Variation at early life stages of Atlantic salmon: Relationships between emergence time and stress coping styles.

Variasjon i tidlige livsfaser hos Atlantisk laks: Sammenheng mellom oppsvømmingstidspunkt og stressmestringsstrategier.

Philosophiae Doctor (PhD) Thesis

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Dept. of Animal and Aquacultural Sciences
Norwegian University of Life Sciences

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TABLE OF CONTENTS

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Jonathan

Ås, December 2010

ABSTRACT/SUMMARY

Vaz-Serrano, J. (2010). Variation at early life stages of Atlantic salmon: Relationships between emergence time and stress coping styles. Philosophiae Doctor Thesis 2011:8, Norwegian University of Life Sciences.

Captive fish are exposed to a variety of stressful situations, which can affect growth rates and promote diseases. Identification and selection for stress resistant fish at early stages of the production cycle could be used as a cost-efficient tool to increase productivity, welfare and to reduce disease susceptibility in aquaculture. In different animal groups, two distinctive sets of behavioural and physiological responses to stress, termed proactive and reactive stress coping styles, have been identified. In salmonid fish, emergence time, i.e. the moment when a larva leaves the spawning redd and starts exogenous feeding, has been shown to be related to growth rates, standard metabolic rates, time of smoltification and social status. In this thesis, I have studied whether variability in emergence time could be coupled to differences in stress coping styles. As well, I have investigated the relationships between other early life traits and emergence time in Atlantic salmon (*Salmo salar*).

In the first part of the thesis, the relationship between hatching time and larval growth were examined. As well, a novel method to sort salmonid larvae according to emergence time was developed to investigate the relationships between family variation in emergence time and egg size, hatching time and larval developmental rate in Atlantic salmon. In the second part of the thesis, fry with different emergence times were screened for divergences in stress coping styles.

The results in the first part of this thesis demonstrate that larvae with a late hatching time had higher post-hatch growth rates, thereby compensating for a delayed hatching time. Furthermore, comparisons between families showed a relationship between variation in egg size and hatching

time, although these traits were not related to emergence time. In addition, families with a faster larval developmental rate reached emergence earlier.

In the second part of this thesis, where the relationship between time to emerge and stress coping styles was investigated, it was shown that fry with an early time to emerge were bolder compared with a late emerging fry. However, differences in emergence time were not associated to other behavioural and physiological traits of the proactive and reactive coping styles, such as standard metabolic rates, social dominance, or post stress cortisol levels. The decoupling between boldness and such traits could be related to the absence of a strong selection pressure at emergence in captive fish.

To conclude, this thesis demonstrates that the rate of development of the larvae, rather than egg size or hatching time, predicts time to emerge from the spawning redds in Atlantic salmon. Furthermore, this thesis presents a novel method to sort salmonid larvae that could improve rearing conditions of domesticated salmon. As well, it was shown that an earlier emergence was related to boldness behaviour, but earlier emergence was not related to other traits of the stress coping styles in domesticated Atlantic salmon. Future studies should examine if selection of fish according to emergence time is related to other production traits, such as disease resistance, growth rates, filet colour, occurrence of deformities or feed conversion ratio.

Keywords: Atlantic salmon, *Salmo salar*, early life traits, hatching time, emergence time, metabolic rate, larval development, stress coping styles.

SAMMENDRAG

Fisk i oppdrett vil være eksponert for en rekke unaturlige miljøforhold som vil innebære stress hvilket vil kunne medføre redusert tilvekst og økt forekomst av sykdom. Hensiktsmessig identifisering og seleksjon for økt stresstoleranse, anvendt tidlig i produksjonssyklusen, vil derfor kunne være et kostnadseffektivt verktøy for bedre sykdomsresistens, økt dyrevelferd og kostnadseffektiv produksjon. Det har i forsøk med ulike dyrearter blitt vist at det grovt sett finnes to ulike mønstre for fysiologiske og adferdsmessige reaksjoner på stress: proaktiv og reaktiv stressmestringstype. Det er blitt vist at det hos laks er en sammenheng mellom oppsvømmingstidspunkt, dvs. tidspunktet når en fiskelarve beveger seg bort fra gytemediet for å finne ekstern næring, og tilvekst, metabolsk rate og sosial status. I denne avhandlingen har jeg studert om variasjon i oppsvømmingstidspunkt kan kobles til forskjeller i stressmestringsstil. I tillegg har jeg undersøkt forholdet mellom andre tidlige livsegenskaper og oppsvømmingstidspunkt hos atlantisk laks (*Salmo salar*).

I den første delen av denne studien ble forholdet mellom klekketidspunkt og larvevekst undersøkt. I tillegg ble en ny metode for å sortere yngel mht oppsvømmingstidspunkt utviklet for å undersøke sammenhengen mellom eggstørrelse, klekketidspunkt og oppsvømmingstidspunkt hos atlantisk laks. I den andre delen av studien ble det undersøkt om yngel med ulikt oppsvømmingstidspunkt domineres av ulike stressmestringstyper.

Resultatene i den første delen av denne studien viser at fiskelarver med sent klekketidspunkt har høyere tilvekst i den første fasen etter klekking, noe som vil kompensere for det sene klekketidspunktet. I familiematerialet ble det dessuten vist en sammenheng mellom eggstørrelse og klekketidspunkt. Det var imidlertid ikke noe signifikant sammenheng mellom klekketidspunkt

og oppsvømmingstidspunkt i dette materialet, men familier med høyest metabolsk omsetning hadde også tidligst oppsvømmingstidspunkt.

I den andre delen av studien, hvor vi undersøkte om oppsvømmingstidspunkt er knyttet til adferd eller fysiologiske egenskaper som er typiske for de to stressmestringstypene, fant vi at yngel som hadde et tidlig oppsvømmingstidspunkt var modigere eller mest uredde. Men vi fant ikke at disse uredde individene også hadde de typiske trekkene til den proaktive stressmestringstypen, som er høyere metabolsk rate, sosial dominans eller lavere kortisolnivå etter standardisert stress. En mulig forklaring på dette kan skyldes fraværet av seleksjon eller stress i oppdrettsmiljøet.

Etter dette må det konkluderes med at metabolsk omsetning, i langt større grad enn eggstørrelse eller klekketidspunkt, påvirker oppsvømmingstidspunkt hos atlantisk laks i oppdrett. Vi har også utviklet en ny metode for sortering av lakseyngel og denne vil kunne brukes til å bedre produksjonsforholdene til oppdrettsfisk. I tillegg ble det vist at tidlig oppsvømmingstidspunkt var relatert til modig oppførsel, selv om tidlig oppsvømmingstidspunkt ikke ble relatert til andre trekk ved stressmestringstypene i opdrettslaks. Ytterligere studier bør undersøke om disse metodene i et seleksjonsprogram også vil kunne gi bedret sykdomsresistens, tilvekst, fôrutnyttelse og kvalitetsegenskaper.

LIST OF PAPERS

This thesis is based on the following papers referred to by their roman numerals in the text:

Paper I

J., Vaz-Serrano, M., Åberg-Andersson, H.M., Gjøen, J.F., Steffensen and E., Höglund. Differences in metabolic and developmental rates after hatching in Atlantic salmon *(Salmo salar)*: Evidence for catch-up growth in yolk sac larvae? *Submitted to Aquaculture*.

Paper II

J., Vaz-Serrano, M. L., Ruiz-Gómez, Ø., Øverli, F. A., Huntingford, H. M. Gjøen and E., Höglund. Self-sorting of Atlantic salmon (*Salmo salar*) based on time to emerge from an artificial redd: a novel method revealing inter family relationships between egg characteristics, larval development and emergence time. *Submitted to Aquaculture*.

Paper III

J., Vaz-Serrano, M. L., Ruiz-Gómez, F. A., Huntingford, H. M. Gjøen, P.V., Skov, Ø., Øverli, and E., Höglund. Consistent boldness behaviour in early emerging fry of domesticated Atlantic salmon (*Salmo salar*): Decoupling of behavioural and physiological traits of the proactive stress coping style. *Submitted to Physiology and Behavior*.

GENERAL INTRODUCTION

1. INTRODUCTION

The exhaustion of the commercial fisheries stocks in the world has lead to an increase in the aquacultural industry over the last decades (Naylor, et al., 2000). Among domesticated fish species, salmonid fish represent one of the largest groups in fish farming (FAO, 2009), although their relatively recent domestication implies that salmonid fish are not totally adapted to captivity. Moreover, husbandry practices in aquaculture expose fish to stressful situations, such as handling, transport or food deprivation, which can compromise the welfare of fish (Huntingford, et al., 2006).

Similar to other vertebrates, fish respond to a challenging situation by activating the stress response (Bonga, 1997). This response is a combination of behavioural and neuroendocrine regulations that helps the individual to recover its homeostasis. In particular, when stress is perceived by an individual, it stimulates a quick release of catecholamines from the chromaffin tissue, accompanied by the activation of the hypothalamic-pituitary-interrenal axis and the subsequent synthesis and secretion of cortisol (Bonga, 1997; Barton, 2002). However, if the stress response is prolonged or repetitive, it could induce loss of appetite, slow growth, immunosuppression and inhibition of the reproduction (Bonga, 1997). Stress is also an economical issue, and approaches that minimize stress are needed when dealing with captive animals.

One way to reduce the amount of stress experienced by domesticated animals is to select for stress resistant animals. In the research field of stress physiology, rainbow trout (*Oncorhynchus mykiss*) selected for high- (HR) and low- (LR) cortisol response to stress has been used as an animal model (Pottinger and Carrick, 1999). Studies within these lines have revealed that selection for stress responsiveness is related to a number of other traits. For example, LR fish is

characterized by a low locomotor response to acute stress, social dominance and bolder behaviour in novel environments. On the other hand, HR fish show a high locomotor response to acute stress, usually become subordinates during social encounters and exhibit a more shy behaviour in novel environments (Øverli, et al., 2005; Øverli, et al., 2007). Moreover, stress coping styles (see further down) have been identified in these lines: LR fish normally displays a proactive coping style and HR a reactive coping style (Schjolden and Winberg, 2007; Øverli, et al., 2007). Further studies with HR and LR trout have demonstrated that selection of fish with lower stress responsiveness leads to reduced feed waste, following a stressful event (Øverli, et al., 2006). In addition, it has been shown that the higher competitive ability of the LR fish promoted growth differences when these lines were reared in co-culture (Pottinger, 2006). Thus, as well as improving welfare in farmed fish, selection for stress resistance could generate economical benefits in aquaculture.

The main events in the early life of fish are: egg fertilization, hatching, first oral ingestion and yolk exhaustion (Kamler, 2002). The relationships between some of these events and associated traits, as well as their relationship to stress responsiveness later in life, are poorly documented. Information about individual stress responsiveness, future growth performance and disease resistance could be revealed by early monitoring of developmental rates. This knowledge could in turn be used to optimize rearing regimes, before fish enters the more cost intensive parts of the production cycle

Exploring an alternative method to long term selection programmes, in this thesis I have examined the relationships between early life traits in domesticated Atlantic salmon (*Salmo salar*), and I have investigated if selection of fish according to the developmental rate of salmonid larvae could be used to predict stress coping styles of captive fish.

2. BACKGROUND

2.1 Relationships between early life traits in Atlantic salmon

In natural populations of salmonid fish, females bury their fertilized eggs in gravel nests known as spawning redds (Ottaway, et al., 1981). After hatching, the larvae stays in the spawning redd and feeds on the yolk sac, which provides nutrients and energy until the larvae are able to search and ingest exogenous feed. The moment when the larvae leave the protection of the spawning redd is known as the "emergence" or "swim-up". The time-span between the first and last larva to emerge from a spawning redd can last 2 weeks or even longer, depending on the temperature (Brännäs, 1995; De Leaniz, et al., 2000; Sundström, et al., 2005). Emergence time usually corresponds to the optimal start feeding conditions of the fry (Northcote, 1978), also known as the first feeding date.

Variations in early life traits significantly affect future traits of salmonid fish. In lake trout (*Salvelinus namaycush*), for example, larger eggs hatched later than smaller eggs (Pakkasmaa and Jones, 2002). However, contradictory results have been reported in other salmonid fish, where no relationship between egg size and hatching time was found (e.g. Kristjánsson and Vøllestad, 1996; Gilbey, et al., 2009). Furthermore, a positive relationship between egg size and larval length has also been demonstrated (e.g. Gilbey, et al., 2005), although it remains to be concluded if size differences are maintained until emergence in salmonid fish. Einum and Fleming (2000) demonstrated that fry from larger eggs emerged earlier than fry from smaller eggs, indicating a positive relationship between egg size, emergence time and fry size in natural populations of Atlantic salmon. This is somehow in contrast to the study done by Gilbey and collaborators (2009), demonstrating that early hatching individuals were larger at the moment of

hatching, but showed a slower growth rate during the larval stage, compared with late hatching individuals of Atlantic salmon. As a consequence, the potential size advantage of hatching early may not persist after emergence and first feeding. What is more, fry that are largest at emergence in nature have a competitive advantage over smaller individuals, which can be reflected in higher growth rates and earlier time for smoltification (Metcalfe and Thorpe, 1992). Thus, relationships between early life traits have been established previously in salmonid fish, although some inconsistencies have been reported.

The study of embryonic and larval developments is a complex exercise due to the difficulties encountered when following individual eggs through all their developmental stages. Isolation of individual eggs and the subsequent monitoring until emergence is not a practical method, given that isolation can modify behavioural and physiological traits of the developing larva (Sloman and Baron, 2010). A way to come around this problem is to rear groups of eggs with a known genetic background. In this thesis, eggs from different families of domesticated Atlantic salmon were reared separately and measures of family average egg size, hatching time and larval development were determined.

