Fish coping with stress

Physiology and behaviour of salmonids with focus on stress coping styles, rearing conditions and conservational releases

Thesis for the degree of Doctor of Philosophy

by

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FISH COPING WITH STRESS - PHYSIOLOGY AND BEHAVIOUR OF SALMONIDS WITH FOCUS ON STRESS COPING STYLES, REARING CONDITIONS AND CONSERVATIONAL RELEASES

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DISSERTATION ABSTRACT

When animals are reared for conservational releases it is paramount to avoid reducing genetic and phenotypic variation over time. Therefore, an understanding of how behavioural and physiological traits affect the performance of an individual both within the captive and the wild environment is required. To preserve threatened populations of salmonids, large numbers of hatchery-raised juveniles are released each year. The captive environment do, however, differ in many aspects from the wild, and the standard hatchery practice, using high animal densities and barren tanks, raise questions concerning stress levels, misdirected selection pressures and a naivety of the released fish when coping with the wild environment.

The overall aim of this thesis is therefore to extend the knowledge-base on stress physiology and behaviour of salmonids with special focus on individual variation, stress coping styles and conservational releases. The objectives are to increase the understanding of how behavioural and physiological traits are related to each other, how they are maintained during juvenile development and how they are modulated by environmental factors like the rearing environment.

The results from the present thesis shows that lowering the animal density and adding structural enrichment to the rearing tanks reduces aggression and stress levels while in captivity, as well as have a positive influence on physiological and phenotypic traits important for survival after release. However, fish reared with in-tank shelter at high densities showed a lower post-release survival during smolt migration. These results highlight the importance of thoroughly examining interaction effects of hatchery alterations before implementation.

Furthermore, salmon showing early emergence from the spawning nest as well as a bold behavioural profile had a lower maximum metabolic rate and aerobic capacity prior to release, compared to those with late emergence and a more cautious behavioural profile. While this may have no clear negative implications for the individual in the hatchery, after release it could result in greater risk of predation caused by higher risk-taking and possible impaired swimming capacity and recovery from exhaustion. These results highlight how the selection pressure might differ between different behavioural profiles and environments.

In rainbow trout, individual variation in systemic cortisol output during stress (HPI-axis reactivity) also had an impact on metabolic rate and on the stress sensitive barrier function of the intestine. HPI-axis reactivity was further linked to other stress related neuronal systems in the forebrain and to behavioural traits.

The findings of this thesis broadens the knowledge-base on stress physiology and behaviour of salmonids and how they are interlinked with and modulated by environmental factors.

In conclusion, the rearing environment, behavioural profile and stress reactivity of an individual will affect its ability to cope with challenges both in captivity and in the wild. There is much to gain in continuing the work on finding hatchery alterations, where a reduced rearing density together with structural enrichment is a promising candidate to implement both increased welfare and post-release performance.

Keywords: Behaviour, Conservation, Cortisol, Intestinal barrier function, Metabolism, Salmonids, Stress, Stress coping styles

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I.   Hatchery tank enrichment affects cortisol levels and shelter-seeking in Atlantic salmon (*Salmo salar*). **2013**. Näslund, J., Rosengren, M., Del Villar, D., Gansel, L., Norrgård, J.R., Persson, L., Winkowski, J.J., and Kvingedal, E. *Canadian Journal of Fisheries and Aquatic Sciences 70: 585-590.*

II. Born to be wild: effects of rearing density and environmental enrichment on stress, welfare and smolt migration in hatchery reared Atlantic salmon. **2016**. Rosengren, M., Kvingedal, E., Näslund, J., Johnsson, J.I., and Sundell, K. *Canadian Journal of Fisheries and Aquatic Sciences 74: 396-405.*

III. High risk no gain: Metabolic performance of hatchery reared Atlantic salmon smolts, effects of nest emergence time, hypoxia avoidance behaviour and size. **2017**. Rosengren, M., Thörnqvist, PO., Winberg, S., and Sundell, K. *Revised version sent to Physiology and Behaviour.*

IV. The brain-gut axis of fish: Rainbow trout with low and high cortisol response show innate differences in intestinal integrity and brain gene expression. **2017**. Rosengren, M., Jutfelt, F., Sandblom, E., and Sundell, K. *Under revision for publication in General and Comparative Endocrinology.*

V.   Cortisol is costly: HPI-axis reactivity in rainbow trout - effects on metabolic rate and behaviour. **2017**. Rosengren, M., PO., Johnsson, JI., Sandblom, E., Winberg, S., and Sundell, K. *Manuscript.*

Supplementary information for paper I, II IV and V

TABLE OF CONTENT

INTRODUCTION

In this thesis I investigate the effects of individual variation and rearing environment on stress physiology and performance of salmonids. The main focus is on Atlantic salmon (*Salmo salar*) reared for release into the wild, but the thesis also includes a study on rainbow trout (*Oncorhynchus mykiss*) as a model species for stress coping mechanisms. The thesis aims at increasing the understanding of how behavioural and physiological polymorphism is structured within a population and how it may affect the vulnerability, performance and survival of the individual. The results also add to the knowledge of "do's and don'ts" when designing rearing environments for conservational releases.

The many faces of salmon

Anadromous salmonids are wide-spread across the northern hemisphere where they inhabit freshwater rivers, estuaries and ocean-pelagic ecosystems. Many salmonid species have high ecologic value and several genera within the subfamily Salmoninae, include species with high economic and sociocultural value such as *Salmo* species (Atlantic salmon and brown trout), *Oncorhynchus* species (rainbow trout and Pacific salmon) and *Salvelinus* (Arctic char) (Froese, 2016). Atlantic salmon is besides from being one of the most highly intense aqua-cultured species, also caught by commercial fisheries and popular in sport-fishing (Nasco 2017). To increase ocean stocks and to save local and endangered populations of salmonids, large numbers of hatchery-raised juveniles are released each year into the wild.

Atlantic salmon life history

Sexually maturing Atlantic salmon migrate from the ocean into freshwater rivers to spawn in the autumn (Fig.1). The female buries the eggs in the river gravel where the embryos develop until they hatch the following spring. Within a few weeks the fry emerge and disperse from the nest to establish a territory. As the fry grow over the summer and autumn they develop into parr. The juveniles stay at the parr-stage typically one to two years (but up to eight years) depending on environmental factors and individual growth rate. The parr-smolt transformation is an adaptive specialization associated with the change from a bottom-living and territorial freshwater fish into a free-swimming pelagic smolt (Fig. 2). Smoltification is initiated when the fish are still in the river and involves morphological changes such as streamlining and

elongation of the body structure (decreased condition factor) and skin silvering. Physiological changes involve e.g. increased Na^+,/K^+-ATPase activity in the chloride cells of the gills, which aid in dealing with the higher osmolality of the ocean. In a river, the fish is hyperosmotic in relation to the surrounding water, creating a net potential driving diffusion of water and ions into the fish. In the ocean the fish is instead hypo-osmotic compared to the water, creating a net potential leading to water loss. To compensate, the fish drinks seawater, absorb ions and water over the intestine and excretes excessive ions over the kidney and gills.
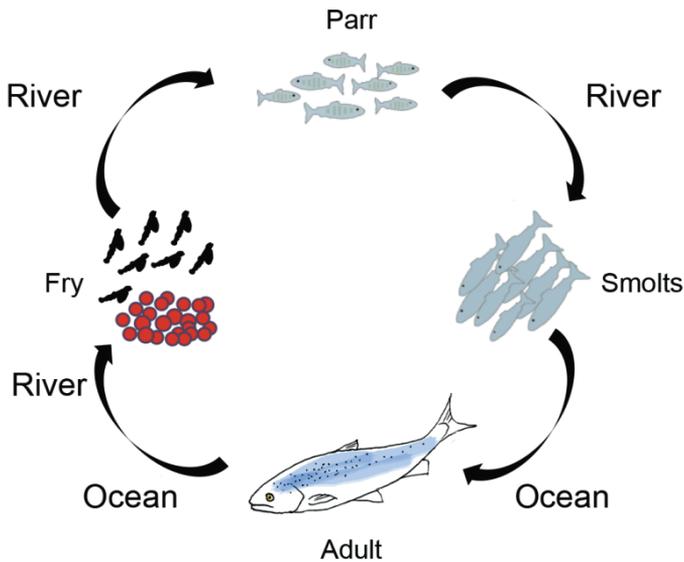
Fig. 1. The Atlantic salmon life-cycle.

Behavioural changes during parr-smolt transformation include decreased territoriality and increased schooling behaviour and swimming activity. The smoltification process is governed by hormones, in particular growth hormone, cortisol and thyroid hormones, which in turn is triggered by an increase in temperature and photoperiod during spring. The determination of whether a parr will respond to the environmental cues and undergo smolt metamorphosis depends on whether it has reached an appropriate size and developmental stage (~10 > cm) (Thorpe et al., 1992). After migration the salmon usually spend one to two years in the ocean, growing and maturing before returning as spawning adults to their home river (McCormick et al., 1998; Thorstad et al., 2012).

Fig. 2. Atlantic salmon smolt, photo: Bjørn Ove Johnsen

Born to be wild

As the list of highly exploited and endangered species is growing world-wide so are supplementation and re-introduction programs for such species, including fish (Barnosky et al., 2011; Fischer and Lindenmayer, 2000; Seddon et al., 2007). While, global production of farmed fish has increased dramatically and now almost matches wild fisheries in yearly yield, many wild fish populations are still under severe pressure due to overfishing or habitat degradation. For salmonid species, anthropogenic effects have negative consequences on both freshwater spawning habitats and ocean survival (Department of Fisheries and Oceans Canada, 2010; Fraser, 2008; National marine Fisheries Service, 2016; FAO, 2016; Naylor et al., 2000; Parrish et al., 1998). Damming a river can both destroy and block the passage to up-stream spawning habitats. Companies running hydroelectric power plants are therefore driven by injunctions to finance and manage the release of juveniles, to compensate for the loss of natural production. There are supplementation programs for Atlantic salmon and the anadromous form of rainbow trout, the steel head trout that releases billions of individuals every year both in Europe and Northern America. In the Baltic sea alone around 5 million Atlantic salmon juveniles are released as smolts each year, which is almost double the amount of natural recruitments migrating from rivers (Brown and Day, 2002; ICES 2015; National marine Fisheries Service, 2016).

