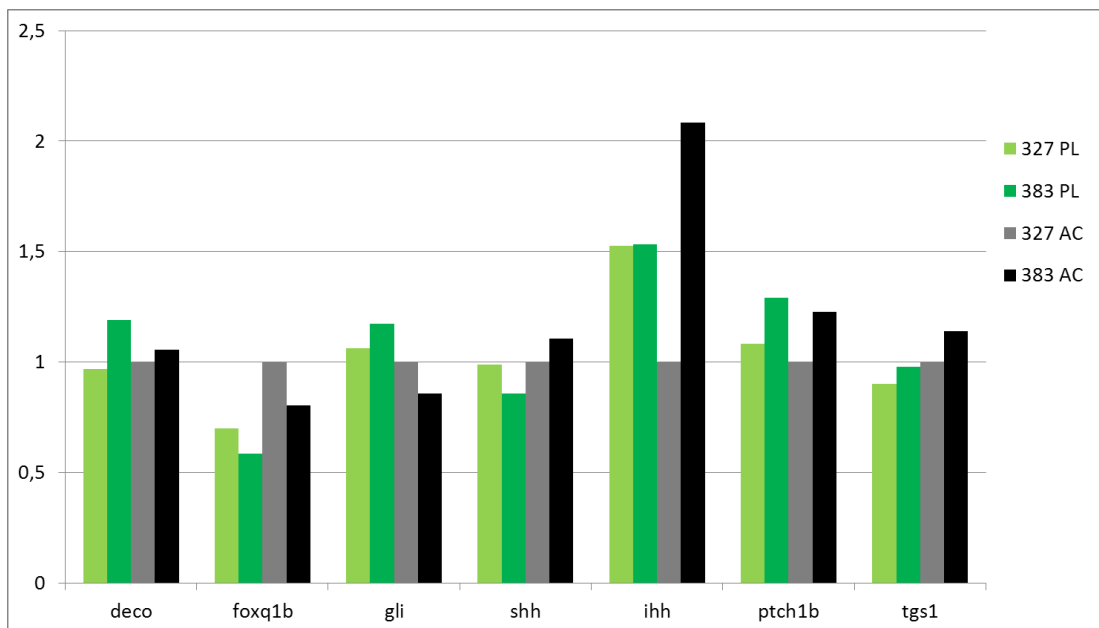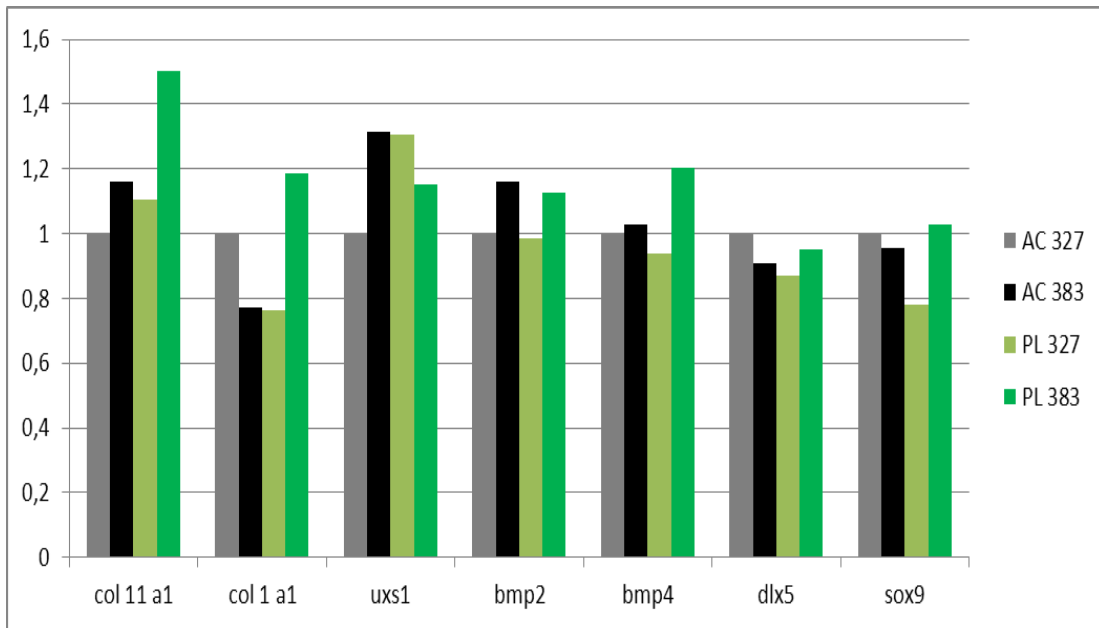

Figure 7 (a): Gene expression results

Figure 7 (b): Gene expression results

4 Discussion

4.1 The differences in morphology can be adaption to habitats

4.1.1 SB-swimming ability

The fin rays of the fish are supported by a caudal skeleton, folded by skin (Gosline. et al. 1997). The basic uses of the fin rays of the tail are swimming and steering (Videler. et al.1993). Therefore any variation of the anatomical features of a tail in a species can be regarded as an adaption to type of swimming in a given environments certain populations of this species are inhabiting. According to Videler, fish that swim faster and longer usually have lunate tails, with large spans and small chords. Whereas the tails of fish which need to maneuver a lot to survive have lower spans and larger surfaces.

From Figure 1 it can be speculated that the tails of SB and LB adults have lower result of AR (span squared divided by the surface) than PL and PI. During the tail development (Figure 6), fin rays of AC and PL separate more than those of SB which makes the span larger.

These different swimming abilities are believed to be adaptations of the different morphs to their habitats. SB and LB live in the stony littoral zone, which makes maneuvering more important than swimming speed. Therefore one can speculate that the tails of the benthic morphs are selected to be W-shape so that it will allocate less energy to survival. While PL live in the pelagic zone of the lake, adaptations of the tail increasing the swimming speed would be beneficial for their live in wide and open habitats.

4.1.2 AC-domestication

AC is a domestic morph and in my study, it starts to ossify at 360DD (Figure 5), which is much later than the other three wild morphs. It can be speculated that the delayed ossification might be related to the domestic environment these fish live in.

According to a study by Price, 1999, the development of domesticated animals differs from wild animals since the living environment affects development. For example affecters of environment such as the quality and quantity of living space, feeding and drinking, social role in the society and so on have been shown to influence development. Moreover the strong artificial selection domestic morphs are subjected to may force them to evolve differently than morphs from the wild (Price. et al. 1999). Research on Mongolian gerblis

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showed that these animals had a faster growth speed in laboratory environment (Mertice M. et al. 1980). In addition, domestic animals become sexually mature earlier (Price. et al. 1999). These changes in domestication can also be considered as an adaption to a new environment. Domesticated animals lack survival pressure, their wild counterparts are subjected to and which has been found to decrease the growth speed.