In this thesis, a method to sort individual larvae according to the time to emerge from an artificial redd was developed. In a hatchery environment, the decision when to initiate first feeding can be complicated. If feed is delivered too early, larvae are not sufficiently developed to ingest solid particles, and feed excess would deteriorate water quality (Sveier and Raae, 1992). On the other hand, a delayed first feeding may cause larvae to starve, which could affect growth and survival rates of fish later in the production cycle (Koss and Bromage, 1990; Yoseda, et al., 2006; Wang, et al., 2010). One way to prevent these unwanted effects is to identify batches of larvae with a different emergence time. By sorting fish by this criterion, initiation of first feeding could be

adjusted for suitable batches of salmonid fry, avoiding water quality problems or starvation. After using this method, sorted fish were DNA-typed for parental assignment and family variation in time to emerge was then related to family differences in egg size, hatching time and larval development.

2.2 Variation in emergence time and stress coping styles.

Individual variation in the time to emerge has been shown to predict social dominance, metabolic rates, growth rates and life history strategies in salmonid fish originating from natural populations. Fry with an earlier time to emerge have been demonstrated to become socially dominant, to have higher metabolic rates, higher growth rates and to reach smoltification earlier, when compared with fry emerging later (Metcalfe and Thorpe, 1992; Metcalfe, et al., 1995; McCarthy, et al., 2003). While it is usually considered that early emerging individuals have a competitive advantage, this benefit may be offset by other factors such as increased predator exposure (Brännäs, 1995; Sundström, et al., 2005) and decreased availability of food early in the season (Sundström, et al., 2004).

In addition to the effect of gaining early access to available territories, some of the traits related to emergence time in salmonid fish indicate general differences in the way individuals respond to challenges. Such differences have been observed in a variety of animal groups and are known as behavioural syndromes (Sih, et al., 2004), stress coping styles (Koolhaas, et al., 1999), temperaments (Réale, et al., 2000) or animal personalities (Wolf, et al., 2007). In particular, behavioural patterns that are expressed during different challenges are often related to each other and across situations, giving rise to behavioural syndromes (Sih, et al., 2004). Moreover, stress coping styles are known as sets of behavioural responses that are clustered with physiological

reactions to challenges, and are constant over time (Koolhaas, et al., 1999). Two broad types of stress coping styles have been identified, the proactive and the reactive stress coping style. Proactive individuals show an adrenaline-based stress response, social dominance, risk-taking behaviour, routine formation and higher standard metabolic rates. On the contrary, reactive individuals are characterized by a cortisol-based stress response, social subordination, risk-avoiding behaviour, behavioural flexibility and lower standard metabolic rates (Korte, et al., 2005; Coppens, et al., 2010; Huntingford, et al., 2010).

Stress coping styles appear to be related to emergence time in natural populations of salmonid fish; early emerging fry have been demonstrated to have a higher standard metabolic rate and to be dominant over late emerging fry (Metcalfe and Thorpe, 1992; Metcalfe, et al., 1995), which are traits of the proactive stress coping style (Korte, et al., 2005; Huntingford, et al., 2010). Moreover, studies using the HR and LR trout model demonstrated that proactive (LR) larvae emerged earlier compared with reactive (HR) larvae, further strengthening the relationship between stress coping styles and emergence time (Åberg, et al., 2010). However, if this relationship is present in domesticated Atlantic salmon is, to my knowledge, unknown. In the second part of this thesis, domesticated fry with a different time to emerge were screened for stress coping styles. Following isolation in a new environment, measures of boldness, standard metabolic rate, social status and post stress cortisol concentrations were taken for groups of fry with an early or late emergence time. In addition, by repeating the measurements after five months, it was investigated if some of these traits were constant over time.

3. THE AIMS

This thesis has two main aims. First, it will attempt to increase the knowledge about early life traits that are related to emergence time in captive Atlantic salmon. Secondly, it will investigate if variation in emergence time can predict stress coping styles.

The specific objectives of this thesis were:

- To examine the relationship between hatching time, metabolic rate and larval development.
- To develop a method to sort individual fry by the time to emerge.
- To investigate if family variation in time to emerge from an artificial redd is related to eggs size, hatching time, or larval development.
- To study if differences in time to emerge are related to stress coping styles in domesticated Atlantic salmon.

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PAPER I

4. DIFFERENCES IN METABOLIC AND DEVELOPMENTAL RATES AFTER HATCHING IN ATLANTIC SALMON (*SALMO SALAR*): EVIDENCE FOR CATCH-UP GROWTH IN YOLK SAC LARVAE?

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Abstract

There are several studies indicating a coupling between early developmental rate and future growth performance in salmonid fish, and techniques to sort fish with respect to time to hatch and larval development have been suggested to decrease size heterogeneity in farmed fish. In the present work, the relationship between hatching time, post hatch larval development and metabolic rate in farmed Atlantic salmon (*Salmo salar*) was investigated. Weight-specific oxygen consumption (VO_2) and development were studied in groups of larvae originating from three families with an early and three with a late hatching time. Measurements were taken at two time points (T1 and T2) with 20 day-degrees in between. The difference between measurements

corresponded to the difference in hatching time between early and late hatching families. Measures of development (the body to yolk weight ratio, the total larval length and the behaviour response to a hypoxic challenge) indicated larval development between T1 and T2, and that early and late hatching groups were equally developed at T1 and at T2. However, VO_2 did not follow this pattern, and late hatching larvae had higher VO_2 compared to early hatching individuals at both T1 and T2. That late hatching larvae reached the same level of development, and had higher metabolic rate at T1 compared to early hatching larvae, suggests that differences in development that are present during hatching can be evened out by post hatch catch-up growth.

Keywords: larval ontogeny, hatching time, yolk sac, oxygen consumption, hypoxia, aquaculture.

4.1. Introduction

In natural populations of salmonid fish, larval developmental rate has been shown to influence future growth and life history strategies (Metcalfe and Thorpe, 1992; Metcalfe et al., 1995). For example, a difference of less than 1 week in the relative timing of first feeding can result in years of difference in the timing of migration (Metcalfe and Thorpe, 1992). However, this effect may be offset by other factors such as predator exposure (Brännäs, 1995; Sundström et al., 2005) and food availability (Sundström et al., 2004). These selective pressures promote variability in developmental rate, which is maintained by nature. If this relationship between variation in larval developmental rate and future performance is present in selected and cultured populations, it could be utilized to predict growth and to optimize production of reared fish. This has been demonstrated in pikeperch (*Sander lucioperca* L.) where sorting fish with respect to

time to hatch has been shown to affect size heterogeneity later on in the production cycle (Steenfeldt et al., unpublished).

Metabolic rate is related to growth rates (Cutts et al., 1998; Alvarez and Nicieza, 2005) and is affected by abiotic factors, such as temperature (Lee, 2003; Peck, 2005), light conditions (Finstad et al., 2004) and oxygen levels (Gruber and Wieser, 1983). Moreover, individual characteristics, such as age, size, developmental stage and activity affect metabolic rates (Spicer and Gaston, 1999; Willmer et al., 2004). Inheritance is also a source of intra-individual variation in metabolic rate (Pough and Andrews, 1984; Garland and Bennet, 1990). This has been demonstrated in salmonid larvae, in which family differences in metabolic rate have been reported (Pakkasmaa et al., 2006; Regnier et al., 2009).

During the yolk feeding stage in fish larvae, the energy from the yolk reserves is mostly directed towards growth and metabolism. The energy absorbed from the yolk sac is invested in forming new tissue and in respiration (Kamler, 2008), which suggests a close association between larval development and metabolism in yolk feeding fish larvae. A number of studies have shown a relationship between variability in time to first feeding and subsequent growth (McCarthy et al., 2003; Einum and Fleming, 2000). Moreover, Gilbey and collaborators (2009) demonstrated a positive relationship between time to hatch and post-hatch growth during the yolk sac stage. This suggests that ontogenic shifts are related to larval development and future growth. In summary, the above data suggest a close link between hatching, metabolic rate and developmental rate in fish, which could be utilized to predict growth performance and to decrease size heterogeneity in farmed fish.

The aim of this study was to investigate the relationship between hatching and post hatch growth in reared larvae of Atlantic salmon (*Salmo salar*). To achieve this, measurements of

development (the body to yolk weight ratio, total larval length, oxygen consumption and behavioural response to a hypoxic challenge) were compared for larvae with different hatching times.

4.2. Material and methods

4.2.1 Experimental fish

During November and December 2008, eggs from 144 families, originating from 144 females and 74 males, were stripped and fertilized at the facilities of Marine Harvest, Øyerhamn in Hardanger, Norway. In February 2009, eyed eggs at 347 day-degrees after fertilization, DDF, were transported from Øyerhamn to the research facilities of the Technical University of Denmark (DTU) at the North Sea Centre in Hirtshals, Denmark. Due to a lack of space, randomly selected batches of eggs from 100 families (80-100 eggs per family) were incubated separately in individual compartments (9 x 9 x 14 cm). Four incubators were used, and each incubator contained 25 individual compartments. Starting at 454 DDF, each family was photographed every 8 hours. Photos taken at 454 DDF were used to estimate egg size. Family specific egg diameter was estimated in a subsample of 20 eggs from each family. Egg sizes and the number of hatched larvae in each picture were analyzed using the free image analysis software ImageJ (http://rsbweb.nih.gov/ij/). A family was considered hatched when 95% of its eggs were hatched. This approach allowed us to analyze a large number of individuals over rather short time during minimal disturbance of the eggs and larvae. Temperature data was logged every 30 min using Tinytag Aquatic 2 (Omni instruments). Temperature during incubation ranged from 5.3 to 10.7 °C.

4.2.2 Experimental set-up.

To investigate differences in metabolic rate and development in larvae with an early or late hatching time, a group with an early and a group with a late hatching time were formed. This was done by randomly sampling three families with an early and three families with a late hatching time among the 20% earliest and the 20% latest hatching families respectively. 15 individuals within each of these six families were again sampled for further studies (see further down). This approach resulted in an early hatching and a late hatching group with significant different time to hatching (t-test; t-value = -8.12; $p < 0.01$) and no measurable overlap in hatching time. Measurements of larval development and metabolic rate were taken during two sessions (T1 and T2) with two days in between. The average DDF for T1 was 575 DDF and for T2 it was 595 DDF. This period of 20 day-degrees between measuring sessions corresponded to the average difference in hatching time between early (487 ± 3.5 DDF; mean ± S.E.) and late (509 ± 1.1 DDF) hatching groups. This approach allowed us to investigate if the difference in early development indicated by time to hatching persisted later during larval development. Mean (± S.E) egg diameter at 454 DDF from early hatching group was 0.66 ± 0.01 cm; whereas egg diameter from late hatching group was 0.69 ± 0.01 cm. This difference was significant (t-test; t-value = -0.30; $p = 0.03$).

At 554 DDF, the early and late hatching groups were transported from the North Sea Centre in Hirtshals (Denmark) to the Marine Biological Laboratory (MBL) at the University of Copenhagen, in Helsingør (Denmark). At the MBL, individual metabolic rate was quantified by closed respirometry at T1 and T2 in six individuals from each family, resulting in sample sizes of 18 early and 18 late individuals at each session (see below). The day after each session, the individual developmental stage was quantified (see below). Larvae were kept at 10 °C and in

darkness throughout the experiment. Eight early and five late hatching individuals died during this process and were removed from the experiment.

4.2.3. Metabolic rate

Weight-specific oxygen consumption (VO_2) was quantified by closed respirometry according to the method described by Steffensen (1989). However, the method was modified for small test animals. Two chambers were constructed from 6.8 ml vials with rubber corks sealing each chamber. Fiber optic cables connected to PreSens Fibox 3 oxygen monitors were inserted through the corks. The chambers were divided into two compartments separated by a plastic mesh mounted into the chambers with silicone. The test subject was placed in the upper compartment and a magnetic spinner was placed in the compartment underneath to assure mixing and preventing development of oxygen layers.

A pilot experiment was performed to minimize the acclimatization period in the respirometer. This was done to allow the analysis of a high number of individuals in a relative short time. In the pilot study, oxygen consumption was observed to increase occasionally during the first 5 minutes after inserting the larvae in the respirometer. After this it stayed stable for measuring periods of up to 3 hours.

Prior to starting the measurements, an early and a late hatching larva were inserted in two respiratory chambers and left undisturbed for 30 minutes. After this acclimatizing period, the respirometer was closed and early and late hatching individuals were measured at the same time in the two chambers for 30 minutes. Larvae were kept in darkness during the oxygen consumption measurements. Subsequently to the oxygen consumption measurements, the larvae were weighed and the wet weight was used to calculate VO_2. Wet weight was used since the

larvae were kept alive for further study (see below). Respirometry data were logged on a PC using the program Labtech Notebook, and calculations of VO_2 were made following Steffensen and collaborators (1984) and Steffensen (1989). The first 10 minutes of oxygen measurements were excluded to avoid disturbance effects of closing the respirometer. Each larva was kept individually over night before the developmental stage was quantified (see below).

4.2.4. Larval development

The developmental stage of each larva was quantified based on the body to yolk weight ratio (see below), total larval length and the behavioural responses to hypoxic conditions.

Larval mobility and responsiveness increase during ontogeny. In the present study responsiveness to hypoxic conditions was used as a measure of development and was quantified by the protocol described by Höglund and collaborators (2008). Briefly, the behaviour of isolated yolk sac larva was studied inside plastic bottles (11.5 x 8 x 3 cm) with water at either 100% (control) or 10% (hypoxia) oxygen saturation in darkness. For each trial, the behaviour of four larvae (two early and two late) was filmed in darkness with an infrared sensitive camera and an infrared light source at the same time, in either hypoxic or control conditions. The time to initiation of avoidance behaviour, defined as larvae moving more than half a body length in one movement (see Höglund et al., 2008), was measured 1 minute after last disturbance (insertion of the larvae). If no movement was recorded within 10 minutes, the time to initiation of avoidance behaviour was set to 600 s. After quantification of the behavioural response, each larva was conserved individually in 95% ethanol, and transported back to the DTU facilities.