The hatchery environment

There are many issues with raising animals in captivity and the current practices in conservational and supplementary rearing of salmonids raise questions concerning stress levels, morphological changes, misdirected selection pressures and the naivety of the released fish when coping with the wild environment (Department of Fisheries and Oceans Canada, 2010; Fraser, 2008; Jonsson and Jonsson, 2006; Parrish et al., 1998)

While it can be argued that the hatchery environment provides an environment lacking some of the daily dangers and challenges of the river such as finding prey and avoiding predators, it is not without stressors. While juvenile wild Atlantic salmon are bottom-living and territorial and take refuge in shelter when threatened, hatcheries provide a structure-less and monotone environment with high densities of fish and no opportunity to escape from conspecifics or human disturbance (Fig. 3). Conspecific aggression, crowding stress and unfavorable husbandry conditions are all known to affect fish welfare and performance. It can lead to negative stress effects and drain energy from health-related processes and disease outbreaks are a common problem in fish farming (Kalleberg, 1958; Murray and Peeler, 2005; Schreck, 1982; Segner et al., 2012).

When rearing animals for release into the wild, there is also the risk of producing phenotypes well adapted for the captive, but not for the wild environment. Due to the great diversity in life-history patterns, salmonids are very plastic in their development and environmental factors can largely influence the functional phenotype (Fleming et al., 1994; Thorpe et al., 1998). The hatchery-reared phenotype has been found to deviate from the wild phenotype in regard to morphology, maladaptive behavior and high prevalence of fin damage (Fleming et al., 1994; Huntingford, 2004; Latremouille, 2003). This has also been shown to translate into fitness effects, where hatchery-reared fish typically show lower smolt migration success, survival and reproductive success in nature. In the Baltic Sea (to give one example) wild smolts are three times as likely to return as a spawning adult compared with smolts released from hatcheries (Huntingford, 2004; Hyvärinen and Rodewald, 2013; Jonsson and Jonsson, 2006; Kallio-Nyberg et al., 2011). For conservational, ethical and economic reasons a shift towards producing a more wild-like smolt has therefore been advocated (Araki et al., 2007; Brown and Day, 2002; Thorstad et al., 2012).

Fig. 3. Left photos show sections of Imsa River, Norway. Right photos show standard hatchery tanks with salmon parr in the supplementary hatchery located at Imsa River, Norway. Photo: Knut Bergesen

What needs to change?

To increase survival rate after release it is important to provide a captive environment that produces robust and healthy smolts, but that also promotes physiological and behavioural polymorphism and that sustain genetic diversity over time (Fraser, 2008). There is, however, a general lack of experimental data showing how alternative designs of captive environments may benefit phenotypic development and post-release performance within conservational release programs (Barnosky et al., 2011; Brown and Day, 2002; Fischer and Lindenmayer, 2000; ICES, 2015; Naylor et al., 2000; Seddon et al., 2007). As the complexity of the natural environment is impossible to mimic in full-scale hatchery facilities, the challenge lies in identifying key modifications of the artificial rearing environment that are practically and economically feasible and that efficiently promote development towards a more wild-like phenotype.

Stress physiology

The word stress is often used loosely as a term to describe both the stressor and the stress response. A stressor can be defined as any factor that may compromise the homeostasis of an individual, which in this broad term could be anything from an unfamiliar environment, conspecific interaction or a bacterial infection (Barton, 2002). A simple stimulus such as a novel sound may thus act as a stressor even though there may be no actual danger connected to it. Upon perception of a stressor a well conserved cascade of events is triggered within the vertebrate body, and a commonly used definition was formulated by Selye (1973) as "The nonspecific response of the body to any demand made upon it".

The stress response can be grouped into primary, secondary and tertiary responses (Fig. 4). The primary response includes neuroendocrine and endocrine changes, such as alterations in neurotransmitter activity and increases in circulating levels of hormones such as catecholamines and corticosteroids. The secondary response includes changes in behavioural, cardiovascular, respiratory, immune and/or cellular processes, all of which are aimed at helping the individual to cope with the stressor at hand. These secondary responses are governed or influenced by the primary responses. If the intensity of the stressor is severe or chronic in nature, the effects from the primary and secondary responses can become maladaptive, leading to tertiary responses, such as decreased reproduction, growth and disease resistance. These effects can be either direct, through e.g. suppressive effects on immune function or indirect through competition between different energy-demanding processes (Barton, 2002; Segner et al., 2012).
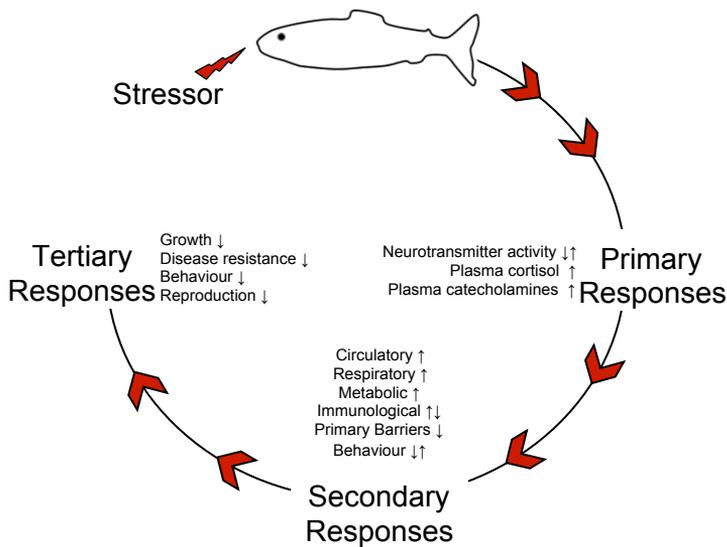
Fig. 4. Upon perception of a stressor, release of stress hormones and alterations in neurotransmitter activity (primary responses) leads to secondary responses. If the intensity of the stressor is severe or chronic in nature, the effects from the primary and secondary responses can switch from adaptive to maladaptive, leading to detrimental tertiary responses, such as decreased reproduction, growth and disease resistance.

Central and peripheral neurotransmitters

Many different neurological pathways are activated during stress, both within the central nervous system (CNS) and directed towards systemic target tissues (Nilsson, 2012). In the teleost brain, stress commonly leads to an increase in neurotransmission of monoamines such as noradrenaline, adrenaline, dopamine and serotonin (Winberg and Nilsson, 1993; Øverli et al., 1999; Øverli et al., 2004; Øverli et al., 2005). These neurotransmitters are concentrated and stored in vesicles, which upon membrane depolarization is released from the pre-synaptic nerve ending into the synaptic cleft, targeting specific receptors on the post-synaptic membrane (Lillesaar, 2011; Nilsson, 2012). They have been shown to control and modulate behaviour such as locomotion, exploration, boldness, temperament and aggression (Lillesaar, 2011; Øverli et al., 1999; Øverli et al., 2004; Øverli et al., 2005). In the peripheral nervous system, sympathetic and parasympathetic autonomic nerves regulate among other things, functions such as such as heart rate,

respiratory perfusion and intestinal function (Holmgren and Olsson, 2011; Nilsson, 2012).

The Hypothalamic-Sympathetic-Chromaffin Cell axis

The chromaffin cells, situated in the head kidney in teleost fish, are innervated by nerve fibres originating from the hypothalamus (Fig. 5). Upon stimulation, the chromaffin cells release catecholamines such as adrenaline and noradrenaline. Systemically, catecholamines acts through adrenoreceptors, where the primary role is to increase oxygen transport through modulation of the respiratory and circulatory systems and to mobilize energy stores, following an increasing energy demand during stress (Barton, 2002; Reid et al., 1998; Wahlqvist and Nilsson, 1980; Wendelaar Bonga, 1997).

The Hypothalamic-Pituitary-Interrenal-axis

The main corticosteroid in teleost fish is cortisol, which is released through the hypothalamic pituitary interrenal axis (HPI-axis), (Fig. 5). The action of the HPI-axis starts with the release of corticotropin-releasing hormone (CRH) from hypothalamic neuroendocrine cells. Release of CRH, in turn, stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. Circulating ACTH reaches the interrenal cells in the head kidney where it stimulates the release of cortisol into the blood stream. Cortisol acts through the glucocorticoid- and the mineralocorticoid receptors, which are abundantly expressed in most tissues, including the teleost brain (Bury and Sturm, 2007; Johansen et al., 2011). Cortisol have many systemic effects during both basal and stressed levels where the primary actions are regulating the osmotic and energy balance, with main target tissues such as gills, liver and intestine. Cortisol can also suppress energy demanding functions that are not essential during a stress episode such as immune activity, growth or reproduction and it can also function as a behavioural modulator (Barton, 2002; Barton and Iwama, 1991; Wendelaar Bonga, 1997).
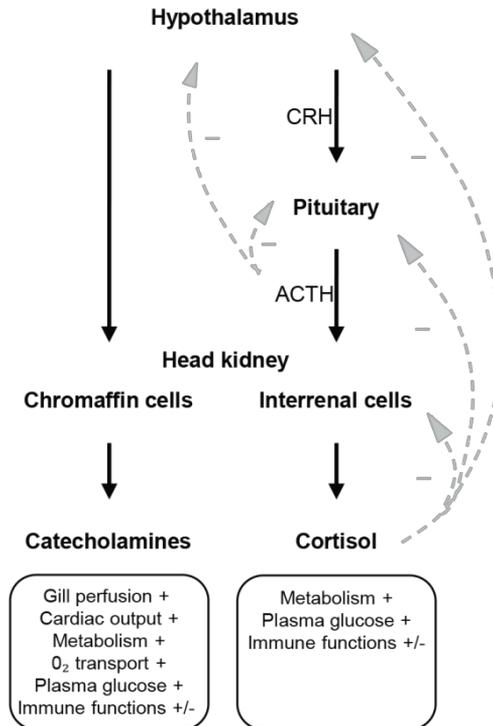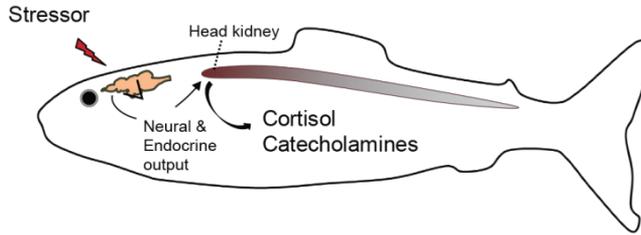
Fig. 5. Generalized image showing the primary, secondary and tertiary stress response. Upon perception of a stressor, catecholamines and cortisol are released from endocrine cells situated in the head kidney. The chromaffin cells release catecholamines upon stimulation of nerve fibres from the hypothalamus. The signalling cascade for cortisol release is initiated in the hypothalamus by the release of CRH which stimulates release of ACTH from the pituitary. ACTH is transported via the bloodstream to the interrenal cells. Cortisol and ACTH have negative feed-back effects on further cortisol release.