4.1.3 LB-size

Large benthic charr has the highest average body length of all morphs in Thingvallavatn: 553 mm, PL has more intermediate length 205 mm and SB is the shortest one 133 mm. My results point out (Table 6) that the speed of development of tails from the fast to slow is: LB, AC, PL and SB. It can be speculated that the length and the skeletal growth speed are positively correlated. This is further supported by the findings in (Snorrason et al. 1993) where it has been shown that in early stages before 1 and 2 years age, the skeletal development of PL proceeds faster than SB. It is likely that these morphs have to form their skeleton fast to be able to quickly develop their swimming abilities. This will lead to an increased survival rate early in life.

4.2 Staining process influence the morphology outcome

During my experiment, I found that staining process can also influence the appearance of samples.

As stated by Walker and Kimmer 2007 it can be difficult to stain bone with alizarin red if the fixation period is too long. Thereforelong fixation time can lead to misinterpreting the begining of ossification. So for Arctic charr the fixition time should be limited to maximum 15-17 hours at 4℃

Besides, colors of the samples might be different if the concentrations of alizarin red and alcian blue dyes are not the same between different staining batches . In order to solve this problem, I made enough staining solution at the same time to make sure staining solution was always in the same condition for staining.

The staining time is not fixable. It depends on the stage of samples and the staining solution. In order to define the optimum staining time I controlled the coloration intensity by staining and checking the coloration quality of one or two samples under the microscope before staining the rest of the fish. Clearing time also needed to be adapted as older fish need more clearing than early embryos. Moreover the shape of the head is easily changed if the samples are kept in glycerol for too long. Tails can also be damaged. It is impossible to define a precise time for the clearing steps even for the same staining batch, so samples had to be checked daily. Furthermore in later stages, tails take much less time

to clear then heads. So for these later stages I first took photos of the tails than continued to clear the samples. It is crucial to follow the samples carefully by the end when they are almost cleared as it takes a very short time between perfect clearing and destroying the samples.