At the DTU, the total larval length (mm) of each larva was measured before the yolk sac was dissected from the body. The total larval length of each fish was not corrected for ethanol

induced shrinkage. Both the yolk sac and body were dried for 2 hours at 45 °C and left at room temperature under an extractor fan over night. After drying, each body and yolk sac was weighed separately. Larval development was quantified by the body to yolk weight ratio, which was determined using the following equation:

$$Body\ to\ yolk\ weight\ ratio = \left(Bw / \left(Bw + Yw\right)\right) * 100$$

where *Bw* is the dry weight (mg) of the larva excluding the yolk and *Yw* is the dry weight (mg) of the yolk.

4.2.5 Statistical methods

All values are presented as mean (± S.E.). Differences between hatching time and measuring session were investigated using a two-way analysis of variances (ANOVA) with weight, egg size, total larval length, body to yolk weight ratio or VO_2 as dependant variables. The post hoc Tukey honest significant difference procedure for multiple comparisons was applied to compare means between different groups. Effects of hatching time, measuring session and oxygen concentration (hypoxia or control) on the time to initiation of avoidance behaviour were investigated using a GLM model, and non-significant terms were removed with a backwards elimination procedure. Time to initiate avoidance swimming behaviour was log transformed to attain normality. Statistical analyses were performed using Statistica version 5.0 (StatSoft, Inc, Tulsa, OK, USA).

4.3. Results

4.3.1 Total wet weight

Early hatching larvae weighed 131 ± 0.6 mg at T1 and 137 ± 0.6 mg at T2, and late hatching larvae weighed 136 ± 0.3 mg at T1 and 134 ± 0.3 mg at T2. The ANOVA did not indicate any differences between the weights of early and late hatching larvae (ANOVA, $F_{(1, 55)} = 0.77$; $p = 0.38$) or between measuring sessions (ANOVA, $F_{(1, 55)} = 1.66$; $p = 0.20$). The ANOVA indicated a significant interaction effect (ANOVA, $F_{(1, 55)} = 4.63$; $p = 0.04$), although the post hoc test did not show any significant effects (for all tests, $p > 0.05$).

4.3.2 Metabolic rate

The ANOVA indicated differences in VO_2 between early and late hatchers (ANOVA, $F_{(1, 55)} = 11.24$; $p < 0.01$) and between T1 and T2 (ANOVA, $F_{(1, 55)} = 37.89$; $p < 0.01$). This was reflected in significantly higher values of VO_2 in late hatchers (Fig. 1) and in significantly higher values observed at T2. No interaction effect between hatching time and measuring sessions was observed (ANOVA, $F_{(1, 55)} = 1.06$; $p = 0.31$).

4.3.3 Larval development

No differences in the body to yolk weight ratio between early and late hatchers were detected (ANOVA, $F_{(1, 55)} = 0.13$; $p = 0.71$). However, differences between T1 and T2 (ANOVA, $F_{(1, 55)} = 24.17$; $p < 0.01$) indicated a higher body to yolk weight ratio at T2. No interaction effect between hatching time and measuring session was observed (ANOVA, $F_{(1, 55)} = 0.29$; $p = 0.59$) (Fig. 2).

The total larval length of the larvae showed the same general pattern as the body to yolk weight ratio. The two-way ANOVA indicated no differences between early and late hatchers (ANOVA, $F_{(1, 55)} = 0.30$; $p = 0.57$), but differences between T1 and T2 were detected (ANOVA,

$F_{(1, 55)} = 49.28$; $p < 0.01$). No interaction effect between hatching time and measuring session was observed (ANOVA, $F_{(1, 55)} = 0.10$; $p = 0.75$). The post hoc test shows that larvae at T2 were significantly longer than those at T1. Late hatching larvae were 21.13 ± 0.04 mm at T1 and 22.31 ± 0.04 at T2, and early hatching larvae were 21.27 ± 0.06 at T1 and 22.34 ± 0.04 at T2.

The mean (± S.E) time to initiate avoidance behaviour between early and late hatching groups was 298.57 (± 48.13) s and 262.97 (± 40.05) s, respectively. For the hypoxic conditions, the mean (± S.E.) time to initiate avoidance behavior in 100% oxygen saturation was 407.94 (± 39.17) s, and in 10% oxygen saturation it was 128.07 (± 29.06) s. Between measuring sessions, the mean (± S.E) time to initiate avoidance behavior at T1 was 335.48 (± 44.71) s, and at T2 was 226.10 (± 41.00) s. The GLM procedure indicated significant effects of hypoxic condition (GLM, $F_{(1, 56)} = 28.62$; $p < 0.01$) and measuring sessions (GLM, $F_{(1, 56)} = 9.24$; $p < 0.01$) on the time to initiate avoidance behaviour. However, no effect of hatching time or the interactions terms were detected, and were removed with a backwards elimination procedure.

4.4. Discussion

In this study, an increase in VO_2 was observed as the larvae developed. Furthermore, larvae originating from late hatching families had higher VO_2 compared to larvae originating from early hatching families. However, differences between early and late hatchers in post-hatch development (i.e., the body to yolk weight ratio, the total larval length and the responsiveness to hypoxic condition) were not observed. This finding suggests that the differences in development indicated by time to hatch were evened out by faster development in late hatching individuals.

In general, larger eggs are considered to develop slower and hatch later (Pauly and Pullin, 1988; Teletchea et al., 2009). Even if the latter studies are based on inter-species comparisons

with large differences in egg size, the present results showing that eggs from the early hatching families were smaller than late hatchers is coherent with this. However, a higher VO_2 was measured in late hatching individuals. In fish larvae, the energy absorbed from the yolk sac is mainly invested in the forming of new tissue and the in respiration (Kamler, 2008), and little energy is spent on other activities (Killen, 2007). Taken together, these differences in developmental rate before hatching can be evened out by a higher post-hatch metabolic and developmental rate.

Several studies have shown a metabolic shift and higher oxygen consumption during hatching (Kamler et al., 1994; Kamler, 2008), and it is possible that these events triggered the higher metabolic rate in late hatching individuals observed in the present study. One must keep in mind that both abiotic and biotic factors influence the timing of hatching, and suboptimal environmental factors, such as low oxygen levels and high temperatures, have been shown to induce premature hatching (Jungwirth and Winkler, 1984; Czerkies et al., 2001). In the present study, eggs and larvae were incubated in well oxygenated water and at a temperature that presumably would not induce premature hatching. Therefore, the observed differences in hatching time were most likely of a biotic nature.

Gilbey and collaborators (2009) showed that inherited factors are involved in both hatching time and post-hatch growth. Furthermore, they found a positive correlation between hatching time and size-specific growth, which suggested that length differences observed just after hatching between early and late hatching larvae did not persist after first feeding. In the present study, the 20 day-degrees difference in hatching time, was not reflected in differences in oxygen consumption, length and development between the two measuring sessions, T1 and T2. Moreover, late hatching individuals showed a generally higher metabolic rate. Taken together,

the results from the present study show the same trend as Gilbey and collaborators (2009), suggesting a catch-up growth in yolk sac larvae. Other studies have showed a rather constant increase in metabolic rate in yolk feeding larvae and eggs (Pakkasmaa et al., 2006; Regnier et al., 2009). In the present study, oxygen consumption and larval development were quantified in a rather narrow interval of developmental time, and further studies of individual developmental and metabolic rate throughout larval development are needed to clarify growth trajectories during early ontogeny in fish.

In a natural environment, inherited components of enhanced growth have been linked to being sired by precocious males (Garant, 2002), but this effect is not associated with farmed populations of salmon because of the different selection of males (Garant et al., 2002). Moreover, the effect of precocious males has also been suggested to be involved in post-hatch growth differences in Atlantic salmon yolk sac larvae (Gilbey et al., 2009). However, the catch-up growth observed in late hatching individuals in the present study contradicts this suggestion and demonstrates that variability in post-hatch growth is present in farmed populations in which the genetic contribution of precocious males is supposed to be low. Furthermore, in natural populations of salmonid fish it is usually considered that early emerging individuals are thought to have a competitive advantage (e.g. Einum and Fleming, 2000), and individual variation in time for swim up and first feeding have been shown to predict social dominance, growth and life history strategies (McCarthy et al., 2003; Metcalfe and Thorpe, 1995; Metcalfe et al., 1992). Still, the relationship between larval development at first feeding and future growth in a hatchery environment is debated (Gilbey et al., 2005). The present study suggests that under captive conditions, larvae of Atlantic salmon selected according to hatching time differ in growth after

hatching. Future studies are needed to clarify if these differences are also reflected in growth performance later in the production cycle.

Metabolic rate in the present study was quantified via closed respirometry with a rather short acclimatization period compared to what is normally used in fully developed fish. This approach was used to allow measurements of a large number of individuals during a relatively short period of time. Closed respirometry is normally not recommended (Steffensen, 1989), but in the present study it was justified since the metabolic rate was low and hence did not cause neither hypoxic nor hypercapic conditions during the trial. In general, fish larvae have more limited metabolic scope than fully developed fish and invest most of their energy to growth (Killen et al., 2007). This suggests that stress-induced effects on metabolic activity are less pronounced in fish larvae than in adult fish. Moreover, no differences in behavioural response to hypoxic conditions (see below) between larvae originating from early and late hatching groups were observed. Furthermore, differences in behavioural response to hypoxic conditions have been shown to be associated with inherited differences in stress responsiveness (Höglund et al., 2008). In the present study, such behavioural differences were not observed between families with early and late hatching times. This result lends further support to our premise that the differences in VO_2 between early and late hatching larvae observed in the present study are more related to resting metabolic rate than stress reactions to the respirometer.

Generally, larval mobility and responsiveness increase during development (Pakkasmaa et al., 2006), and our results support this. The findings of the present study shows that larvae at T2 tended to have a generally shorter lag time before moving half of their body length compared to T1. Moreover, hypoxic conditions induced a decrease in this lag time, an effect that was not related to VO_2. This suggests that factors other than metabolic rate are involved in this response,

which is in agreement with studies showing that avoidance behaviour to hypoxic conditions is associated with stress responsiveness in yolk sac larvae (Höglund et al., 2008) and that the larvae and adult fish show avoidance behaviour to hypoxic water layers (Weltzien et al., 1999; Petersen and Petersen, 1990; Petrosky and Magnuson, 1973; Magnuson et al., 1985). Differences between the two measuring sessions in larval response to hypoxic conditions were not detected in the present study, indicating that this response occurs earlier in the development than was examined in this study.

4.5. Conclusions

The present study illustrates family differences in time to hatch of Atlantic salmon, which suggests an inherited component in individual developmental rate. However, faster post-hatch growth and development in larvae originating from late hatching families evened out this difference. This finding demonstrates that differences in growth occur after hatching, and further studies are needed to investigate how this affects future performance of salmonid fish.

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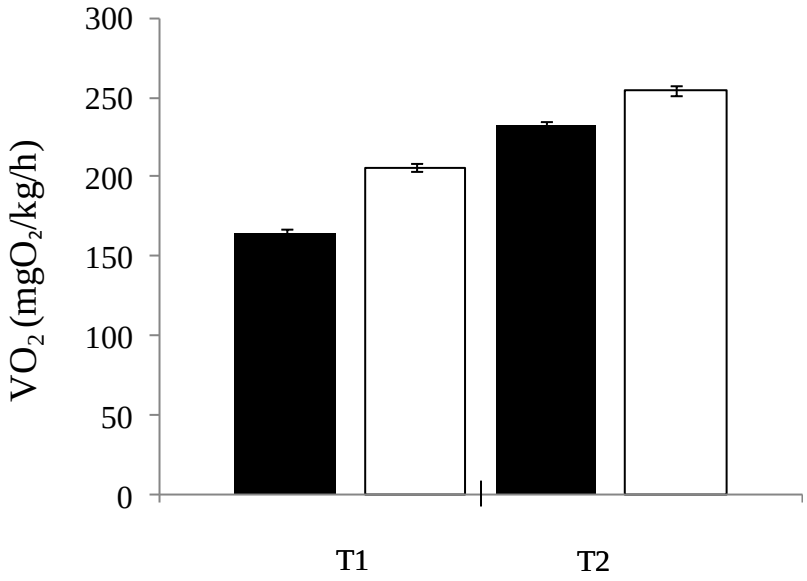
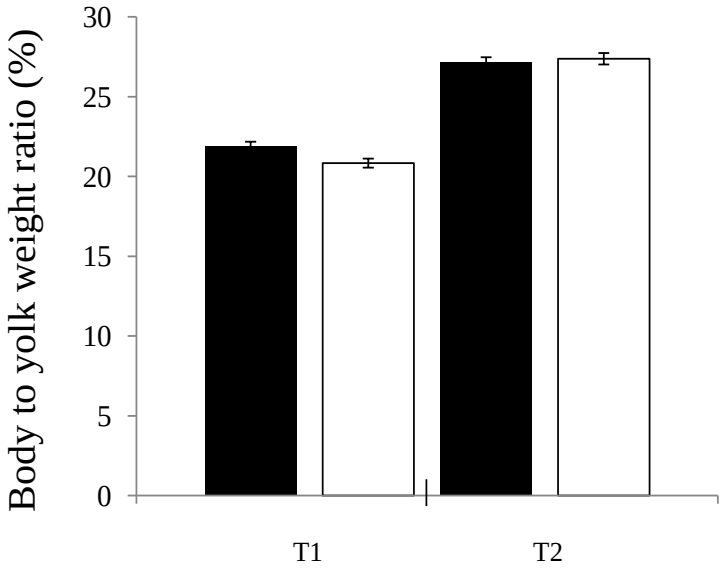
Fig. 1. Weight-specific oxygen consumption (VO_2) in yolk sack larvae originating from Atlantic salmon (*Salmo salar*) families with an early (■) or late time to hatching (□). VO_2 was measured at two time points: 560–570 (T1) and 590–600 (T2) day-degrees after fertilization. N =15–16 individuals in each group in each session. No significant interaction was found between the hatching time and the measuring sessions (see text).