Coping with stress

In both captivity and in the wild, fish are subjected to a wide range of stressors, which if severe or chronic can lead to tertiary effects, where the

stress response itself, as described earlier can initiate negative physiological or behavioural responses (Barton, 2002; Pickering and Duston, 1983; Strange and Schreck, 1978). In both fish and mammals, prolonged or severe stress has been shown to impair decision making and spatial memory, decrease growth and reproduction, compromise immunity and lead to outbreak of disease (Gaikwad et al., 2011; Gregory and Wood, 1999; Murray and Peeler, 2005; Schreck, 1982; Segner et al., 2012; Wright and Conrad, 2005). Within a population there is, however, often large variation in how well individuals cope with a specific stressor and tertiary responses to stress are also most severe when an individual is faced with uncertainty and/or lack of control (Koolhaas et al., 2010; Weiss, 1968). As exemplified by a study by Weiss (1968), individuals which are offered a "coping response" during stress may also be less affected. In this famous study, one group of rats was thought to believe that they controlled the offset of electric shocks given to them, while the other group was not offered this perception. The second group lost more weight and developed more gastric ulcers compared to the group that could perform a "coping response", even though the intensity of the physical stressor (the electric shocks) was the same between the groups.

Animal personality

There is also large inter-individual variation in how individuals perceive and respond to potential stressor. Some people enjoy taking risks, such as skydiving while others don't. Upon facing a challenge some individuals are also more prone to respond with aggression or active avoidance, while others will take on a more submissive or passive strategy. The occurrence of such individuality in temperament and behaviour has not only been found in humans, but across animal taxa, in groups as diverse as insects, rodents, fish and crustaceans (Koolhaas et al., 1999; Koolhaas et al., 2010; Wilson et al., 1994).

In fish, behavioural suites of dominance, boldness, aggression and exploration has also been found to correlate with physiological factors such as stress hormone reactivity and brain serotonergic activity (Thörnqvist et al., 2015; Øverli et al., 2005; Øverli et al., 2007). Within different research fields, this phenomenon of suites of linked behavioural and physiological traits has acquired different names and labels and research on animal personalities, behavioural syndromes and stress coping styles are now published in ecological, conservational, psychological and medical research. The criteria for these different "terms" largely overlap and they are all defined by repeatable intrinsic

characteristics that do not include non-repeatable variation determined by e.g. recent experience or current physiological state (Careau et al., 2008; Koolhaas et al., 1999; Koolhaas et al., 2010; Wilson et al., 1994; Wolf et al., 2007).

The evolutionary basis for the establishment and maintenance of this type of polymorphism is that seemingly opposing traits can have similar selective value. The fitness benefit of a certain trait is also dependent on its environmental context, which in a natural environment inevitably changes over time. This has caused concern as to whether the hatchery environment can have a negative impact on genetic and phenotypic diversity. Previous studies on salmonids reared for release have shown that the hatchery environment promotes aggressive and dominant behavioural strategies, leading to selection for risk-prone phenotypes (Johnsson et al., 1996; Metcalfe and Thorpe, 1992; Sundström et al., 2004). A study by Vaz-Serrano (2011), on the other hand showed relaxed selection pressure within the hatchery, which instead could lead to decreased selection for diverging behavioural types. Both scenarios can have a negative impact on the polymorphism and performance of a population and also over time, decrease its evolutionary potential.

Stress coping styles

The term "stress coping style" is often used in medical and physiological research, where the main focus is to search for intrinsic links between the physiological and behavioural profile during stress.

A distinction is often made between two divergent stress coping styles, the proactive (active coping) and the reactive (passive coping). The behavioural repertoire of proactive individuals is often characterized by a "fight or flight" reaction when perceiving a stressor, involving active avoidance, bolder behavioural profile, high aggression, dominance and quick development of routines. The reactive stress coping style is instead characterized by a "conservation-withdrawal" response when challenged, involving immobility and "freeze" behaviour , more risk sensitive behaviour (shyness), low aggression and subordination, and a more flexible behavioural profile (Koolhaas et al., 1999; Øverli et al., 2007). Differences in adrenergic, serotonergic and dopaminergic mechanisms within the CNS as well as in adrenergic and corticoid humoral output has been linked to diverging stress coping styles (Brelin et al., 2005; Koolhaas et al., 2010; Schjolden et al., 2006; Thörnqvist et al., 2015; Øverli et al., 2005; Øverli et al., 2007).

Both genetic and ontogenetic factors appear to impact the formation and maintenance of diverging stress coping styles within a population. Hormonal stress reactivity, aggression, boldness and exploratory behaviour are all traits that have been shown to be partly controlled by genetic components (Dingemanse et al., 2002; Koolhaas et al., 2011; Pottinger and Carrick, 2001; Øverli et al., 2007). The timing of when salmonid siblings swim up from the spawning nest to establish a territory has e.g. been coupled to diverging stress coping styles. Fish that emerge late show stronger activation of the brain serotonergic system during stress compared to early emerging fry, whereas early emerging fry have been shown to be bolder and more aggressive than late emerging fry (Metcalfe and Thorpe, 1992; Thörnqvist et al., 2015). While early emerging fry have been shown to suffer from higher rates of predation, in a hatchery environment their aggressive and dominant "personality" can lead to a higher acquisition of resources. Early emerging fry have been shown to obtain higher feed rations, growth, resulting in a higher proportion of early emergers reaching smoltification within their first year compared to late emerging fry with a slower development (Brännäs, 1995; Metcalfe and Thorpe, 1992). This has raised concern as to whether early emerging fry have a selective advantage in the hatchery which might skew and lower the variation of released phenotypes (Metcalfe and Thorpe, 1992).

HPI-axis reactivity

Large repeatable inter-individual variation in circulating levels of plasma corticosteroids during stress exist in a wide range of vertebrates, including several fish species (Castanheira et al., 2013b; Koolhaas et al., 2010; Pottinger et al., 1992). Corticoid reactivity has repeatedly been linked to certain behavioural traits and to the concept of stress coping styles, suggesting evolutionarily conserved correlation among multiple traits. High responding individuals (HR) generally have traits linked to a reactive stress coping style, such as a shy behavioural profile, subordination and low aggression whereas low responding individuals (LR) instead generally show a more proactive personality with a bolder behavioural profile, high aggression and dominance ranking. For rainbow trout, HPI-axis reactivity has been found to be a heritable trait where linked physiological and behavioural traits expressed in the parental generation persist in their progeny (Castanheira et al., 2013b; Koolhaas et al., 2010; Pottinger and Carrick, 2001; Ruiz-Gomez et al., 2011; Schjolden et al., 2005; Øverli et al., 2005).

The intestinal barrier function

The intestine is an organ that is known for being sensitive to stress. This is well documented in mammals and the body of information growing also for fish (Segner et al., 2012; Söderholm and Perdue, 2001; Velin et al., 2004; Weiss, 1968). Both acute and chronic stressors such as crowding, hypoxia, high rearing densities and social stress can impair the integrity of the intestinal epithelium of fish. Stress induced negative effects on the intestinal barrier function has also been shown to lead to tertiary effects such as increased pathogen entry and infection rate (Fridell et al., 2007; Niklasson et al., 2011; Olsen et al., 2005; Peters, 1982; Sundh et al., 2010).

While providing the individual with an efficient nutrient and fluid transport, the intestinal barrier needs to be selective, preventing entrance of pathogens and detrimental substances. This selective barrier can be divided into three functional parts, the extrinsic, the intrinsic and the immunological barrier (Fig. 6). The outermost layer towards the lumen is the extrinsic barrier, constituted by mucus containing e.g. antimicrobial substances. The mucus, continuously secreted by goblet cells in the intestinal epithelium, protects and removes harmful agents from the epithelium while commensal bacteria in the lumen further protect the epithelial cells against foreign microbes and pathogens. The intrinsic barrier is constituted by a single layer of innervated epithelial cells called enterocytes, attached to each other by tight junctions. The tight junctions are transmembrane-protein-complexes connecting the enterocytes to each other at the apical end of the epithelium. The cytosolic side of the tight junctions is also attached to the actin-ring of the enterocyte cytoskeleton.
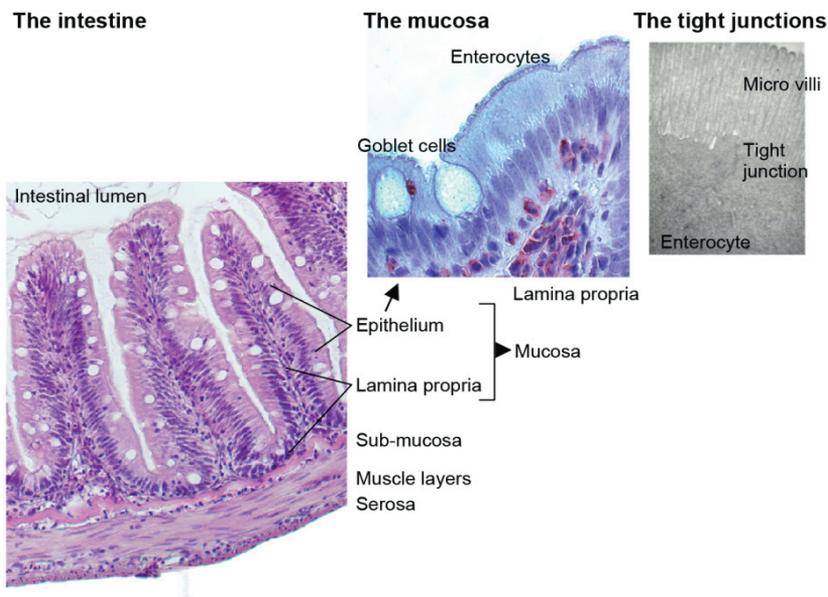
Fig. 6: Cross section of Atlantic salmon anterior intestine showing gross morphology as well as some functional components of the intestinal primary barrier. Courtesy of Kristina Sundell and Henrik Sundh.

Together the enterocytes and the tight junctions form a continuous barrier that regulates both transcellular and paracellular diffusion of molecules, thus constituting the principal component of the intestinal primary barrier (Beck and Peatman, 2015; Schneeberger and Lynch, 2004; Sundell and Sundh, 2012). The immunological barrier, is comprised of various immune cells (e.g. macrophages, lymphocytes and mast like cells) mainly situated in the lamina propria and sub-mucosa underneath the epithelium (Jutfelt, 2011; Press and Evensen, 1999; Schneeberger and Lynch, 2004; Segner et al., 2012)

Some of the negative effects on the intestine caused by stress are thought to be conveyed via the local immune system and/or the brain-gut axis, which is a term often used in mammalian literature to describe the bi-directional communication between the CNS and the intestine (Aziz and Thompson, 1998; Segner et al., 2012). Top-down signals of the axis are conveyed via efferent autonomic nerves and systemic release of corticosteroids and catecholamines, while bottom-up signals are conveyed via afferent sensory fibers (Aziz and Thompson, 1998; Holmgren and Olsson, 2011).