4.3 Gene expression results

4.3.1 Ihh and col11a1

Indian hedgehog (Ihh) is a secretory ligand member of the conserved Hedgehog signaling pathway, involved in chondrocyte proliferation and differentiation. More importantly, Ihh stimulates alkaline phosphatase (ALP) activity during bone development and osteoblast differentiation. The osteoblast differentiation of mesenchymal cells by Ihh is implemented through up-regulation of Runx2, which is an essential transcription factor for osteoblastogenesis (Shimoyama et al., 2007). Moreover, specific expression pattern of Ihh has been already reported in the regenerating fin and craniofacial cartilages of zebrafish (Smith et al., 2006; Avaron et al., 2006). The second differentially expressed gene, Col11a1 has an essential role in skeletal morphogenesis (Li et al., 1995). In particular, the formation of cartilage collagen fibrils and cartilage cohesive properties can be dramatically affected in the absence of collagen XI. However, during zebrafish embryonic development the role of col11a1 activity is more prominent in craniofacial cartilage morphogenesis (Baas et al., 2009). Col11a1 also shows a high degree of expression during zebrafish fin development. A recent study suggests a potential role for col11a1 in structural adaptation of the vertebral architecture in Atlantic salmon (Wargelius et al., 2010).

4.3.2 How to improve

Ihh expression at 383 DD in AC is approximately two times that of 327 DD (figure 7), however, the expression of Ihh in PL at both stages is similar. Col11a1 has shown a drastic increase in expression at the later stage in PL than earlier stage, but no great difference in AC. From Figure 6, AC starts to ossify at 360DD and PL at 346DD. Considering the time from gene expression to detectable phenotypic changes, those genes might have been expressed before the ossification, i.e. between 327DD and 383DD. The expression results suggest that ihh might have more pronounced role in tail development of AC, and col11a1 in PL tail development, Therefore, conducting a study with more embryonic stages, with more distinct Arctic charr morphotypes, together with biological replicates for each morph and embryonic stage is an inevitable future plan to improve upon my preliminary study. Ideally the functional analysis through the suppression of ihh and col11a1 expression in both morphs might even be more informative to shed light on their specific roles during caudal fin chondrocyte development.

5 Conclusions

1. Heads start to ossify earlier than the tails.

2. The ossification begins from the middle and extents to the two sides gradually

3. The fin starts to ossify from the middle fin rays.

4. There are small fin rays visible.

5. The fin rays of SB appear to be different from the other three morphs.

6. SB tails have the presence of an L-shaped bone in earlier stages, later in development the L-shaped bone tends to become straighter.

7. The development speed of LB is the highest, while SB is the lowest. PL and AC appear to have similar development.

8. Two out of fourteen of the studied candidate genes (col11a1 and ihh) appear to be differentially expressed between AC and PL in 383 DD.

References

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Appendix

Table I qRT-PCR primer sequences

Primer name	Sequence (5'->3')
eIF5A1 F	GGCTTCGTGGTGCTGAAG
eIF5A1 R	CCATGTGGACCTTAGCGTG
ub2l3 F	CGAGAAGGGACAGGTGTGTC
ub2l3 R	ACCAACGCAATCAGGGACT
dlx5 q1 F	CGCAAACGCAGGTCAAGAT
dlx5 q1 R	GCCATAGGGTCGCTGGAG
shh q1 F	AAAGCAGAAAACTCCGTTGC
shh q1 R	TCGCCAACTCTGAGGTCTTT
bmp4 q2 F	ACCACGAAGAGCACATGGA
bmp4 q2 R	CTCTGGGATGCTGCTGAGAT
dlx5 q1 F	CGCAAACGCAGGTCAAGAT
dlx5 q1 R	GCCATAGGGTCGCTGGAG
col1a1 F	CAGAGGTGACCAAGGAGTCAAGGGT
col1a1 R	CACCAGGAGAGCCAGCAGGG
col11a1 F	TTGCTGGAACTGATGGCTCTGCG
col11a1 R	ATGGACCAGGAGGACCAGGCT
decorin F	F-CCAAAGGTCTGCCCTCCTCCCT
decorin R	TAGACCCAGCTTAGCCAGGTTCTTG
foxq1b F	CCACAACCTGTCGCTGAACG
foxq1b R	CATCCAGTAGTTGTCCTTGCCC

gli-like F	AGAAGAAGGAGTTCGTGTGTCGC
gli-like R	GCATCGGTGTGGTTTCTCCC
ihh F	F-CAGGAGGACAAGGGCGTGTTT
ihh R	CCAGTGGGCGAGGTCATGC
ptch1 F	GAGGAGTTCACCCGTAAAGGCGT
ptch1 R	CCGCCAGGAGGAACCAGTGT
tgs1a F	GAGTGCTGCGACTGTGTGG
tgs1a R	TAGAGTTCCTCCACCGTGCT
uxs1 F	CCCCCTGTGAAGTTCCTCTC
uxs1 R	GCCGCCCGTTATCAAAATCCT