Hatching time (ANOVA, $F_{(1,55)} = 11.24$; $p < 0.01$)
Measuring session (ANOVA, $F_{(1,55)} = 37.89$; $p < 0.01$)

Fig. 2. Body to yolk weight ratio (%) of larvae originating from Atlantic salmon (*Salmo salar*) families with an early (■) or late time to hatching (□). The body to yolk weight ratio is the weight proportion between the body excluding the yolk and the body including the yolk. Larvae were sampled at 570–580 (T1) and 600–610 (T2) day-degrees after fertilization DDF. N =15–16 individuals in each group in each sampling session. No significant interaction was found between the hatching time and the measuring sessions (see text).



Hatching time (ANOVA, $F_{(1,55)} = 0.13$; $p = 0.71$)
Measuring sessions (ANOVA, $F_{(1,55)} = 24.17$; $p < 0.01$)

PAPER II

5. SELF-SORTING OF ATLANTIC SALMON (*SALMO SALAR*) BASED ON TIME TO EMERGE FROM AN ARTIFICIAL REDD: A NOVEL METHOD REVEALING INTER FAMILY RELATIONSHIPS BETWEEN EGG CHARACTERISTICS, LARVAL DEVELOPMENT AND EMERGENCE TIME.

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Abstract

Egg characteristics, larval development and emergence from the spawning redd have all been related to future performance in salmonid fish. However, it is still poorly documented how these factors are related to each other. In the present study, a new method for sorting fish with respect to the time to emerge from an artificial redd was used to investigate the relationship

between family variation in the time to emerge and larval development of Atlantic salmon (*Salmo salar*). As well, the relationships between emergence time, egg diameter and hatching time on the genetic level were studied. The results show that earlier emergence time was associated with faster larval development; however, egg diameter and hatching time were not related to emergence time. These findings suggest that hatching time and emergence time are not coupled in salmonid fish. The method presented in this study shows that emergence from an artificial redd could be used to predict and optimize future growth in aquaculture.

Keywords: embryonic development, larval development, swim-up, salmonid fish, aquaculture.

5.1. Introduction

There are three major events in the early life history of fish: fertilization, hatching and first feeding. It has been found that these events, and its relationships with other traits, influence future performance of fish. For example, egg size is related to embryonic development and time to hatch, as well as larval growth of fish (Kamler, 2002; Kamler and Kato, 1983; Pakkasmaa and Jones, 2002). The time to emerge, i.e. when salmonid larvae leave the spawning redd and start exogenous feeding, is related to future growth, social dominance and smoltification (McCarthy, et al., 2003; Metcalfe and Thorpe, 1992; Metcalfe, et al., 1995). Taken together, this suggests a link between ontogenic shifts and future traits. Thus, easily distinguished ontogenic milestones could potentially offer an early selection criterion for future growth performance, which could be utilized for optimization of rearing conditions in aquaculture. However, it is not well understood how characteristics such as egg size, larval development and emergence time are related in salmonid fish. This could be due to the difficulty of monitoring individual eggs from hatching to

emergence. Some studies have followed families from egg to emergence instead (Gilbey, et al., 2005).

The aim of this study was to investigate family variation in the time to emerge from an artificial redd, and to determine if this variation is related to egg size, hatching time and larval development in Atlantic salmon (*Salmo salar*). To achieve this, a novel method for sorting salmonid larvae with respect to the time to emerge from an artificial redd was developed. Family variation in time to emerge was thereafter related to the mean egg diameter, the hatching time and the larval development. The advantages of sorting fish according to the time to emerge from an artificial redd in captive fish are discussed.

5.2. Material and methods

5.2.1 Experimental fish

During November 2008, eggs from 144 families, originated from 144 females and 74 males, were stripped and fertilized over a period of 3 weeks. Families with different fertilization dates were incubated at different temperatures in order to synchronise their development by attaining equal day-degrees after fertilization (DDF). In February 2009, 80-100 eyed eggs per family were transported from the hatchery of Marine Harvest, Øyerhamn, Hardanger, Norway, to the research facilities of the Technical University of Denmark (DTU) at the North Sea Centre in Hirtshals, Denmark, where the experiments were carried out. 30 eggs from each family were separated and sorted according to the emergence time (see below). At the same time, 50-70 eggs from 100 of these 144 families were randomly selected and incubated in individual incubators (9 x 9 x 14 cm). In these families, the mean egg diameter and hatching time were recorded (see below). In addition, 20 larvae from each family were conserved in 95% ethanol at three different

sampling times (534, 619 and 703 DDF, denoted hereafter as T1, T2 and T3, respectively) to investigate their larval developmental rate.

5.2.2 Egg diameter, hatching time and developmental rate

In order to minimize stress, each family was photographed every 8 hours. Using those photographs, the egg diameter (at 454 DDF) and hatching time were estimated. The free image analysis software ImageJ (http://rsbweb.nih.gov/ij/) was used to measure the egg diameter of 20 randomly selected eggs from each family. From each photograph, the number of larvae that had hatched was also counted. Hatching time was measured as the DDF when 95% of the eggs in each family had hatched. Temperature data was recorded every 30 min using Tinytag Aquatic 2 (Omni instruments). The minimum and maximum temperatures during incubation were 5.3 and 10.7 °C.

In order to determine the developmental rate from each sampling time, the total length (mm) of every larva conserved in ethanol was measured, after which the yolk sac was dissected apart from the larval body. The total length of each fish was not corrected for ethanol induced shrinkage, but this factor was assumed to be constant between families. Afterwards, both the yolk sac and the body larva were dried during 2 hours at 45 °C and left at room temperature over night under an extractor fan. The following day, each body larva and yolk sac was weighed. The body to yolk weight ratio, defined as the weight proportion between the larva excluding the yolk and the larva including the yolk, was calculated for each sampling time (T1, T2 and T3) using the following formula:

$$Body\ to\ yolk\ weight\ ratio = (Bw/ (Bw + Yw))*100$$

where, *Bw* is the dry weight of the body larva and *Yw* is the yolk sac dry weight.

When all the calculations were done, the mean total larval length and the mean body to yolk weight ratio were calculated for each family.

5.2.3 Emergence time

Eggs from the 144 families were randomly divided in three flat-screen incubators. 30 randomly selected eggs from each family were placed inside the incubators. Batches of eggs were reared until hatching to facilitate removal of unviable eggs. At 654 DDF, these three batches where moved to three incubators designed for sorting fish in respect to the time to emerge. These incubators consisted of an incubation area (45 x 70 x 14 cm), where one layer of golf balls was placed to provide the larvae with a shelter that imitated natural gravel. The incubation area was separated by a wall from a section with a PVC tube that ended in a collecting net underneath. Water flow was directed towards the collecting net (Fig 1). The incubators were covered by a layer of expanded polystyrene to avoid light induced stress and minimize disturbance. The larvae were sorted by the time to emerge since they were carried downstream to the collecting net when they approached to the surface to search for endogenous nutrition. During the first seven days after the larvae were inserted in the incubators, only 5 - 20 individuals per incubator (0.34 – 1.36 % of the initial number of eggs) were collected daily. These individuals had a considerable amount of yolk remaining and were re-introduced to the incubators. At 718 DDF, the number of individuals in the collecting nets with a small yolk sac increased to 40-50 in all the incubators; therefore the sorting of individuals in respect to emergence time started. After 17 days (at 870 DFF) no larva remained in the incubators and 85.3 % of the eggs had hatched successfully and reached emergence (Fig 2). During emergence, the minimum and maximum water temperature was 7.2 and 10.4 °C, respectively.

The first 25% (early fraction) and the last 25% (late fraction) of individuals that emerged from the incubators were subjected for further analysis. The remaining larvae were preserved in 95% ethanol once they emerged from the incubators following emergence. The fry were reared in six 30 l tanks, each tank containing the first or the last emerged fry of each incubator. At 890 DDF, the fish were transferred to 60 l tanks. The fry were fed with larval feed START (Biomar) to satiation using automatic feeders. The pellet size was increased subsequently following the manufacturer recommendations. At 1257 DDF, fish from the early and late fractions were counted and fin clipped. The fin samples were preserved in 95% ethanol and later DNA-typed for parental assignment using 9 micro-satellite markers. Thereafter, the actual size of each fraction was back-calculated based on the numbers of individuals that died between emergence and fin clipping. 37.3% of early emerged fry died, whereas only 9.4% of late emerged fry died. The mortalities were taken into account when calculating the family variation in emergence time using the formula:

$$\textit{Family emergence index} = (\textit{Early} + (\textit{Early} * 0.373)) - (\textit{Late} + (\textit{Late} * 0.094))$$

where *Early* and *Late* are the number of individuals observed in each fraction respectively during fin clipping.

In total, data on the egg size, the hatching time, the total length, the yolk body weight ratio and the emergence time were obtained for 100 families of Atlantic salmon. To minimize possible biases induced by the different incubation temperatures applied after fertilization, 3 families fertilized at November 13, 2008 and 6 families fertilized at December 13, 2008 were excluded from the analysis. The remaining 91 families included in the analyses were fertilized between November 19 and November 27, 2008. Another 6 families that had at least one measurement missing were also excluded from the analysis. The remaining families (n = 85)

were divided according to the *family emergence index* into the following fractions: families with the 25 % highest index were labelled as early emerged families, the families with 25 % lowest index were labelled as late emerged families and the middle fraction (50%) was labelled as the intermediate fraction. This resulted in 21 families categorized as early, 42 as intermediate and 22 as late emerged families.

5.2.4 Statistics

A Pearson correlation coefficient between the egg diameter and the DDF until hatching was used to assess the relationship between the egg diameter and the hatching time. A one-way analysis of variances (ANOVA) was applied to determine the effect of egg diameter and hatching time on the time to emerge. General linear models (GLM) procedures followed by Tukey post hoc tests were performed to investigate if inter family variation in emergence time was related to larval development using the total length and the body to yolk weight ratio at T1, T2 and T3. All the statistics were performed using the SAS 9.1 statistical package for personal computers.

5.3. Results

5.3.1 Relationships between the egg diameter, the hatching time and the emergence time

A positive correlation was present between the mean egg diameter and hatching times of the families included in this study (Pearson correlation; $R = 0.4$; $p < 0.01$).

The families with an early, intermediate or late emergence time did not differ in the mean egg diameter (one-way ANOVA, $F_{(2, 82)} = 2.4$; $p = 0.1$) or in hatching time (one-way ANOVA, F

$_{(2, 82)}$ = 0.1; p = 0.93). Mean values (± S.E) for the egg diameter and hatching times for families with different emergence time are presented in Table 1.

5.3.2 Relationships between the total larval length, the body to yolk weight ratio and the emergence time

In general, total larval length increased between T1, T2 and T3 (GLM, $F_{(2, 246)}$ = 1404.2; p < 0.01). Furthermore, the early emerged families were longer compared to the intermediate and the late emerged families, although the intermediate and the late emerged families had similar total larval length (GLM, $F_{(2, 246)}$ = 10.8; p < 0.01; Fig 3). These length differences between the families with different time to emerge were independent of the sampling times (GLM, $F_{(2, 246)}$ = 1.7; p = 0.14). The mean values (± S.E.) are presented in Table 2.

Regarding the body to yolk weight ratio, the same pattern was observed as in the total larval length. The body to yolk weight ratio increased between the sampling times (GLM, $F_{(2, 246)}$ = 668.1; p < 0.01). Moreover, the early emerged families had a higher body to yolk weight ratio compared to the intermediate and the late emerged families. As well, the intermediate emerged families had higher body to yolk weight ratio than late emerged families (GLM, $F_{(2, 246)}$ = 11.9; p < 0.01; Fig 4). However, the differences in the body to yolk weight ratio between families with different emergence time was independent of the effect of sampling times (GLM, $F_{(2, 246)}$ = 1.2; p = 0.32). The mean values (± S.E.) are presented in Table 2.

5.4. Discussion

In the current study, a novel method for sorting yolk sac larvae of salmonid fish according to the time to emerge from an artificial redd was introduced. By applying this method,

it was shown that the inter family variation in emergence time is related to the larval development, but not to the hatching time or to the mean egg size. The results suggest that hatching time and emergence time are not coupled in salmonid fish.

In the present study, there was a positive relationship between the egg diameter and hatching time. This result is coherent with previous findings, where smaller eggs tend to hatch earlier (Kamler, 2002). However, neither the egg diameter nor the hatching time was related to the emergence time. Unpublished results from our research group indicate that the differences in development present at hatching can be compensated by catch up growth in the late hatching larvae during the yolk sac stage. Together with the results presented in this study, this suggests that the timing of two major events in the early ontogeny of salmonid fish, hatching and time to emerge, are in fact not tightly coupled.

Regarding the larval development, the present results indicate that the families with the fastest growing larvae emerged from the artificial redd at an earlier time point. Since the larval development is closely related to the metabolic rate (Vaz-Serrano et al., unpublished), this could indicate that the larva emerging earlier may also have higher metabolic rates. Previously, higher metabolic rates have been related to a higher potential for fast growth and earlier smoltification age (Metcalfe and Thorpe, 1992). If differences in growth continue after emergence, selecting fry according to the time to emerge could be used to optimize rearing regimes in aquaculture, before fry enters the most cost intensive part of the production cycle. However, Gilbey and collaborators (2005) reported that pre- and post-first feeding growth was unrelated in Atlantic salmon, suggesting that the advantages gained during the yolk sac phase may disappear as the fish grows on exogenous food. However, that study was carried out in a hatchery environment, where larvae had plentiful food supply; therefore differences in growth could be present when

there is competition for resources, as occurs in nature. Hence, further studies are needed to investigate if the described differences in growth are still present after emergence.

In the present work, it was shown that all larvae had emerged from the incubators by 152 day-degrees, i.e. approximately 17 days at 8.9 °C. Similar results have been found in laboratory conditions in salmonid fish (Brännäs, 1995; Sundström, et al., 2005), as well as in nature (De Leaniz, et al., 2000) under similar temperature conditions. This suggests that the method to sort fish by the emergence time presented here is likely reflecting a natural phenomenon (emergence time from a salmonid redd).