Metabolic rate

All of the energy demanding processes that occur in the body of an animal can be summed up to calculate an energy budget. The input (energy intake) and output (energy expenditure) of this budget will not only vary depending on the environment the animal lives in, different life stage requirements and challenges it face but also on its behavioural profile (Burton et al., 2011; Careau et al., 2008; Tort, 2011).

At rest, an animal spends energy on the most essential processes such as cell and organ-system maintenance and basic immune function. Excess energy reserves, beyond those used for maintenance can be used for locomotion, growth and reproduction (Tort, 2011).

For ectotherms, the energy output needed to sustain basal functions is defined as the standard- or resting metabolic rate. The standard metabolic rate (SMR) can be approximated by measuring the oxygen consumption at a resting and post-absorptive state, at a given acclimation temperature (Burton et al., 2011). The maximum aerobic metabolic rate (MMR), which in most cases is measured during or after strenuous exercise, provides a measure of the oxygen transporting capacity of an individual. The aerobic MMR is largely dependent on the efficiency of gas exchange and the cardiovascular system (Claireaux et al., 2005; Norin and Clark, 2016). During maximal burst swimming, the muscles, however, rely on both aerobic and anaerobic metabolism. The MMR is therefore not only a critical determinant of aerobic swimming capacity, it also determines the need for using anaerobic mechanisms. For the individual, reaching a high MMR during exhaustive exercise may thus improve the swimming capacity and reduce the post-exercice recovery rate (Claireaux et al., 2005; Kieffer, 2000). The difference between the MMR and the SMR of an individual gives the aerobic scope (AS), which can be used as a proxy for the capacity to perform various oxygen-demanding processes simultaneously (Burton et al., 2011; Clark et al., 2013; Norin and Clark, 2016). A high AS has e.g. been shown to correlate to a high growth potential in fish, at least when food is abundant (Auer et al., 2015a; Auer et al., 2015b)

The behaviour of an individual will ineviatably affect energetic expenditure, where high activity and aggression are metabolically demanding actions. A more passive and shy behavioral profile should thus enable a life-style with comparatively lower energy requirement and metabolic rates (Careau et al., 2008; Korte et al., 2005). Individuals showing characteristics such as boldness, high aggression and dominance

have been revealed to have both a higher SMR and routine metabolic rate in comparison to more shy and subordinate individuals (Killen et al., 2011; Killen et al., 2012; Lahti et al., 2002; Metcalfe et al., 1995). The causal relationship between metabolic rate and behavioural characteristics is however not always clear as the metabolic rate can affect behaviour and vice versa. While an intrinsically bolder and more aggressive behavioural profile may increase total metabolic costs, these behavioural characteristics may also lead to higher feed intake which in turn can cater for a higher metabolic rate. On the other hand will an intrinsically higher metabolic rate demand a higher energy input, which in turn will enforce risk taking while searching for food (Careau et al., 2008).

One physiological system which is linked to both behavioural modulation and energy translocation is the HPI-axis, suggesting that this system also may function as a link between personality traits and metabolic rate (Barton, 2002; Boeck et al., 2001; Wendelaar Bonga, 1997). Following a fight for social dominance in brown trout, losers showed an increase in SMR compared to winners. This effect was suggested to be caused by higher levels of cortisol in subordinate individuals (Sloman et al., 2000). Furthermore, while several studies have investigated the relationship between behavioural characteristics and SMR, other metabolic factors such as composite metabolic rate (total oxygen consumption over an extended period), MMR and aerobic scope (AS) and their relation to behaviour and stress coping is not as well understood.

AIMS

The overall aim of this thesis is to extend the knowledge-base on stress physiology and behaviour of salmonids and how they are interlinked with and modulated by environmental factors.

The specific aims of the thesis are:
- **To assess how different rearing environments impacts stress levels, welfare and survivability of Atlantic salmon reared for conservational releases.**

- **To investigate how the early life-stage phenotype of Atlantic salmon is linked to behavioural stress coping and physiological performance traits in later life-stages**

- **To explore intrinsic links between hormonal stress reactivity and relevant and related behavioural and physiological traits in salmonids.**

Implementation and specific objectives
- **Paper I & II** was designed to evaluate the effects of decreased rearing density and added structural enrichment to the tanks of juvenile Atlantic salmon, with the incentive to produce a more wild-like and robust smolts for release into the wild. The outcomes were evaluated through measurements of primary stress responses, the intestinal barrier function as a secondary stress marker, conspecific aggression levels and adaptive behavioural traits such as shelter seeking and smolt migration.

- **Paper III** was designed to investigate how early and late emergence from the spawning nest of newly hatched salmon affect the behaviour and physiology at later juvenile life-stages in hatchery reared salmon. Early and late emerging individuals were tested repeatedly for risk-taking during their juvenile development. Prior to release as smolts, the metabolic rate and aerobic capacity was evaluated in two ecologically relevant behavioural extremes (groups based on emergence and risk taking).

- Based partially on the results from **papers I**, **II** and **III**, a fourth experiment was designed to look for causal links between hormonal stress reactivity, expression of selected stress related neuronal components in the CNS, intestinal stress sensitivity, metabolic rate, boldness and activity levels. The results from this experiment are divided into two papers, where **paper IV** covers the brain-gut axis and **paper V** covers the metabolic rate and behaviour of rainbow trout with low and high HPI-axis reactivity.

METHODOLOGICAL CONSIDERATIONS

This section will provide an overview and discussion of the methodology used in the studies which comprise this thesis. For a complete and more detailed description of the specific procedures and experimental protocols, the reader is referred to the individual papers.

Experimental animals

Two species of salmonids were used as experimental animals: Atlantic salmon (**papers I, II, III)** and rainbow trout (**papers IV, V**). As the first three papers are based on experiments performed to evaluate issues concerning supplementary release of Atlantic salmon, the choice of species needs no further explanation. For paper **IV** and **V**, rainbow trout was chosen as a model species for stress coping mechanisms and to enable comparison with previously published articles on HPI-axis reactivity in rainbow trout. The rainbow trout is also a robust fish to keep in the laboratory and its close phylogenetic relationship with other ecologically and commercially important salmonid species makes it a relevant and commonly used species for biological and physiological studies.

Atlantic salmon (*Salmo salar*)

The hatchery in **paper I** and **II** is located at the river Imsa, on the Norwegian west coast, south of Stavanger. It was founded as a research facility in the 1970's to study salmon reproduction and migration and the effect of supplementary release. The research station is driven by The Norwegian Institute for Nature Research, NINA. Imsa is a relatively small river and the research station has full monitoring of ascending and descending fish in the river through a "wolf trap" positioned in the river outlet. The eggs and milt for the hatchery stock are collected from wild parents migrating from the ocean into the river, which sustains a small population of wild Atlantic salmon.

The hatchery in **paper III** is situated in Älvkarleby by River Dalälven which empties into the Baltic Sea on the Swedish east coast (Fig. 7). The hatchery was founded upon the construction of a hydroelectric power plant in 1915, 10 km from the river mouth and it is now a joint

supplementary facility and research station, driven by Swedish University of Agricultural Sciences, SLU.
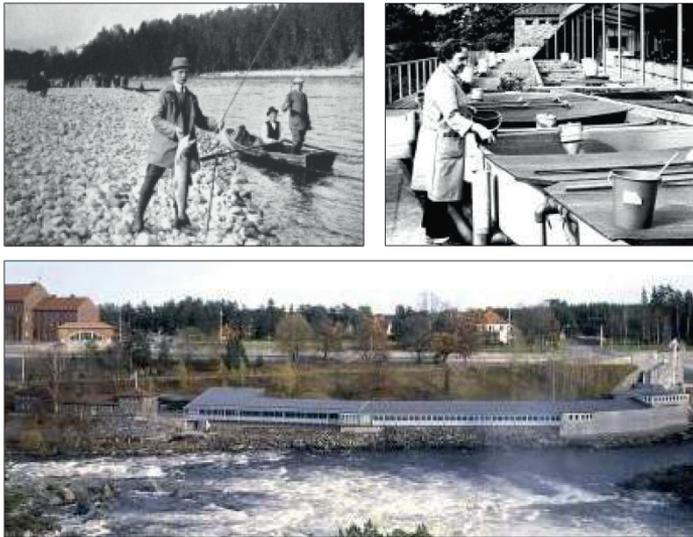
Fig. 7. Photographs from the Älvkarleby hatchery, River Dalälven, Sweden. Top left photo shows sports fishing of Atlantic salmon in the river in the early 1900s, photo: Vattenfall. Top right photo show hatchery tanks in the 1960s, photo: Bjarne Ragnarsson. The bottom photo shows the current hatchery and research facility, photo: Håkan Jansson.

Although Atlantic salmon spawn naturally downstream from the power plant, the wild reproduction in this river system is minimal and the eggs and milt for the hatchery stock are collected from sea-run adults released from the hatchery as smolts.

Rainbow trout (*Oncorhynchus mykiss*)

The rainbow trout used for this thesis in **papers IV** and **V** originated from Vänneåns sport fishing hatchery situated 180 km south of Gothenburg. The rainbow trout is native to North America and Russia but is frequently farmed in both freshwater and seawater for either release for angling or consumption, all over the temperate regions of the world (Stanković et al., 2015). The cultivated strains in Europe originates from the anadromous form of the *Oncorhynchus mykiss* species, the steelhead trout, but are now named after the wild landlocked version, the rainbow trout, since they have been cultivated for generations in freshwater.

How to evaluate fish performance and welfare?

The ideal approach for testing the performance and/or survivability of different phenotypes reared in different environment or that have diverging stress coping styles would be to follow their development on a large time-scale. For hatchery fish this can be done by releasing the fish as smolts and then to monitor their survival at sea and the subsequent return rate to the spawning grounds. However, as this approach is time-consuming and acquires a very large number of fish (sea survival rate is normally <5 %), it is from an ethical and practical perspective preferable to work with smaller sample sizes before large-scale implementation of e.g. changes in rearing environment design. Instead studying different welfare markers and fitness- and performance correlates, can give an indication of how competitive a phenotype is and/or how suitable a rearing environment is. The approaches used in this thesis are summarized and discussed below.