Sveier and Raae (1992) investigated the potential use of incubators with substrate, and they achieved higher growth rates when larvae were reared in artificial turf incubators. In the presented method, artificial turf substrate was replaced by golf balls, which worked successfully at higher densities (personal observations). As well as improving incubation conditions by resembling natural spawning conditions, the method presented here could be used to sort individual fish and predict future growth in salmonid fish reared under hatchery conditions. Furthermore, initiation of first feeding is based on the average developmental stage of the reared larva. As demonstrated by this study, differences between the first and the last larvae to emerge can last more than two weeks, and can cause early emerging fish to starve, affecting their growth and survival (Sveier and Raae, 1992). By using the method presented here, first feeding could be adjusted for suitable batches of fry, thus increasing overall growth and survival during the start-feeding period.

Approximately 5 weeks after all fry came out of the incubators, higher mortalities were observed among fry with an early emergence time. The observed mortalities are not expected to be a consequence of starvation, since food was provided right after emergence. In semi-natural

conditions, reduced survival was also observed in early emerged fry (Sundström, et al., 2005), although in the last study mortalities were suggested to be caused by higher predation at the beginning of the emergence season. The absence of predators here suggests that other factors may be the cause of differential mortalities between the early and late fractions. Individuals with an early time to reach first feeding are known to be more aggressive (Metcalfe, et al., 1995), and it is possible that the higher mortality in the early fraction could be related to more pronounced social competition in the early fraction. Another not exclusive possibility is that the faster development in early emerged fry may reduce the survival of early emerged fry.

In conclusion, the method presented in this study is a promising tool for sorting salmonid fish with respect to the time to emerge from an artificial redd. By applying this method in a commercial salmon hatchery, advantages such as enhanced incubation conditions and increased growth could be achieved in salmonid fry. Further studies are needed to investigate if growth differences between early and late emerged fry continue after emergence.

Acknowledgments

We are grateful to Marine Harvest for its contribution of the experimental fish. As well, we thank to the staff at DTU Aqua for assistance with the rearing of the fish. This study was supported by The Research Council of Norway.

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Fig 1. Design of the incubator used to sort Atlantic salmon larvae with respect to the time to emerge from the artificial redd.

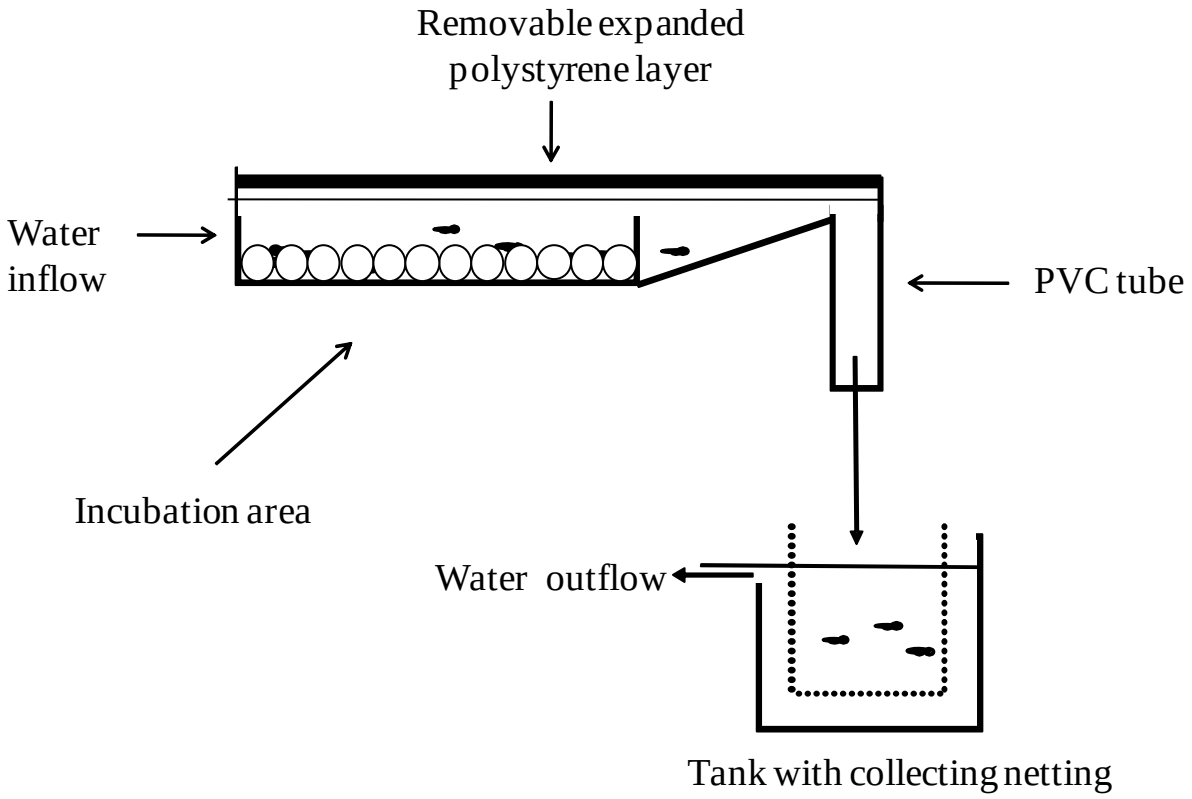

Fig 2. The number of individuals that emerged from the three incubators which allowed the larvae to swim freely to a collecting tank when nearly all the yolk sac was consumed. Each column represents one incubator at one sampling time. Emergence started at 718 DDF (i.e. the March 21, 2009) and was finished at 870 DDF (i.e. April 7, 2009).

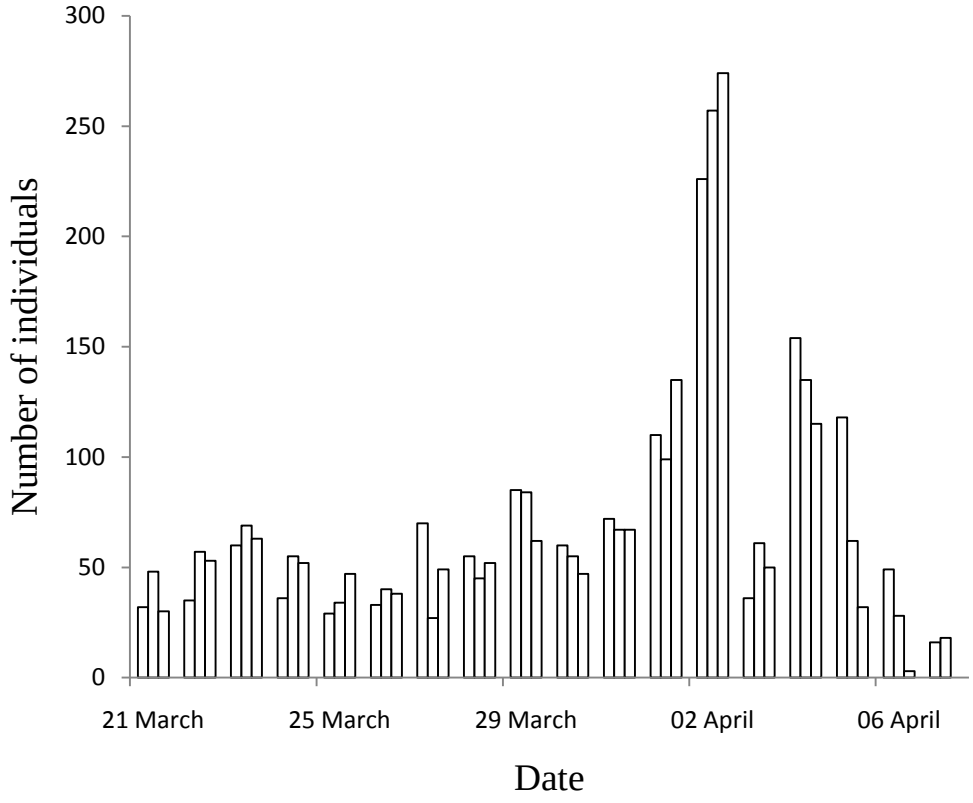

Fig 3. The average total larval length (mm) (± S.E.) of yolk sac larvae in the families with an early (□), intermediate (▩) or late (■) emergence time. The values represented are averaged from the three sampling times (T1, T2 and T3). N= 21 early, 42 intermediate and 22 late emergence families at every sampling time. Lower cases indicate significant differences ($p < 0.05$). Significant differences were shown between the emergence time and between the sampling times; although no interaction effect was observed (see text).

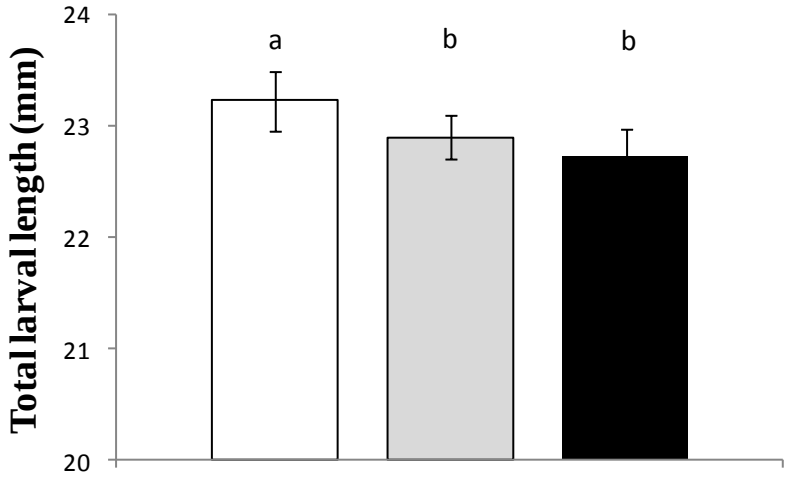

Fig 4. The average body to yolk weight ratio (%) (± S.E.) of yolk sac larvae in the families with an early (□), intermediate (■) or late (■) emergence time. The values represented are averaged from the three sampling times (T1, T2 and T3). N= 21 early, 42 intermediate and 22 late emergence families at every sampling time. Lower cases indicate significant differences ($p < 0.05$). Significant differences were shown between the emergence time and between the sampling times; although no interaction effect was observed (see text).

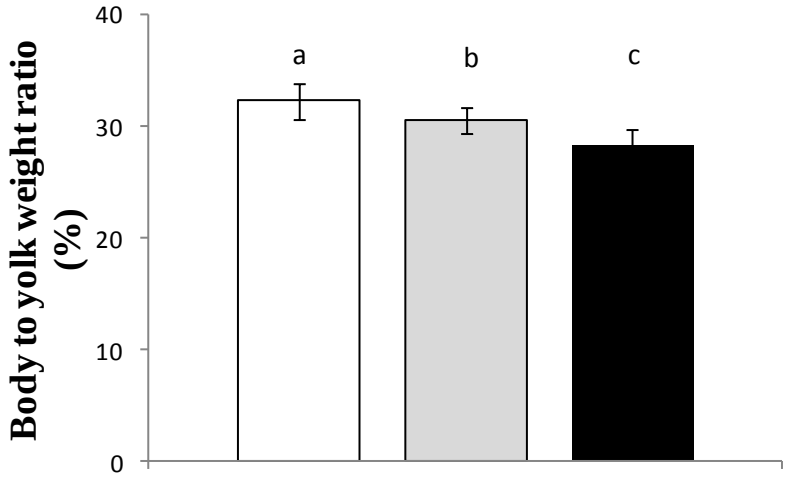

Table 1. The average values (± S.E.) of the egg diameter (mm) and hatching time, measured as day degrees since fertilization (DDF), of families with different emergence time. No differences in the mean egg diameter and the hatching time were shown for families of Atlantic salmon (*Salmo salar*) with different emergence time (see text).

Emergence time	**N**	**Egg diameter (mm)**	**Hatching time (DDF)**
Early	21	6.8 ± 0.1	497.1 ± 1.2
Intermediate	42	6.7 ± 0.1	496.5 ± 0.9
Late	22	6.8 ± 0.1	496.6 ± 1.2

Table 2. The average values (± S.E.) of the total larval length (mm) and the body to yolk weight ratio (BYR) (%) between larvae from families of Atlantic salmon (*Salmo salar*) with an early, intermediate or late time to emerge from an artificial redd. The larvae were collected at three different sampling dates: 534, 619 and 703 DDF (denoted as T1, T2 and T3).

	Emergence time					
	Early		**Intermediate**		**Late**	
Sampling time	**Length**	**BYR**	**Length**	**BYR**	**Length**	**BYR**
T1	20.6 ± 0.1	19.4 ± 0.4	20.1 ± 0.1	18.1 ± 0.4	20.3 ± 0.1	17.4 ± 0.5
T2	23.5 ± 0.1	29.6 ± 0.8	23.3 ± 0.1	28.1 ± 0.6	23.0 ± 0.1	25.7 ± 0.9
T3	25.6 ± 0.2	47.8 ± 1.0	25.3 ± 0.1	45.4 ± 1.1	24.9 ± 0.2	41.8 ± 1.5

PAPER III

6. CONSISTENT BOLDNESS BEHAVIOUR IN EARLY EMERGING FRY OF DOMESTICATED ATLANTIC SALMON (*SALMO SALAR*): DECOUPLING OF BEHAVIOURAL AND PHYSIOLOGICAL TRAITS OF THE PROACTIVE STRESS COPING STYLE.

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Abstract

Individual variation in the way animals cope with stressors has been documented in a number of animal groups. In general, two distinct sets of behavioural and physiological responses to stress have been described: the proactive and the reactive coping styles. In comparative studies using natural populations of salmonid fish, some characteristics of stress coping styles seem to be coupled to the time of emergence of fry from spawning redds. In the

present study, behavioural and physiological traits of stress coping styles were compared two and five months after emergence in farmed Atlantic salmon (*Salmo salar*), using individuals with an early or late time to emerge. Initially, compared with late emerging individuals, early emerging individuals showed a shorter time to resume feeding after transfer to rearing in isolation. Resumption of feeding after isolation was suggested to be related to boldness behaviour, rather than hunger, in the present study. This observation was repeated five months after emergence, demonstrating behavioural consistency over time in this trait. However, in other traits of the proactive and reactive stress coping styles, such as social status, resting metabolism or post stress cortisol concentrations, early and late emerging individuals did not differ. Therefore, this study demonstrates that boldness in a novel environment is uncoupled from other traits of the proactive and reactive stress coping styles in farmed salmonid fish. It is possible that this decoupling is caused by the low competitive environment in which fish were reared. In natural populations of salmonid fish, the higher selection pressure at emergence could select for early emerging individuals with a proactive coping style.

Keywords: Swim-up, cortisol, oxygen consumption, dominance, animal personalities.

6.1. Introduction

Individual differences in the way animals respond to a challenge are often referred to as behavioural syndromes, coping styles, temperaments or animal personalities (Wolf, et al., 2007), and have been suggested in mammals (Koolhaas, et al., 1999), birds (Groothuis and Carere, 2005), reptiles (Øverli, et al., 2007) and fish (Øverli, et al., 2002). In particular, behavioural syndromes are characterized by behavioural responses that are correlated across different situations (Sih, et al., 2004), whereas the term stress coping styles is often used to describe suits

of behavioural and physiological responses to challenges that are constant over time (Koolhaas, et al., 1999). Such behavioural and physiological differences have been clustered into two characteristic responses, termed proactive and reactive stress coping styles (Koolhaas, et al., 1999). Proactive individuals are characterized by a fight-flight adrenaline-based stress response, social dominance, routine formation and bolder behaviour. Reactive individuals, on the other hand, show a freeze-hide cortisol-based stress response, social subordination, behavioural flexibility and a more shy behaviour (Koolhaas, et al., 1999; Korte, et al., 2005; Coppens, et al., 2010).

Several studies have suggested that the degree to which different characteristics correlate to each other may depend on the context in which they are expressed and on the magnitude of the selection pressure (Bell and Stamps, 2004; Huntingford, 2004; Huntingford and Adams, 2005; Bell and Sih, 2007; Brelin, et al., 2008; Conrad and Sih, 2009; Evans, et al., 2010). For example, in sticklebacks (*Gasterosteus aculeatus*), bolder males were more aggressive towards other individuals when predation pressure was high, whereas this correlation was absent when the predation pressure was low (Bell and Sih, 2007). Similar findings were observed in song sparrows (*Melospiza melodia*), where the correlation between aggression and boldness varied between populations adapted to human presence and populations in the wild (Evans, et al., 2010). Fish domestication has also been shown to affect the relationship between behavioural and physiological traits (Huntingford, 2004; Huntingford and Adams, 2005; Brelin, et al., 2008; Conrad and Sih, 2009). Moreover, the relation between traits in an individual can be produced by both selection and by behavioural plasticity (Bell and Sih, 2007). In rainbow trout (*Oncorhynchus mykiss*), for example, it has been demonstrated that the energetic status of an

individual can affect the relationship between physiological and behavioural traits (Ruiz-Gomez, et al., 2008).

Stress coping styles have been suggested to be related to the energy utilization and metabolic rate of an individual (Careau, et al., 2008). In accordance with this, fast-growing individuals tend to have higher metabolic rates, show higher levels of aggression when fighting over food resources and bolder behaviour while foraging in the presence of a predator (Biro and Stamps, 2008). In the common carp (*Cyprinus carpio*), individuals displaying a risk taking behaviour had higher metabolic rates and lower stress responsiveness, compared with individuals showing a risk avoidance behaviour (Huntingford, et al., 2010). A link between stress coping styles and metabolism may exist in salmonid fish, where individuals with higher metabolic rates grow faster and are more likely to become dominant, compared with individuals with a lower metabolic rate (Metcalfe and Thorpe, 1992; Metcalfe, et al., 1995). All this suggests that the proactive coping style is associated with a more costly strategy, whereas the reactive type is characterized by an energy conserving strategy, as suggested by Korte and collaborators (2005).

In Atlantic salmon (*Salmo salar*), the time to emerge from the spawning redds may vary as much as 2 weeks (Brännäs, 1995). Unpublished results from our lab suggest that early emergence in salmonid fish is associated with a higher metabolic rate during the larval phase. Moreover, early emerging fry have been demonstrated to have higher metabolic rates and to be more dominant over late emerged fry (Metcalfe and Thorpe, 1992). This indicates that timing for emergence could be related to stress coping styles. However, it is still unknown if such differences are present in domesticated fish and if differences are maintained over time.

Therefore, the aim of this study was to examine the relationship between emergence time and selected traits indicative of the stress coping styles in domesticated Atlantic salmon. Larvae

were incubated in artificial spawning redds, which allowed fry to be sorted with respect to the time to emerge. Two months later, resumption of feeding after isolation, standard metabolic rate, post stress cortisol concentrations and social status were compared between fractions of early and late emerging fry. In addition, the aim was to investigate whether differences between early and late fractions of fish were consistent over time and thus, differences detected in this first set of experiments were re-investigated five months after emergence.

6.2. Material and methods

6.2.1 Study material and experimental design

Atlantic salmon larvae were sorted according to the time to emerge from artificial redds. A total of 4320 eggs were reared until hatching and then hatched larvae were placed in three incubators with artificial redds, which allowed fry to swim freely to a collecting net when their yolk reserves were nearly emptied. Emergence occurred between March 21 and April 7, 2009, and fish were sorted into different fractions according to the time of emergence from the artificial redds. Approximately, 3686 fry emerged from the incubators and the first and last 25% of emerging fry were separated for future investigation and reared in triplicates in 60 l holding tanks. Fish were fed with automatic feeders at a daily ratio corresponding to 4% of the biomass per day for the duration of the experiments.

During May-June 2009, two sets of experiments were carried out. In the first set of experiments, the time taken to resume feeding after isolation, standard metabolic rate (SMR) and whole body post stress cortisol concentrations after confinement stress were measured. From each of the early and late fractions, 19 individuals were randomly selected and isolated for 5 days in ten 20 l observation aquaria with a removable opaque dividing wall in the middle of the

tank. Out of these selected fish, 8 individuals from each fraction were used as unstressed controls (see below).

In the second set of experiments, 9 pairs of fish with a similar body mass (within pair difference < 5%) composed of one early and one late emerging individuals were isolated for 7 days in the same observation aquaria as described above. Following isolation, the dividing wall was carefully removed and fish were allowed to interact for 24 h after which social status was determined (see below).

A third set of experiments was conducted during September 2009 to investigate if the behavioural differences detected in the two first sets of experiments were consistent over time. For this experiment, 16 early and 16 late emerging individuals were chosen from the holding tanks and isolated in 180 L glass aquaria. One late emerging individual died during the experiment and was removed from the analysis. Each aquarium was divided in 4 compartments by three opaque PVC walls so that 4 fish could be tested in the same aquarium. Fish were fed by hand for 10 days, and the time to resume feeding after isolation was recorded for each fish.

6.2.2. Resumption of feeding, metabolic rate and post stress cortisol concentrations.

Since measurements of SMR were performed on two fish simultaneously (see below), one early and one late emerging fry were randomly selected from the holding tanks and isolated each day (Mean body mass ± S.D, 1.6 ± 0.4 g for early and 1.4 ± 0.4 g for late emerging individuals, $t = 1.4$, $d.f = 20$, $p = 0.5$). Following transport to the observation aquaria, fish were hand fed once a day *ad libitum* for 5 days. For each fish, resumption of feeding after isolation was quantified by measuring the number of pellets consumed and the latency to eat the first pellet in each day. If no pellets were consumed within 3 minutes, the latency was set to 180 s. At

the end of each feeding observation, the uneaten pellets were removed from the tank. The temperature during the tests was 12 ± 1 °C, which was similar to the water temperature in the holding tanks.

After isolation for 5 days, fish were fasted for 48 hrs, before the oxygen consumption (MO_2) was measured. The recording was performed over an 18 hour period, using computerized intermittent flow-through respirometry, as described by Steffensen and collaborators (1984). Respirometers were constructed from glass cylinders sealed at both ends with a rubber stopper. A fibre optic oxygen sensor was inserted through one rubber stopper into the chamber, and oxygen saturation was registered every second. A blunt needle with a length of an 18G was inserted through each stopper, connected to a length of Tygon tubing for flushing the respirometer using a peristaltic pump (Ismatec SA, Switzerland). A horizontal piece of plastic mesh was glued in place in each chamber approximately one third from the base. The test subject was placed above the mesh, while a miniature magnetic stirrer below ensured complete mixing of the water volume in the respirometer. Measurements of MO_2 were performed in 16 minute intervals. Each interval consisted of a flush period lasting 240 s followed by a 120 s waiting period to reach steady-state conditions and terminated by a 600 s measurement period during which the decline of the water oxygen tension inside the respirometer was recorded using data acquisition software (AutoResp 4, Loligo Systems).

Following MO_2 measurements, individuals were returned to the behavioural observation aquaria, where they were fed and allowed to recover for 48 hrs, before being subjected to a confinement stress test. Individuals were confined for 30 min, in 50 ml containers with aerated water. Immediately after confinement, fish were exposed to a lethal dose of MS 222, carcasses were frozen on dry ice and stored at -80 °C until further analysis of the whole body cortisol

concentrations. The unstressed controls were isolated and fed following the same protocol as described above, with the modification that fish were euthanized and frozen immediately after 5 days in isolation.

6.2.3 Social dominance

To investigate the relationship between time to emerge and social status, pairs of fish, consisting in one early and one late emerging fry, were isolated and hand fed for 7 days. Individuals were marked by upper or lower fin clipping. After the isolation period, the dividing wall separating the fish was lifted and the individuals were allowed to interact. This resulted in escalated fights in all pairs, and aggressive acts such as bites, chases and approaches were observed. This behaviour has been previously observed in salmonid fish before establishment of a hierarchy (Øverli, et al., 1999; Øverli, et al., 2004). Social interactions were video recorded for 60 min. After the observation period, the aggression was unidirectional and dominant and subordinate fish were clearly distinguishable. The dominant-subordinate relationship was verified the next morning by behavioural observations during feeding. It was observed that the dominant fish swam around the aquarium and responded to the offered pellets, whereas the subordinate fish were immobile in a corner and did not respond to food.

6.2.4. Consistency over time

The above experiments, performed two months after emergence, indicated differences in the resumption of feeding after transfer to isolation between fish with an early and late time to emerge (see results). Consequently, it was investigated if the observed differences were still present five months after emergence. The mean body mass (± S.D) of the fish in this test was

29.1 ± 4.8 g for early and 29.7 ± 5.4 g for late emerging fish (t = -0.3, d.f = 29, p = 0.7). The water temperature during this experiment was 10 ± 1 °C, which was similar to the temperature in the holding tanks.

6.2.5. Cortisol assay

Frozen salmon carcasses were sent to the Department of Comparative Physiology, Evolutionary Biology Centre, Uppsala University, Sweden. Each carcass was homogenized in a volume of PBS equivalent to the fish body mass. Five volumes of ethyl acetate were added to the fish homogenate, vortexed and centrifuged at 2000 rpm for 5 minutes. The supernatant was transferred to a borosilicate tube and dried under nitrogen. The pellet was resuspended in 100 µl ethyl acetate for fractionation by thin-layer chromatography (TLC) using a modification of the method described by Denver (1998). Briefly, the samples were loaded on TLC silica gel 60 glass plates (250µm). The plates were then run in a mobile phase of toluene:cyclohexane (1:1) for approximately 1 hour. Plates were air-dried and placed in a second mobile phase of chloroform:methanol (98:2) for approximately 1 hour. Again the plates were air-dried and thereafter the first 2 cm from the loading line from each lane was scraped off into a borosilicate tube and extracted overnight with 5 ml ether. The next day the ether-silica was spun at 1300 rpm for 5 min and supernatant transferred into a new borosilicate tube and dried with nitrogen. The position of the cortisol spot was located by using H3-cortisol spiked samples with a mean recovery rate of 80 %. Samples were redissolved in 500 µl of ethyl acetate and the concentration of cortisol was analysed by radioimmunoassay (RIA).

6.2.6. Statistical analysis

All values are presented as mean (± S.E.) unless otherwise is stated. In the first set of experiments, general linear model (GLM) procedures followed by a Tukey HSD posthoc test was used to investigate the differences between early and late emerging individuals in the number of pellets consumed and in latency to eat the first pellet during the 5 day isolation period. These observations were log transformed to attain normality. It was tested for the effects of the number of days in isolation, the emergence time and also the combined effect of days in isolation and time to emergence on the number of pellets consumed and on latency to eat the first pellet. Further on, a t-test was used to evaluate group differences in MO_2. In addition, a two-way ANOVA followed by a Tukey HSD posthoc test was used to analyse effects of stress and emergence time on cortisol concentrations between the unstressed controls and confined individuals with an early or late time to emerge. Measures of post stress cortisol concentrations were log transformed to achieve normality. Pearson correlation coefficients and p-values were calculated to correlate the measurements of resumption of feeding, MO_2 and post stress cortisol levels made on the same individuals. In the second set of experiments, a chi test was used to investigate whether proportions of individuals becoming subordinate or dominant differed among individuals grouped as early or late emerging individuals. Resumption of feeding after transfer to isolation in the third set of experiments was analysed following a similar GLM procedure as described for the first set of experiments. All the statistics were performed using the SAS 9.1 statistical software package.

6.3. Results

6.3.1. Resumption of feeding, metabolic rate and post stress cortisol concentrations.

In generally, the number of eaten pellets increased significantly with the days in isolation ($F_{(4, 100)} = 8.8$, $p < 0.01$; Fig 1a). Furthermore, early emerging individuals consumed more pellets than late emerging individuals in isolation ($F_{(1, 100)} = 7.2$, $p < 0.01$; Fig 1a), and this effect was independent of the number of days spent in isolation ($F_{(4, 100)} = 1.3$, $p = 0.3$).

The latency to eat the first pellet decreased during the 5 days in isolation ($F_{(4, 100)} = 12.1$, $p < 0.01$; Fig 1b) and early emerging individuals took less time to eat the first pellet, compared with late emerging individuals ($F_{(1, 100)} = 8.4$, $p < 0.01$; Fig 1b). This effect was independent of the number of days in isolation ($F_{(4, 100)} = 1.5$, $p = 0.2$).