Physiological assessments

Stress hormone levels: Plasma cortisol concentration is a frequently used stress marker in fish (Barton, 2002). The magnitude and extent of a cortisol response to acute stress generally reflects the severity and duration of the stressor (Barton and Iwama, 1991). Plasma cortisol levels in resting or unstressed teleost fish usually measure below 10 ng/mL, while peak post stress cortisol levels characteristically span between 40-300 ng/mL in salmonids (Barton and Iwama, 1991; Iwama, 1998). If the stressor is transient, the levels generally start to decrease within a few hours, depending on the severity of the stressor. During long-term stress,

plasma cortisol levels can be sustained at an elevated level for days and weeks (Pickering and Pottinger, 1989). However, also during sustained and chronic stress, plasma levels tend to decline over time, either stabilizing at an elevated level above what is usually considered basal or even declining down to levels measured at rest (Barton and Iwama, 1991; Fridell et al., 2007; Sundh, 2009). This effect is generally caused by negative feed-back mechanisms on the HPI axis and/or by acclimation effects (Barton and Iwama, 1991). Through these desensitization mechanisms, a state of chronic stress has also been shown to decrease the sensitivity of the HPI-axis, resulting in lower cortisol response towards acute stressors in chronically stressed individuals (Barcellos et al., 1999; Madaro et al. 2015). The latency of the HPI-axis to excrete cortisol into the bloodstream following the perception of a stressor is beneficial during sampling, where the plasma levels can remain largely unaffected by sampling, if performed swiftly and within the window of cortisol excretion, <4 min (Gamperl et al., 1994). Anaesthetizing the fish using Metomidate also provides protection against cortisol elevation, as it blocks the interrenal cells from excreting cortisol, once the fish is anaesthetized (**papers II, III, IV,V**)(Olsen et al., 1995).

Plasma cortisol measurements were performed in all papers. It was measured during basal (undisturbed) conditions, to allow comparison with post-stress levels and/or to observe elevations above what is normally considered basal (**papers I, II, IV**). Post-stress plasma cortisol measurement was also performed to examine possible differences in cortisol release (**all papers**) and to separate a population of rainbow trout into two distinct groups with low responding and a high responding HPI-axis reactivity (**papers IV, V**).

Intestinal barrier function: The physiological function of the intestine primary barrier can be measured in live epithelium using the *in vitro* Ussing chamber technique (Ussing and Zerahn, 1951). The method allows for studies of both active transport and passive diffusion rates over the epithelium and it is also a commonly used method to assess pathophysiological conditions and mechanisms in mammals (Santos et al., 2001; Saunders et al., 1994; Saunders et al., 1997).

The intestinal epithelium functions as an electrical circuit where the active ion transport mechanism of the enterocytes, mainly driven by $Na^+,/K^+$-ATPases, creates a potential difference across the apical and basolateral side of the epithelium. The transepithelial potential can be measured by placing two electrodes on each side of the epithelium and by

manipulating the active transport mechanisms (by briefly adding an external source of voltage) other properties such as the electrical resistance of the epithelium can be calculated. The transepithelial electrical resistance (TER) provides a measurement of the ion fluxes moving across the epithelium, which due to the differences in strength of the three resistors included in the electrical circuit of the intestinal epithelium (apical cell membrane, basal cell membrane and tight junctions) is mainly conveyed via the paracellular route. Subsequently, measurements of transepithelial resistance mainly reflect the permeability and tightness of the tight junction proteins (Loretz, 1995; Sundell and Sundh, 2012)

In **papers II** and **IV**, the TER was used to estimate the paracellular permeability of charged molecules, while the apparent permeability (P_{app}) of the hydrophilic molecule mannitol was used to estimate passive diffusion of uncharged, water soluble and inert substances over the paracellular route. Nutrient transport rate was assessed using an exemplifying amino acid, L-lysine.

As stress is known to have a negative impact on the intestinal barrier function in both mammals and fish, the integrity of the intestinal epithelium was used as a secondary stress marker in **papers II** and **IV**. In addition, as even small changes and decreases in intestinal integrity can affect e.g. antigen and pathogen translocation rates, the results are also used to discuss possible differences in disease resistance between groups (**papers II, IV**) (Fridell et al., 2007; Sundh, 2009; Velin et al., 2004).

Metabolic rate: To gain knowledge of the energy requirements and aerobic capacity and performance among experimental groups, SMR, MMR, and AS was measured in **papers II** and **V**. In **paper V**, an approximation of the composite (total) oxygen consumption was also calculated.

Oxygen consumption was measured using optical oxygen sensors and an intermittently closed respirometry system. The size of the respirometer chambers limits swimming and exploration behaviour while still allowing the fish to exhibit some spontaneous locomotion (i.e. turning around) to reduce the sensation of confinement. There is a trade-off between the size (volume) of the respirometer and the size of the fish and an appropriate water volume: body volume ratio has to be maintained in order to get accurate measurements.

The SMR was calculated from the lowest measuring values to exclude measuring points during spontaneous activity, while the MMR was measured directly upon termination of a 5 min long chase protocol.
The composite oxygen consumption was calculated in **paper V** using a linear trapezoidal method for calculations of "the area under the curve" (AUC)

$$AUC = \sum_{n=0}^{N-1} = \tfrac{1}{2} \cdot (f_n + f_{n+1}) \cdot (\Delta_x)_n$$

$f = mg\ O_2\ consumed \cdot kg^{-1} \cdot 5^{-1}\ min$

$\Delta_x = 1$

The AUC method was chosen as it provides an estimate of the total oxygen consumed during a given time period rather than just providing individual mean values of total oxygen consumption (which sometimes is presented as routine metabolic rate, in addition to SMR and MMR). The use of this method not only aids in presenting the data in a physiologically relevant manner but it also provides a higher resolution of the data and no assumptions regarding the distribution of data need to be made at this point in the calculations, compared to when using average values. Furthermore, while SMR is a standardized tool for measuring the metabolic cost of basic physiological functions, it is based on the lowest points in a data set and is thus based on extreme values (Clark et al., 2013). The true metabolic requirement of an individual is found when it is measured over a longer time scale and should preferably cater for both the stress and behavioural profile of that individual (Careau et al., 2008). While placing the animal in a respirometer chamber will inevitably restrict behavioural choices, adding calculations of the composite oxygen consumption will still give added value, providing a data set with higher resolution and complexity.

Hematology and cardiac ventricle measurements: Hematocrit, hemoglobin levels and cardiac functionality are all critical components in the oxygen transporting cascade. During exercise and stress, contraction of the spleen, which holds a red blood cell reservoir increases hematocrit and hemoglobin levels and thus the oxygen carrying capacity of the blood (Pearson and Stevens, 1991; Wendelaar Bonga, 1997).
Hematocrit and hemoglobin concentrations were measured in **papers III** and **V**. The mean corpuscular hemoglobin concentration (MCHC) is

calculated by dividing the hemoglobin concentration by the hematocrit. A low MCHC can thus be a sign of either low hemoglobin production or a swelling of the red blood cells. As catecholamines are known to stimulate the Na^+/H^+-exchanger in the red blood cells during stress (leading to cell swelling) a low MCHC can be a sign of an increased adrenergic output (Nikinmaa, 1982).

Smolt status: The smolt status was evaluated to determine whether an individual is "ready" to migrate from freshwater into the more saline seawater. The standard hatchery practice is to evaluate smolt status through visual examination of skin silvering index and swimming behaviour. When the smolts are ready to migrate, they start to exhibit down-stream migration-like behaviour, swimming with, rather than against the water current in the tanks (**papers II, III**). It can also be evaluated through measurements of plasma cortisol levels (**paper II**) and through measurement of $Na^+,/K^+$-ATPase enzyme activity in the gills, which increases during smolt development as a pre-adaptation to sustain ionic homeostasis in seawater (**paper III**) (McCormick, 1993; Staurnes et al., 1993).

Telencephalon gene expression: Within the CNS, cortisol is known to act via both mineral- and glucocorticoid receptors (Schjolden et al., 2009; Sørensen et al., 2013). Another component within the CNS suggested to be interlinked with the HPI-axis is the serotonergic system (Chaouloff, 2000; Winberg and Nilsson, 1993; Winberg et al., 1997). The majority of previous studies that have looked at links between behavioural characteristics, stress reactivity and the serotonergic system have, however, mainly looked at the concentration of serotonin and its metabolites (Andersson and Höglund, 2012; Schjolden et al., 2006; Øverli et al., 2005). Other factors affecting the nerve signaling transduction are the amount of receptors conveying the signal and re-uptake pumps which clear the synaptic cleft from serotonin (Lillesaar, 2011). mRNA levels of serotonergic receptors and pumps and corticoid receptor levels were therefore measured in the telencephalon (**paper IV**). Measuring mRNA levels shows the translation levels of a specific gene and can thus provide an approximation of the levels of the expressed protein (e.g. a receptor). It can also show whether a specific gene is up- or downregulated following a specific treatment.

Growth: For salmonids, growth is generally considered an adequate fitness-correlate as it affects other life history traits such as survival and reproduction (Friedland et al., 2009; Jonsson et al., 1996). Growth rate is dependent on food consumption, intestinal up-take and metabolic rate and as all of these processes can be affected by stress, impaired growth can also be a sign of impaired welfare and health (Wendelaar Bonga, 1997). Specific growth rates were monitored in **all papers**.

Behavioural assessments

Fin damage: Fin damage on the dorsal fin is primarily caused by aggressive biting by conspecifics and can thus be used as a tool to study social interactions in large groups of fish (Hoyle et al., 2007; Turnbull et al., 1998) (**papers I, II**). While fin damage is a common concern in hatchery-reared salmon, the prevalence of fin damage in wild parr and smolts are low in comparison (MacLean et al., 2000). Aggressive biting not only causes irreparable structural damage to the fins which can reduce swim capacity but wounds are also susceptible to infection by pathogens (Latremouille, 2003; Webb, 1977).

Shelter seeking: Taking refuge in shelter is a natural element in the behavioural repertoire of wild juvenile salmonids and adding shelter to the rearing tanks may thus increase the welfare of the fish (Kekäläinen et al., 2008; Sneddon et al. 2016). Displaying active shelter-seeking upon release could further increase survival of hatchery fish as they typically experience high predation (Aarestrup et al., 2005). Adding structural enrichment to otherwise barren tanks has also been shown to enhance spatial cognition in salmonids (Näslund and Johnsson, 2014).
For **paper I**, a shelter seeking arena was therefore designed, allowing for visual observation of shelter seeking propensity in a novel environment. The fish were placed on one side of a tank divided by a mesh, with a small opening through which the fish could swim. On the other side, there was a sheltering structure (plastic tubing). The opening was elevated 15 cm from the bottom so that fish had to actively swim upwards to pass through the opening in the mesh and reach the shelter. The experimental protocol and arenas was also used for **paper II**.

Smolt migration success: When the fish had developed into smolts ready to migrate to the ocean (**paper II**), they were released 1000 m up-stream from the Imsa river outlet. The migration success of the different experimental groups was then monitored through the capture rate of fish

entering a so-called wolf trap in the river outlet. In comparison to using telemetry tags or PIT-tag antennas at different locations in the river, this method does not provide high resolution data of individual migration speed (Barbin Zydlewski et al., 2001; Hyvärinen and Rodewald, 2013). However, the fish do not need to be implanted with larger internal tags which might affect post-release performance. The method also provides a reliable measurement of survival and migration success of all released fish, as the whole water body exiting the river passes through the trap and it is emptied and checked for fish daily, all year round.