No differences in MO_2 were found between individuals with different emergence time ($t = - 0.1$, $d.f = 20$, $p = 0.9$). Mean values (± S.E) of MO_2 were 164.6 ± 8.6 mg O_2/kg/h for early and 165.4 ± 13.4 mg O_2/kg/h for late emerging individuals.

Significant differences were found in post stress cortisol concentrations between the confined individuals and the unstressed controls ($F_{(1, 34)} = 6.0$; $p = 0.02$), where the unstressed controls had lower cortisol concentrations (Fig 2). However, no differences in post stress cortisol concentrations were observed between early and late emerging individuals ($F_{(1, 34)} = 0.1$; $p = 0.8$; Fig 2). Furthermore, the interaction effect between the stress condition (confined vs. unstressed) was independent of the emergence time ($F_{(1, 34)} = 0.9$; $p = 0.4$).

Although there was a significant negative correlation between the total number of pellets eaten and the mean time to eat the first pellet, these two measurements were not correlated to MO_2 or post stress cortisol levels (Table 1).

6.3.2 Social dominance

After 24 hrs of social interaction, 5 early and 4 late individuals had become socially dominant. This frequency difference was not significant ($\chi^2 = 0.22$; d.f = 1; p = 1).

6.3.3 Consistency over time

Similar to the first set of experiments, the number of pellets consumed increased with the time in isolation ($F_{(1, 290)} = 22.8$, $p < 0.01$; Fig 3a). In addition, five months after emergence the early emerging individuals still consumed more pellets than the late emerging individuals during the isolation period ($F_{(9, 290)} = 11.4$, $p < 0.01$; Fig 3a). The observed difference between the early and late emerging individuals was also independent of the isolation days ($F_{(9, 290)} = 1.7$, $p = 0.1$).

The latency to eat the first pellet decreased with the days after being transferred ($F_{(1, 290)} = 23.9$, $p < 0.01$; Fig 3b). Again, early emerging individuals took less time to eat the first pellet offered compared with the late emerging individuals ($F_{(9, 290)} = 9.0$, $p < 0.01$; Fig 3b), and the differences in the latency between early and late emerging individuals were independent of the number of days in isolation ($F_{(9, 290)} = 1.4$, $p = 0.2$).

6.4. Discussion

In the present study, two month-old fry of Atlantic salmon with an early time to emerge from artificial redds, consumed more pellets and took less time to eat the first pellet during isolation, compared with late emerging fry. Thus, early emerging fry resumed feeding faster after isolation in a new environment. Furthermore, this behavioural characteristic was consistent over time, since it was also expressed five months after emergence. Considering the fact that salmonid larvae leave the spawning redds in order to start exogenous feeding, this suggests that initial

individual differences in the ability to exploit a new environment are still present five months after emergence. Early and late emerging fry, however, did not differ in MO_2, post stress cortisol concentrations or social status, traits that are known to differ in the proactive and reactive coping styles (Koolhaas, et al., 1999; Huntingford, et al., 2010).

Time to resume feeding in a new environment has been used as a measure of boldness behaviour in salmonid fish (Øverli, et al., 2006; Øverli, et al., 2007; Ruiz-Gomez, et al., 2008). Consequently, a faster time to resume feeding after transfer and isolation suggests a bolder behaviour in fry with an earlier emergence time in the present study. Time to resume feeding in a novel environment, however, involves potentially anxiogenic anorectic effects (novelty and transport) as well as traits related to hunger (metabolic rate and capacity to process food) (Øverli, et al., 2007). In the present study, bolder behaviour was observed in individuals with a faster time to reach emergence and first feeding stage. This suggests that boldness could be related to larval development and yolk conversion ratio. Considering that salmonid fish which consume their yolk sac faster may also have higher metabolic rates (Vaz-Serrano, unpublished) and greater capacity to process food after switching to exogenous feeding, it cannot be excluded that the bolder behaviour in early emerging fry is related to hunger, rather than to overcome anxiogenic anorectic effects. However, differences in metabolic rate have been related to food processing time and energy conversion (Millidine, et al., 2009), and the absence of a difference in metabolic rate between early and late emerging fry indicates that boldness in this study is related to the time to overcome the anxiogenic effect of being transported and isolated.

In previous studies, resumption of feeding after isolation has been associated with locomotor activity during acute stress, social dominance and post stress cortisol concentrations in salmonid fish (Øverli, et al., 2004; Øverli, et al., 2006; Kittilsen, et al., 2009). Furthermore, these

traits have been proposed to be an indicator of stress coping styles (Øverli, et al., 2007). In a recent study, the standard metabolic rate of an individual has also been suggested to be related to its stress coping style (Huntingford, et al., 2010), where a higher metabolic rate reflects a more active, aggressive life style, such as the proactive stress coping style. In the present study, we could not detect any differences between early and late emerging individuals in post stress cortisol concentrations or in metabolic rates. Furthermore, the social status of an individual was not related to the emergence time. This is in contrast to studies on natural populations of Atlantic salmon, where traits such as higher metabolic rate, probability to become socially dominant and willingness to take more risks in the presence of predators were observed in individuals that emerge early from the spawning nests (Metcalfe and Thorpe, 1992; Brännäs, 1995), suggesting a relation between some traits of the stress coping and time to emergence.

The uncoupling between boldness and other stress coping styles traits in fry with an early or late emergence time observed in the present study, might be a consequence of the domestication of fish. In wild populations of salmonid fish, a high selective pressure is present during emergence from the spawning nest and establishment of new territories (Bardonnet, et al., 1993; Milner, et al., 2003; Einum, et al., 2006). The lack of feeding territories, the presence of predators, the variable environmental conditions and/or the aggressions by a conspecific could results in a co-selection of suits of traits forming the stress coping styles. Furthermore, both genetic and environmental factors (e.g. social interactions and previous exposure to stress) contribute to extensive inter-individual variation in how stressful experience affects behaviour and physiology (Carere and Eens, 2005; Frost, et al., 2007; Korzan and Summers, 2007). Considering that a newly emerged individual may be naive to aggressive behaviour and social competition, the initial defence and establishment of territories could have profound effect on

stress reactivity and behaviour. The bolder behaviour of the early emerging individuals may give them a competitive advantage, shaping their personality towards a more proactive stress coping style. In hatchery reared fish, on the other hand, this dramatic selection during emergence is absent.

According to several views, the selection pressure present in captive animals can be either directional or relaxed (Huntingford, 2004; McPhee, 2004; Huntingford and Adams, 2005; Conrad and Sih, 2009). Some studies have demonstrated that hatchery reared fish are bolder and more aggressive than wild reared fish (Huntingford, 2004; Huntingford and Adams, 2005), suggesting that the selection in captive fish is directed towards a bolder and aggressive behavioural type. However, there are studies suggesting that behavioural variability increases as a consequence of a lack of selection pressure against maladaptive behaviours in captive animals (McPhee, 2004; Conrad and Sih, 2009). Furthermore, this hypothesis predicts a high individual variability as well as a lack of relationship between the behavioural type and other traits, like growth and survival (Conrad and Sih, 2009). This was demonstrated in captive Arctic char (*Salvelinus alpinus*), where boldness towards natural predator cues was not related to growth (Laakkonen and Hirvonen, 2007). In the present study, no correlation was found between the resumption of feeding, metabolic rate or post stress cortisol levels (Table 1), indicating that a suit of correlated traits is not present in the studied fish. Therefore, these findings could be an indication that the selection in captive fish is relaxed rather than directional. However, this view is still debated since other studies have shown behavioural syndromes and stress copying styles in captive fish (Brelin, et al., 2005; Huntingford and Adams, 2005; Silva, et al., 2010; Van de Nieuwegiessen, et al., 2010).

In summary, early emerging individuals of Atlantic salmon were bolder than late emerging individuals and this behavioural trait was consistent over time. This is coherent with studies in natural populations that suggest a relationship between stress coping styles and emergence time in salmonid fish. However, emergence time was not related to other behavioural and physiological characteristic of the stress coping styles. This decoupling could occur in fish reared under hatchery conditions, where competition for resources is less severe than in nature, and further studies are needed to investigate if the differences in stress coping styles between early and late emerging individuals are present in wild populations of salmonid fish.

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Fig. 1. Measures of the average (± S.E) number of pellets consumed [a] and the average (± S.E) latency (s) to eat the first pellet [b] by early (●) and late (○) emerging individuals, during isolation for 5 days. N = 11 for each early and late emerging time individuals. For both measures, significant differences were shown between individuals with different emergence time and between isolation days (see text).

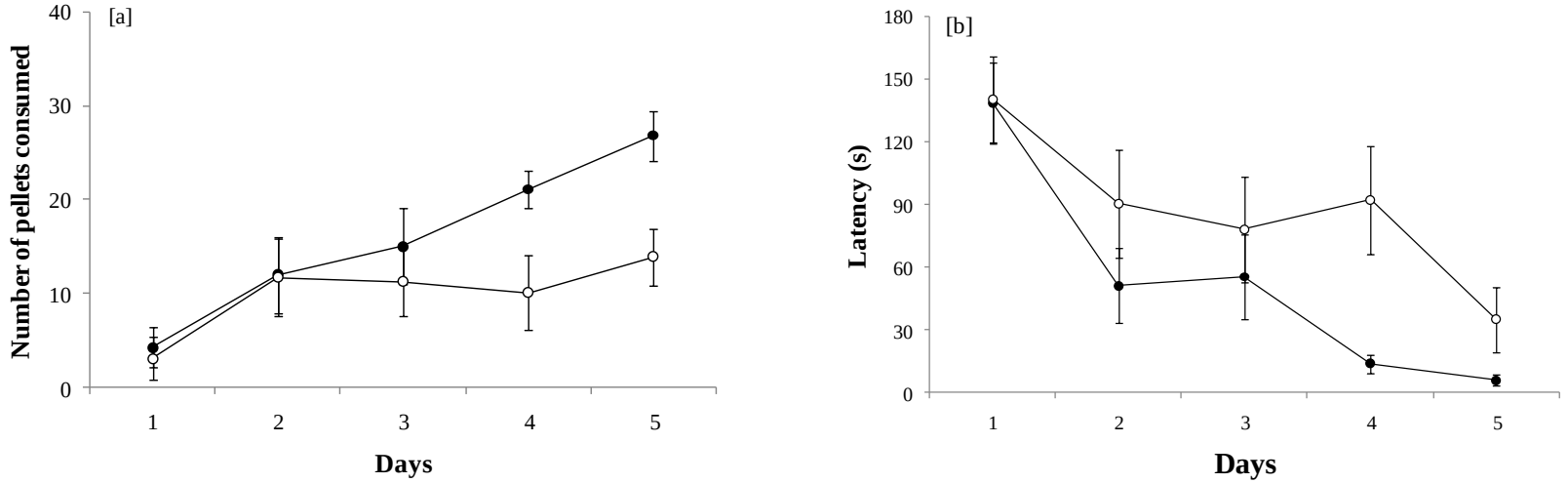
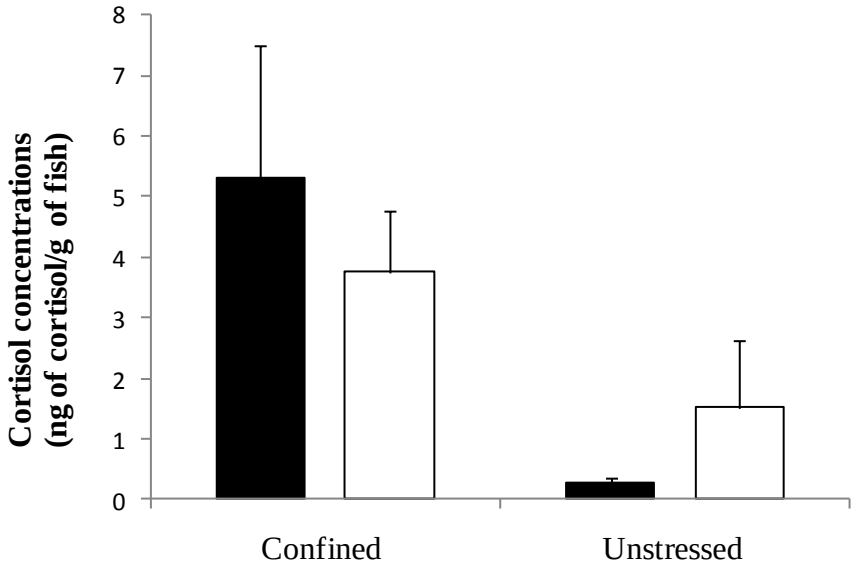


Fig 2. Average (± S.E) post stress cortisol concentrations (ng of cortisol/mg of fish) of individuals with an early (●) and late (○) time to emerge, in confined and unstressed conditions. Significant differences were found between stressed and unstressed conditions, although no differences were found between early and late emerging time. In addition, no differences were found in the interaction between conditions and time to emerge (see text).



Confined vs unstressed ($F_{(1, 34)} = 6.0$; $p = 0.02$)
Early vs late ($F_{(1, 34)} = 0.1$; $p = 0.8$)

Fig 3. Measures of the average (± S.E) number of pellets consumed [a] and the average (± S.E) latency (s) to eat the first pellet [b] by early (●) and late (○) emergence time individuals during isolation for 10 days, four mounths after the first trial. N = 16 for each early and 15 for late emerging time individuals. For both measures, significant differences were shown between individuals with different emergence time and between isolation days (see text).

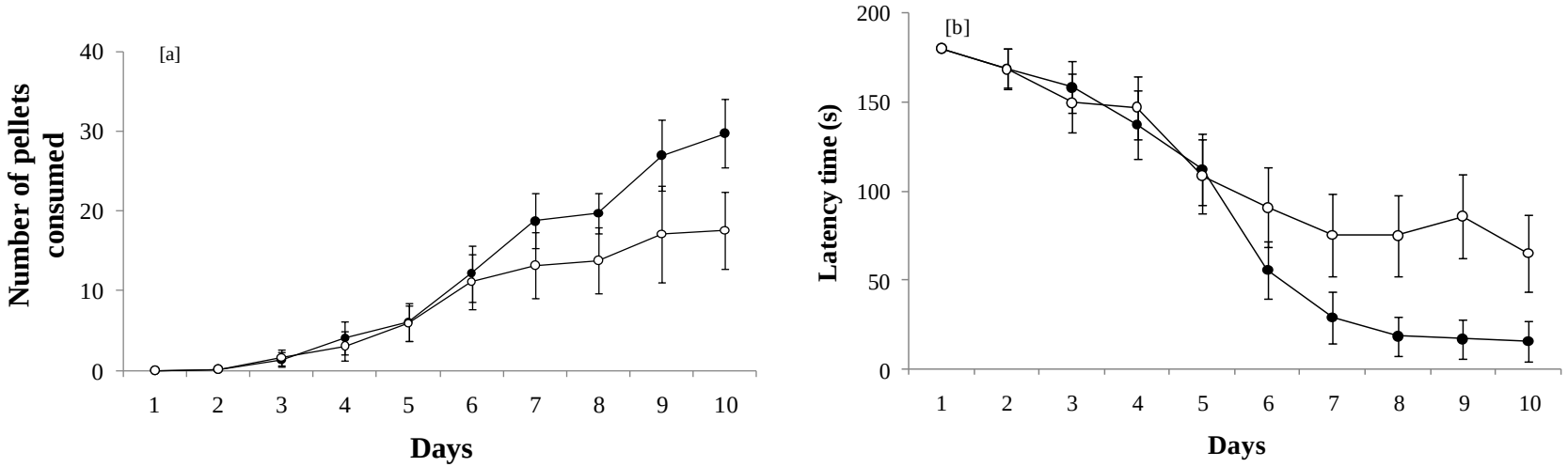


Table 1. Pearson correlation values (R) between the total number of eaten pellets, the time to eat the first pellet, the standard metabolic rate (SMR) and post stress cortisol concentrations. Values marked in bold are statistically significant ($p < 0.05$).

	Number of pellets	Time to eat the first pellet	SMR	Post stress cortisol concentrations
Number of pellets		**-0.88**	0.04	0.05
Time to eat the first pellet			0.05	-0.07
SMR				-0.37
Post stress cortisol concentrations				

GENERAL DISCUSSION AND CONCLUSIONS

7. GENERAL DISCUSSION.

7.1. Relationships between early life traits in Atlantic salmon (Papers I and II)

A positive relationship was shown between egg size and hatching time in Paper II. Families with bigger eggs hatched later than smaller eggs. In general, interspecies variation in freshwater fish suggests that embryonic development is slower in larger eggs than in smaller eggs (review by Teletchea, et al., 2009). Taken the results presented in Paper II together with a study performed by Pakkasmaa and Jones (2002), it suggests that this relationship between egg size and hatching time still exists on interfamily level in salmonid fish. However, there are a few studies where such relationship could not be detected (Gilbey, et al., 2009; Kristjánsson and Vøllestad, 1996). One of the reasons for this discrepancy in the results between these studies could be the low number of families included in the studies. In Paper II, family specific data was presented from 85 families, and in the study by Pakkasmaa and Jones (2002) data were collected from 40 families. Gilbey and collaborators (2009) and Kristjánsson and Vøllestad (1996) conducted their studies based on an even smaller data collection with eggs from 2 half-sib families and 7 families, respectively. It is possible that a bigger sample size could increase the statistical power in the studies performed by Gilbey and collaborators (2009) and Kristjánsson and Vøllestad (1996) revealing similar relationships as in paper II.

In Paper II it was shown that families with an early, intermediate or late emergence time did not differ in hatching time, suggesting a decoupling between hatching time and time to emerge. This is somehow coherent to the study performed by Gilbey and collaborators (2009), where it was demonstrated that individual variation in hatching time was not related to the developmental stage of the larvae later in ontogeny. This decoupling between hatching time, larval development

and emergence time is further supported by the results in Paper I, where individuals originating from late hatching families had reached the same stage of development as early hatching individuals, by the time the measurements were done. Furthermore, late hatchers had a higher metabolic rate compared to early hatchers. Taking in consideration that fish larvae use most of the energy available in the yolk sac for growth and respiration processes (Kamler, 2008), this suggests that the higher metabolic rate observed in late hatchers in Paper I reflects a higher developmental rate in these individuals, resulting in a higher catch-up post hatch growth in these larvae.

7.2 Sorting method (Paper II)

A method to sort salmonid larvae with respect to the emergence time was presented in Paper II. Moreover, the emergence of all the larvae from the incubators revealed a temporal distribution lasting 17 days, at 8.9 °C. Similar findings have been reported in salmonid larvae reared both under natural and laboratory conditions and at similar temperature ranges (Brännäs, 1995; De Leaniz, et al., 2000; Sundström, et al., 2005). Thus, similarities with these studies suggest that the pattern of emergence observed in Paper II corresponds to that seen in natural populations.

Several studies in Atlantic salmon have demonstrated that the incubation of eggs in a substrate that resembles a natural incubation environment is advantageous for the development of eggs, and can improve future growth rates (Hansen and Torrissen, 1985; Hansen and Moller, 1985; Sveier and Raae, 1992; Peterson and Martinrobichaud, 1995; Bamberger, 2009). By allowing larvae to emerge from the spawning redd, stress could be minimized, and the negative effect that stress may cause on the growth of developing larvae could be reduced (Bamberger, 2009).

Therefore, the method presented in Paper II, not only improved incubation conditions of reared salmonid eggs, but offered a novel technique to sort larvae with respect to emergence time.

7.3. Emergence time and stress coping styles (Paper III)

In natural populations of salmonid fish, variation in emergence time has been observed to be related to metabolic rates, growth rates, life-history traits and social status (McCarthy, et al., 2003; Metcalfe, et al., 1995; Metcalfe and Thorpe, 1992). Furthermore, some of these traits indicate that variation in emergence time is coupled to differences in stress coping styles. In the second part of this thesis, it was investigated if stress coping styles are related to emergence time in domesticated Atlantic salmon.

The results in Paper III demonstrate that early emerging fry resume feeding after isolation faster in a new environment, compared with late emerging fry. This behaviour was observed again five months after emergence. In several studies in salmonid fish, resumption of feeding after isolation has been used as a measure of boldness behaviour (Øverli, et al., 2006; Øverli, et al., 2007; Ruiz-Gomez, et al., 2008). Taken together, this indicates that early emerging fry are bolder than late emerging fry, and that this difference is consistent over time. However, if boldness behaviour in early emerging fish is stable through the whole ontogeny, needs further investigation. Moreover, differences in emergence time were not related to other traits where the proactive and reactive stress coping styles show contrasts, such as metabolic rate, social status, or post stress cortisol concentrations (Koolhaas, et al., 1999; Huntingford, et al., 2010).

A number of studies have suggested that selection in hatchery reared fish is directed towards bolder and more aggressive individuals, compared to the wild types (Huntingford, 2004; Huntingford and Adams, 2005;). These studies have also suggested that fish expressing shyer

and non-aggressive traits may be less frequent in hatchery environments (Huntingford and Adams, 2005). An alternative view suggests that the selection in aquaculture is relaxed rather than directional. This hypothesis is based on the idea that the behaviours expressed in farmed animals are a result of a lack of selection pressure against maladaptive behaviours (McPhee, 2004; Conrad and Sih, 2009). For example, in nature, there is a trade-off between the time spent vigilant for predators and the time spent foraging (Brown, 1999). However, in captivity, the expression of the predator avoidance behaviour of a given individual would not affect its survival. This might result in higher behavioural variance, by a decrease in selective pressures reducing the expression of this behaviour (McPhee, 2004).

Correlations between different behavioural traits are known as behavioural syndromes (Sih, et al., 2004), and the absence of predators could generate a decoupling between such correlations. This has been demonstrated in sticklebacks (*Gasterosteus aculeatus*), where boldness and aggression were correlated in populations under a high predation pressure, but this correlation was absent in populations with a low predation pressure (Bell and Sih, 2007). As mentioned above, early and late emerging fry differed in boldness, but did not show contrasts in other traits differentiating the proactive and reactive stress coping style. This decoupling between boldness and other traits of the proactive and reactive stress coping styles could be caused by a relaxed selection in hatchery reared fish, compared to undomesticated fish.

In salmonid fish, there is high selective pressure during emergence from the spawning redds and establishment of territories (Bardonnet, et al., 1993; Milner, et al., 2003; Einum, et al., 2006), and the timing of these events has been associated with individual traits such as aggressiveness, social status and metabolic rate (Metcalfe and Thorpe, 1992;Metcalfe, et al., 1995). The lack of feeding territories, the presence of predators, the variable environmental conditions and/or the

aggressions by a conspecific could result in a co-selection of suits of traits forming the stress coping styles. Furthermore, there are some studies suggesting that the selection during emergence and establishment of territories results in a bimodality in the expression of some traits. For example, a bimodal distribution of individual growth potential in individuals that succeed to establish a territory has been reported (Titus and Mosegaard, 1991). Moreover, this bimodality has been associated with social dominance (Titus and Mosegaard, 1991), indicating that the natural selection during emergence can result in two co-existing strategies, such as the proactive and reactive stress coping styles. The selection pressure during emergence is absent in hatchery reared fish, and it is possible that the decoupling between boldness and other traits is a result of a relaxed selection. On the other hand, experiences during social interactions or stressful events have been known to modify the behaviour and the physiology of an individual (Carere and Eens, 2005; Frost, et al., 2007; Korzan and Summers, 2007). Thus, in natural populations of salmonids, the period after emergence and establishment of feeding territories, can have an effect on the behaviour and physiology of the fry. The bolder behaviour in early emerged individuals may confer them with an advantage when defending feeding territories, determining their personality to a proactive stress coping style.

In summary, the absence of a strong selection during emergence may create the decoupling between emergence and stress coping styles in domesticated salmonid fish. Further studies are needed to investigate if the decoupling of behavioural and physiological traits in Paper III are a result of more a relaxed selection and/or a socially induced modulation of behavioural and physiological traits in domesticated fish. However, it is important to keep in mind that the decoupling between traits that identify the stress coping styles does not seem to be universal for domesticated fish. There is a number of studies demonstrating behavioural syndromes and

coping styles in farmed fish (Brelin, et al., 2005; Huntingford and Adams, 2005; Silva, et al., 2010; Van de Nieuwegiessen, et al., 2010).

7.4. Early life traits and traits expressed after emergence (Papers I, II and III)

In Paper II, a positive relationship between larval developmental rates and emergence was demonstrated. Taken together with the results in Paper III, which show a consistent bolder behaviour in early emerging fry, this suggests a relationship between faster larval developmental rate and bolder behaviour in fry. As suggested in Paper I and II, faster larval development could be related to a higher metabolic rate. However, in Paper III, early emerging fry did not differ in standard metabolic rates compared with late emerging fry. These findings suggest that a metabolic shift occurs between the larval and the fry stages. In fish, the physiological mechanisms involved in energy conversion and growth differ between the larval and the fry stages (Urho, 2002). Larvae use the energy stored in the yolk sac for growth (Kamler, 2008). Growth using exogenous feed sources, on the other hand, requires many more behavioural, physiological and biochemical processes, such as foraging behaviour, appetite, feed uptake and energy allocation (Gilbey, et al., 2005; Kamler, 2008). However, how these more complex processes affect the metabolic shifts occurring, needs to be further examined. Individual studies may reveal relationships in metabolic rate occurring between endogenous and exogenous feeding in fish.

According to the relaxed selection hypothesis, the selection pressure in hatchery reared fish is not directed towards a particular phenotype; although there could be some selection pressure present. A higher mortality in the early emerging fraction was reported in Paper II, which was suggested to be related to a higher aggression and/or a higher growth rates in this fraction.

Higher growth rates could give rise to metabolic induced damages, such as developmental impairments that can lead to death (Metcalfe and Monaghan, 2003). However, in Paper III, individual differences in aggression or standard metabolic rates were not related to the variation in emergence time. In general, after 5 weeks of rearing in communal tanks, the higher mortalities observed in early emerged fry, compared to late emerged fry suggests that there is some selection present in a hatchery environment.

7.5. Future studies

During the course of this thesis, fish from the same families were reared in parallel in the Marine Harvest breeding facilities. In the summer 2010, an immune challenge test to infectious pancreas disease (IPN) was performed on these fish. In the spring 2011, fish will be slaughtered and the quality of the end product (filet colour and fat content) and the frequency of deformed fish will be recorded. With the data collected by Marine Harvest, genetic correlations between the emergence time and all the other traits recorded could be further studied. In addition, the effect of separating rearing of fish with different emergence time on growth performance and on feed conversion ratio could be further investigated.

8. CONCLUSIONS

The following conclusions can be drawn from this thesis:

I. Differences in hatching time were compensated for at later stages by a faster development in late hatching larvae of Atlantic salmon.

II. Families of domesticated Atlantic salmon with bigger eggs hatched later compared with families with smaller eggs. However, these traits were not related to emergence time in the same families.

III. Larval developmental rate predicted emergence time, where families with a faster larval developmental rate emerged earlier. The faster larval development in these larvae is suggested to be coupled to a higher metabolic rate.

IV. A novel method to sort salmonid larvae according to the time to emerge from artificial redds was presented. This method could be used to improve rearing conditions of larvae in hatchery reared salmonid fish.

V. Earlier emergence was related to a bolder behaviour in early emerging fry and this behaviour was consistent over time, compared with late emerging fry.

VI. No relationship was shown between emergence time and other traits that are known to differ between the proactive and the reactive stress coping styles. It is possible that this decoupling is a result of a more relaxed selection and/or an induced modulation of behavioural and physiological traits in domesticated fish.

VII. Further work should investigate the relationships between emergence time and other production traits, such as growth rates, feed conversion ratio, disease resistance, quality of the end product and frequency of deformities.

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