Boldness: Risk-taking and boldness of individual fish was measured using two different techniques. In **paper III**, an hypoxia avoidance set-up was used where decreasing oxygen levels was used as a trigger for exploratory and bold behaviour by moving from a familiar "home tank" into an oxygenated but illuminated "unfamiliar tank" (Laursen et al., 2011). As the behavioural screening is done in groups (~10 fish) individual decisions could possibly be affected by other group members. The method has, however, been validated and shown to correlate to risk taking and boldness in fish (Castanheira et al., 2013a; Thörnqvist et al., 2015).

In **paper V**, an open field arena was used to measure general activity levels and boldness (time spent in centre of the tank) of individual fish, using over-head video recording. The fish were placed in individual, square barren tanks. Individual swimming activity was then recorded and quantified as swimming activity, rapid swimming activity (set to >1 body length/s, inactivity, and duration in centre. As the fish were introduced into a novel tank, the swimming activity is probably influenced not only by general activity levels, but also by exploration propensity and boldness of the fish (Toms et al., 2010).

Basic terminology

High density: The term high density is used to describe the standard density of 150 fish/m^2 (**papers I & II**). At smolt release 150 fish/m^2 corresponds to a mean density of ~4.5 kg/m^2 or 14.5 kg/m^3 which is the standard hatchery practice for 1+ Atlantic salmon hatchery smolts for this locality (Imsa hatchery) as well as other hatcheries in Europe (Brockmark et al., 2007; Roberts et al., 2011).

Low density: The term low density is used to describe the experimentally lowered density used in **papers I & II** (50 fish/m^2),

which corresponds to a density of ~1.5 kg/m² or 4.8 kg/m³ at smolt release. This was based on previously published studies seeing positive effects on e.g. post-release performance using the same level of density reduction (Brockmark et al., 2007; Brockmark et al., 2010). As a reference point the River Imsa has been shown to hold a natural smolt density of 1.3-8.8 kg/1000m² (Jonsson et al., 1998).

Barren tank: Tank void of physical structure (Fig. 8).

Structurally-enriched tank: Tank with added physical structure that provides shelter and increases structural complexity (Fig 8).
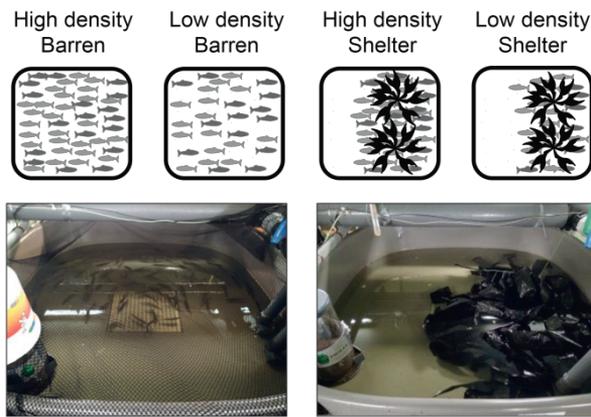

Fig. 8. Photographs of tanks containing Atlantic salmon parr living at low density in barren tanks (left photo) and at low density with shelter (right tank) as well as a schematic picture of the experimental setup used in paper II. Photo: Joacim Näslund.

Basal conditions: Undisturbed conditions (no human contact or other interference the last 24 h and no handling (netting, transport) two weeks prior to sampling.

Stress conditions: Caused either unintentionally by maintenance/handling or intentionally to evoke a stress response.

Standard metabolic rate (SMR): Oxygen consumption at an inactive and post-absorptive resting state.
Maximum metabolic rate: Maximum rate of oxygen consumption during/following exhaustion.

Routine metabolic rate: Mean oxygen consumption over a given time period allowing for spontaneous activity.

Composite metabolic rate: Total oxygen consumption over a given time period.

Fry: Developmental phase following emergence from the spawning nest. During this phase (in the wild), the juveniles disperse from the nest and typically establish a territory.

Parr: The fry quickly develop into parr with camouflaging vertical stripes. The parr typically stay in the river 1-3 years before initiating smoltification.

Smoltification: Morphological, behavioural and physiological pre-adaptation to seawater, transforming the river-dwelling parr into a smolt.

Smolt: Juvenile salmonid physiologically and behaviorally ready to migrate from the freshwater-river into the ocean.

Welfare: There is no universal definition for animal welfare. In this thesis welfare is defined as a state when animals are free from disease, discomfort and detrimental stress. This definition is based on three main methodologies; the feelings-based welfare approach, stating that the individual should not experience discomfort through pain and fear, the function-based approach, stating that the individual should be provided with the resources it needs to sustain homeostasis and health and the nature-based approach which states that the individual should also be provided with the resources it needs to sustain a natural behavioural repertoire (Segner et al., 2012; Sneddon et al., 2016).

The brain-gut axis: Describes the bi-directional communication between the CNS and the intestine (Aziz 1998).

RESULTS & DISCUSSION

Born to be wild

Stress hormones and aggression

Higher levels of plasma cortisol were found in parr living in barren tanks compared to those living in tanks with shelter (see Fig. 9A; **paper I**). They levels were comparable to those typically seen after long-term stress in salmonids (Fridell et al., 2007) and since no tank effects were apparent, this could be a sign of chronic stress. As the HPI-axis reactivity can decrease during chronic stress (Barcellos et al., 1999; Madaro et al. 2015) the parr were also exposed to a confinement stress test and subsequent cortisol measurements. However, no signs of such a desensitization effect on the HPI-axis were found in any of the groups (**paper I**).

To investigate if an elevated cortisol level is a general feature for fish living in barren hatchery tanks, a more elaborate sampling protocol was conducted for **paper II**, with four consecutive samplings from the parr to the smolt stage (Dec, Jan, Feb and May). The only result supporting the findings of **paper I** was found in January where an elevation above resting levels was present in two out of three of the low density barren tanks (**paper II**). At all the other samplings, the levels were below what is usually considered to be basal, except in May when an expected rise in cortisol was found in all groups due to smolt development (Iwama, 1998; Sundell et al., 2003). As the fish density in **paper I** correspond largely with the low density treatment in **paper II,** the result suggests that keeping fish at low density in barren tanks could result in higher incidence of sporadically elevated cortisol levels. This could be triggered by episodic elevations of conspecific competition in low density tanks, as decreasing density generally increases benefits linked to territoriality (Johnsson et al., 2014). When breeding animals for release into the wild, such an effect is not necessarily negative, as competing for resources is a natural part of the territorial river-dwelling salmonid parr (Kalleberg, 1958). Brown trout kept at natural densities during the hatchery phase, had for example higher dominance status, grew and survived better in a natural stream than a group kept at higher densities (Brockmark and Johnsson, 2010).

If the higher plasma cortisol levels in the low density barren tanks indeed was caused by conspecific interaction this was, however, not translated into aggressive biting as this group had lower levels of fin damage compared to the high density group (see Fig. 9D; **paper II**).

The higher cortisol levels in low density barren tanks may also be an effect of how the fish respond to and perceive potential stressors and disturbances. In general, a stimulus becomes a stressor when an individual is faced with uncertainty and/or lack of control (Millidine et al., 2006; Weiss, 1968). In this sense may both in-tank shelter and a higher animal density potentially shield the fish from outer disturbance. Both physical structure and high fish density will not only obstruct the view but also provide an opportunity to cope with the stressor by hiding behind conspecifics or actively seeking shelter. Simply having access to shelter has been shown to lower the metabolic cost by 30% in juvenile salmon, probably due to lower levels of vigilance (Millidine et al., 2006). Furthermore, the hypothesis that in-tank shelter protects against outer disturbances was clearly supported by the results from the human-induced stress test in **paper II,** (see Fig. 9C). Following five minute of severe in-tank disturbance, the fish in the barren tanks had twice as high levels of plasma cortisol (irrespective of density) compared to the shelter-enriched tanks.

Dorsal fin condition was improved both by adding in-tank shelter (**papers I**, **II**) and lowering fish density (see Fig. 9D**; paper II**). A lower density may favour a stronger hierarcial structure and increase familiarity between individuals which can have a preventive impact on displaced and general aggresion levels and thus decrease fin biting (Johnsson, 1997). In-tank structure in the way used in **papers I** and **II** both restricts visibility and provides concealment which can decrease conspecific interaction but it may also lower the benefits of being aggressive through limiting the possibility to e.g. monopolize food (Höjesjö et al., 2004; Jobling, 1985). These results also fits nicely with the growth data from **paper II,** showing that structural enrichment limited the growth of larger individuals compared to smaller.

This indicates that both a lowered rearing density and adding structural enrichments has the potential to lower aggressive interactions in the hatchery, which could aid in promoting phenotypes with a wider spectrum of physiological and behavioural strategies (McDougall et al., 2006).

Shelter seeking

Parr reared at low density with shelter were more likely to actively seek shelter when placed in a novel environment (see Fig. 9B; **papers I, II**). Giving the fish access to shelter may thus not only increase welfare while in the hatchery, it may also impact adaptive behavioural choices after release. Transport and release into a stream or the ocean is a stressful event and previous studies on Pacific salmon have shown that acute stress can have a negative impact on vigilance and antipredator behaviour, leading to decreased survival (Mesa, 1994; Olla et al., 1992). Displaying active shelter-seeking behaviour at release could thus be advantageous for the immediate survival of the smolts which are often experiencing a high predation pressure (Aarestrup et al., 2005; Kekäläinen et al., 2008).

However, as pre-smolts, all treatment groups were less inclined to use shelter both within the rearing tanks and during the shelter seeking trial, with no differences between treatments (**paper II**). This is probably a result of a general increase in activity during this life-stage as the fish are changing from bottom living parr into free-swimming smolts (Thorstad et al., 2012).

Intestinal integrity and smolt migration

While adding shelter at first showed uniformly positive results, the dimension of different densities revealed the importance of examining interactive effects of captive environmental alterations (**papers I, II**). When the intestinal barrier function was tested just prior to release as smolts, individuals raised at high density (in both barren and shelter tanks) showed significantly lower intestinal integrity compared to the low density groups (see Fig. 9E; **paper II**). In addition to indicating a reduction in welfare, an impaired intestinal barrier may compromise disease resistance through an increased risk of pathogen entry (Berg, 1995; Velin et al., 2004). In Atlantic salmon smolts, mild chronic stress at the freshwater stages has been shown to increase disease susceptibility and mortality upon transfer to seawater (Fridell et al., 2007).

Born to be wild-Main findings
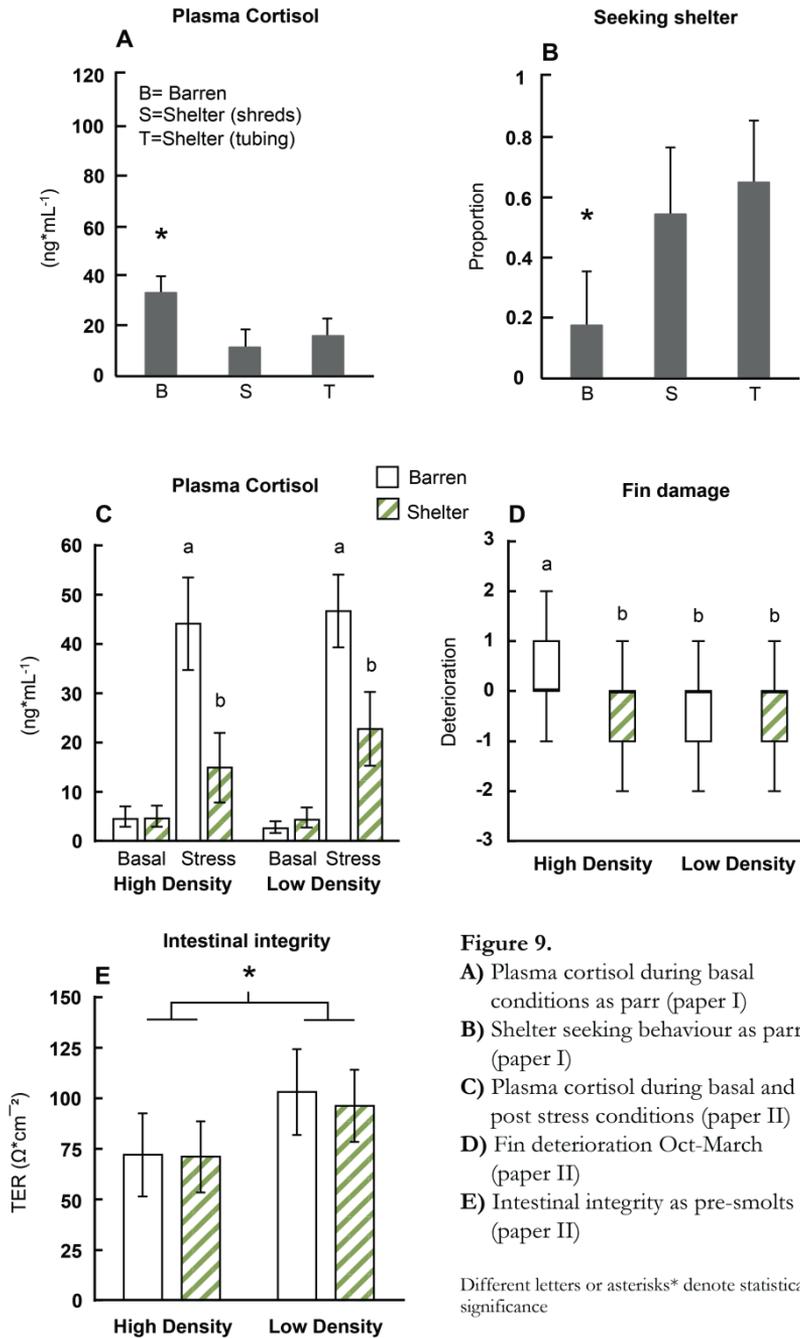
Figure 9.

A) Plasma cortisol during basal conditions as parr (paper I)

B) Shelter seeking behaviour as parr (paper I)

C) Plasma cortisol during basal and post stress conditions (paper II)

D) Fin deterioration Oct-March (paper II)

E) Intestinal integrity as pre-smolts (paper II)

Different letters or asterisks* denote statistical significance

In addition, the combination of shelter and high density did not only eradicate the positive effects on shelter seeking behaviour as parr, it also had negative effect on smolt migration success (see Fig. 10; **paper II**). Smaller fish and fish from the shelter tanks were less likely to reach the river outlet when allowed to migrate downstream in the river. The lower migration success in fish from shelter tanks was however mainly driven by the fish from the high density shelter tanks, showing inferior migration success across all size classes. While previous studies on interaction effects between rearing density and in-tank structure on hatchery performance are limited they also indicate that combining structural enrichment with high fish density might not be the best approach (Brockmark et al., 2007; Brockmark et al., 2010). Atlantic salmon reared in the combination high density and in-tank shelter, grew less, had more fin damage, and lower survival in seawater as smolts, compared with a group reared at low density and in-tank shelter (Brockmark et al., 2007). Brown trout reared with in-tank structure at high densities also showed impaired shelter-seeking after a simulated predator attack and markedly lower survival in a natural stream, compared with a low density shelter group (Brockmark et al., 2010). It thus seems that the combination of structural enrichment and high rearing density should be used with care.
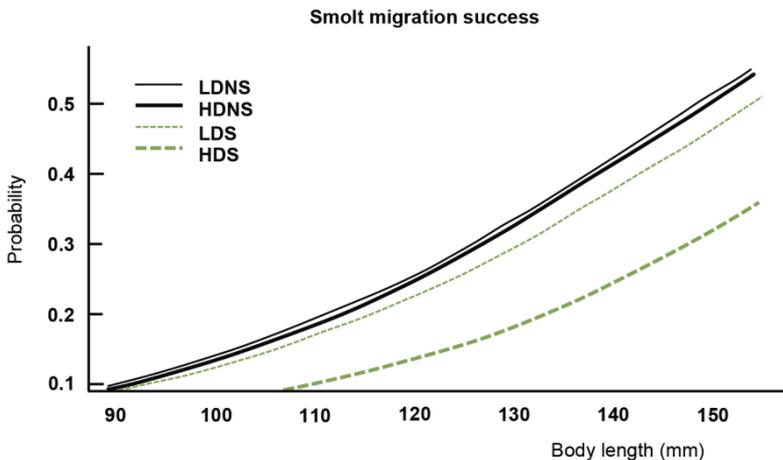

Fig. 10. Smolt migration success probability potted against body length. Migration success was significantly lower for smaller fish and for fish reared with in-tank shelter, especially at high density. **HD** = High Density, **LD** = Low Density, **NS** = No Shelter**, S = **Shelter

Conclusions – Born to be wild I

Density and enrichment alterations will not only affect aggression and stress levels while in captivity, but will also influence physiological and phenotypic traits important for survival after release. Encouraging effects of decreasing the salmonid rearing density below standard has been found before, with positive effects on fin damage, adaptive behaviour, post-release growth and survival (Brockmark et al., 2007; Brockmark et al., 2010; Brockmark and Johnsson, 2010; Larsen et al., 2015a). Smaller group sizes have also been shown to play an important role in acquisition of familiarity in fish, leading to decreased aggression, increased vigilance and improved anti-predator behaviour (Griffiths, 2004; Griffiths and Magurran, 1997; Johnsson, 1997).

Moreover, the results show that having access to shelter can decrease the impact of human-induced stressors. In conservation rearing programs, there is an incentive to minimize human contact and domestication (Carter and Newbery, 2004; Rodriguez et al., 1995) and it has been shown for a wide range of species that opportunity for concealment is important for optimal well-being (Morgan and Tromborg, 2007).

The negative effects on migration success when combining a high rearing density together with structural enrichment, however, highlight the importance of thoroughly examining the effects of hatchery design alterations before implementation on a large scale. It is possible that the lower migration success of the group reared at high density with shelter (**paper II**) was caused by prolonged stress, resulting in maladaptive post-release behaviour (Brockmark et al., 2007; Gaikwad et al., 2011; Teixeira et al., 2007). This may have been an effect of the design and placement of the sheltering structures within the tanks, covering 50 percent of the floor area. As the majority of fish were dwelling within the shelters at any given moment, this led to a structure-induced increase in density, which in turn may have amplified negative effects of an already high rearing density.

Furthermore, the lower intestinal integrity found in the high density tanks and the higher level of fin damage found in the barren high density tanks could lead to higher disease susceptibility. Both the skin and the intestine functions as an infection route for pathogens (Segner et al., 2012). An impaired intestinal barrier caused by stress has previously been shown to induce higher viral infection rate and subsequent mortality upon transfer to seawater in Atlantic salmon smolts (Fridell et al., 2007; Sundh et al., 2009). Fin damage and other skin injuries have also been shown to negatively affect return rates in both Atlantic salmon and Brown trout,

possibly caused by infections (Petersson et al., 2013). As the intestinal epithelium plays an active role in maintaining the salt and water balance of the smolt when it reaches the ocean, disturbances in the intestinal barrier may also induce osmoregulatory problems (Sundell and Sundh, 2012). In a study on Atlantic salmon, a higher rearing density led to lower survival rate during a seawater challenge test, even though both low and high density fish showed the same upregulation of gill Na^+,K^+-ATPases, suggesting that the higher mortality might be caused by impaired barrier functions (Brockmark et al., 2007). Thus the results from **papers I** and **II** suggest that an impaired disease resistance and/or impaired osmoregulation might be one potential factor causing the generally low sea survival of released fish from hatcheries.

Stress coping

Nest emergence, boldness and performance - Born to be wild II

Early and late nest emergence in Atlantic salmon could not be linked to any differences in growth rate or in behaviour, when tested using a hypoxia avoidance trial, either at the parr or the pre-smolt stage (**paper III**). Instead were active avoidance and a more risk-prone behavioural profile more often found in smaller individuals. While smaller fish often display a more cautious behaviour profile (Killen et al., 2012), this is not always the case (Brown and Braithwaite, 2004; Larsen et al., 2015b). Life history theory predicts that in species that suffer high mortality early in life (i.e. wild salmon) the willingness to take risks should decrease with increasing size and age (Roff, 1992), which could induce a more cautious behavioural profile in larger fish. In support of this, the size-effect at the parr-stage was weaker at the later pre-smolt stage. Furthermore, since smaller individuals generally have a higher mass specific metabolic rate (**paper III**) as well as smaller energy reserves, they need to forage more often, which can result in a generally more active and bolder behaviour profile (Brown and Braithwaite, 2004; Larsen et al., 2015b).

At the smolt stage, individuals that showed early nest emergence as well as a bolder behaviour profile during both the parr and pre-smolt stage (Early+Bold group) had a lower maximum metabolic rate and aerobic scope compared to a group that consisted of individuals with late emergence and passive avoidance (Late+Shy group), (see Fig. 11; **paper III**).

The Early+Bold group also had a higher level of red blood cell swelling, suggesting a higher adrenergic output during stress. Higher reactivity in

the brain chromaffin axis has been found in brown trout with a bold and proactive stress coping style (Brelin et al., 2005; Brelin et al., 2008). This result can also be compared to earlier studies from the selectively bred LR and HR trout strains (bred on HPI-axis reactivity), where the LR strain which generally has a bolder personality also show earlier nest emergence and a higher systemic release of catecholamine during stress compared to the HR strain (Andersson et al., 2013; Schjolden et al., 2006). However, in the present thesis (**paper III**) no association between emergence, boldness and HPI-axis reactivity was found as there were no differences in post stress plasma cortisol levels between the Early+Bold and the Late+Shy group.
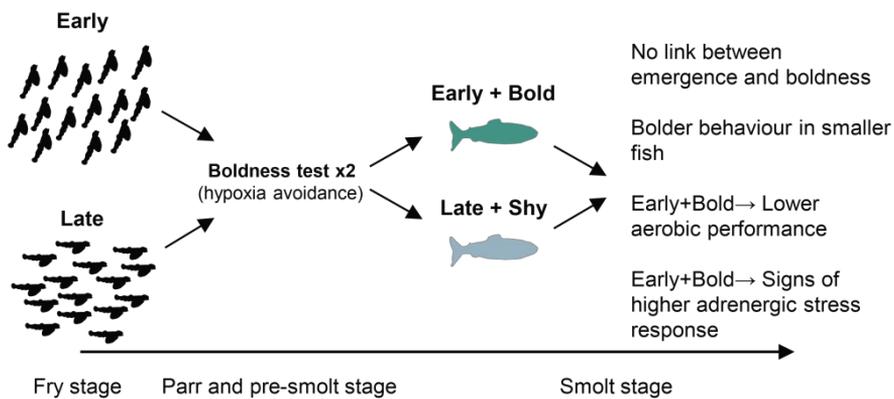

Fig 11. Image showing the experimental protocol in paper III and the creation of the Early+Bold and Late+Shy experimental groups together with some concluding results

The results highlight how different selection pressures in captive and wild fish may be manifested. In captivity, the lower maximum metabolic rate and aerobic scope linked to the Early+Bold group may have no clear negative effects. However, after release higher risk-taking linked to a lower aerobic capacity to escape from a predator attack, could clearly be disadvantageous. In the wild, bold individuals may run a higher risk of predation, suggesting a high selection pressure on Early+Bold individuals following release, as mortality rates during smolt releases are largely caused by predation (Huntingford, 2004; Johnsson et al., 1996; Teixeira et al., 2007; Thorstad et al., 2012).

Behaviour, Metabolism and HPI-axis reactivity

A link between HPI-axis reactivity and metabolic rate was found in **paper V**. High responding (HR) individuals had a significantly higher composite metabolic rate (higher total oxygen consumption) as well as a tendency for a higher SMR compared to low responding (LR) individuals (see Fig. 12).
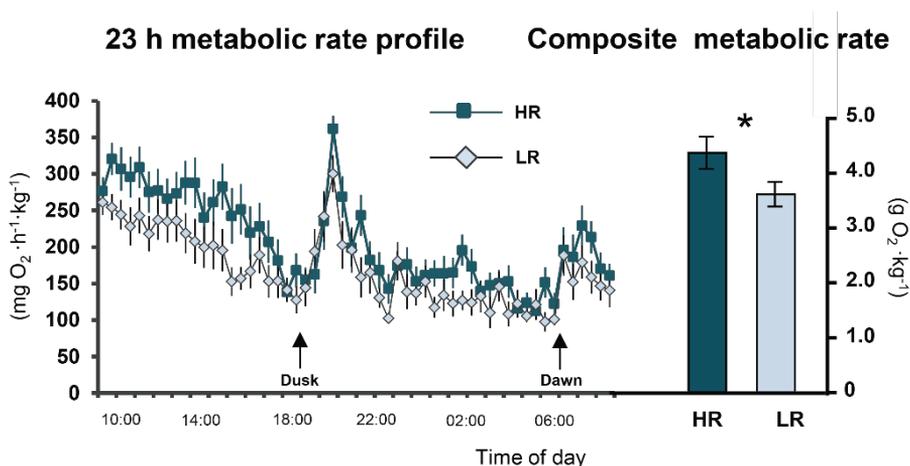

Fig. 12. A) Oxygen consumption profile of LR and HR fish over 23 h. **B)** Composite oxygen consumption (MO_2) in LR and HR fish calculated using   a linear trapezoidal method (AUC). The bars show mean ± SE, Asterisk (*) shows statistical significance ($p < 0.05$).

These results are in contrast to what might be expected as earlier studies have found links between a high metabolic rate and high aggression, dominance and boldness which are behavioural traits often found in LR individuals (Biro and Stamps, 2010; Castanheira et al., 2013b; Killen et al., 2011; Pottinger and Carrick, 2001). However, to our knowledge this is the first study looking at metabolic rate directly linked to HPI-axis reactivity in rainbow trout. Knowing how cortisol is involved in energy translocation it seems probable that the result is driven by the HPI-axis and a higher cortisol output in HR fish. Placing the fish in the respirometer will inevitably result in a stress reaction with possibly sustained higher cortisol levels over the course of the measuring period (24 h). Exogenous cortisol treatment in fish has indeed been shown to increase metabolic rate and depresses growth rates at a given feed intake, inducing a higher cost of living (Boeck et al., 2001; Gregory and Wood, 1999; Morgan and Iwama, 1996). A higher metabolic rate has previously

been found to be positively correlated to weight loss during food deprivation in European seabass, and for the LR and HR rainbow trout strains, a sustained stress exposure (7 day transport) led to a significantly larger weight loss in HR compared to LR fish (Killen et al., 2011; Ruiz-Gomez et al., 2008). Interestingly, in both studies, the weight loss was also coupled to long-term increases in risk-taking, lasting up to a year in the HR fish (Killen et al., 2011; Ruiz-Gomez et al., 2008). Taken together with the results from **paper V**, this suggests that high HPI-axis reactivity can induce a higher metabolic rate, which during prolonged stress or food shortage can lead to decreased growth and even behavioural-modulation effects.

In **paper V**, the LR fish showed a tendency for higher activity levels compared to HR fish during an open field trial, which could be a sign of a more exploratory and bold behavioural profile. Interestingly, a significant positive correlation between plasma cortisol and burst swimming was found within the LR group. Previous studies have shown that the effect of cortisol on fish behaviour is time- and context-dependent, as exemplified by Øverli et al. (2002), where short term exogenous exposure (1 h) lead to behavioural activation while long term exposure (24 h) instead lead to behavioural inhibition. The results from **paper V** thus add to this complexity, showing that the behavioural effects of cortisol might not only depend on e.g. exposure time but also on the innate stress reactivity of the individual.

The HR fish also had higher mRNA levels of corticoid- and serotonergic receptors and re-uptake pumps in the telencephalon compared to LR trout (**paper IV**). Both activation of corticoid receptors and the serotonergic system within the CNS has been shown to induce passivity and inhibit spontaneous locomotion in fish (Schjolden et al., 2009; Winberg and Nilsson, 1993). A higher expression of serotonergic receptors can induce a stronger postsynaptic 5-HT response, while a higher level of serotonergic pumps leads to a more rapid transient effect of released serotonin, through faster clearance. However high pump activity also enables the reuse of serotonin, suggesting that the HR group might have a higher serotonergic turn-over rate in the synaptic cleft compared to the LR group (Winberg and Nilsson, 1993). These structural functionality differences, fits well with the notion of HR/reactive individuals as being more behaviorally flexible and more responsive to environmental stimuli compared to LR/proactive individuals (Koolhaas et al., 1999; Koolhaas et al., 2010; Moreira et al., 2004; Ruiz-Gomez et al., 2011; Sørensen et al., 2013). These results are also interesting to link to a

previous study performed on early and late emerging fish (originating from the same population of Atlantic salmon as in **paper III**). The study showed that late emerging fish had a more cautious behavioural profile as well as higher lower levels of a serotonergic receptor in the forebrain compared to early emerging fish (Thörnqvist et al., 2015).

The Brain-gut-axis

Both rearing conditions and HPI-axis reactivity influenced the intestinal barrier function (see Fig. 9E & Fig. 13; **papers II, IV**). A higher rearing density and a lower HPI-axis reactivity (LR fish) were linked to lower intestinal integrity when measured during basal conditions. There was also a negative correlation between plasma cortisol and intestinal integrity within the LR group, suggesting sensitivity towards the hormone during basal conditions for this group.

Even though no sign of chronic elevation of plasma cortisol was found in the high density groups in **paper II**, a lower intestinal integrity can be a sign of prolonged stress and impaired welfare (Segner et al., 2012; Sundh, 2009). During long-term, low-intensive stress, negative feed-back mechanisms on the HPI-axis can generate a decrease in plasma cortisol levels over time even though the stressor is still present (in this case a high density rearing environment) (Dickens and Romero, 2013; Segner et al., 2012). Social stress and high rearing densities have been shown to lead to habituation of the HPI-axis but still render negative effect on the intestinal barrier in teleosts (Peters, 1982; Sundh et al., 2009).

For the LR and HR fish, assessment of the intestinal integrity following handling and stress exposure also revealed that HPI-axis reactivity can induce differences in how the intestinal primary barrier respond to stress. Following a 23 h handling protocol, LR fish had an unexpected improved intestinal integrity, while the HR fish showed a reduction in integrity compared to the LR group (**paper IV**).
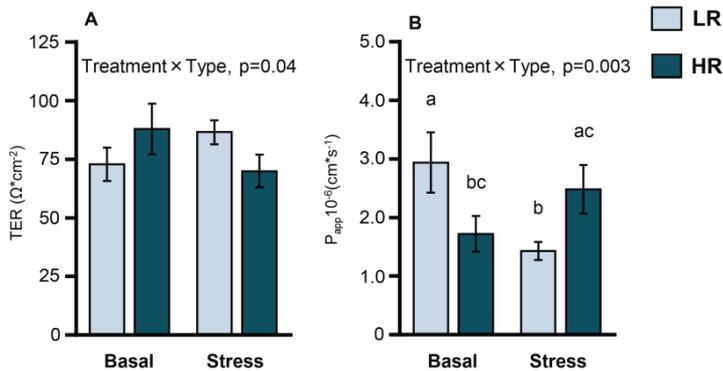
Fig. 13. Intestinal barrier function of the posterior intestine in LR and HR fish under basal conditions and after stress exposure. **A)** TER= transepitelial resistance, **B)** P_{app}= permeability of the paracellular marker molecule, ^{14}C-mannitol.
Bars show averages± SE, statistical significance of main effects are shown by exact p-values and for pairwise comparisons through different letters.

While both acute and long term stressors negatively affect the intestinal barrier in fish (as seen in the HR fish), the proximate mechanisms are not fully understood (Olsen et al., 2005; Segner et al., 2012; Sundh, 2009). Hypothetically, effects on all of the components outlining the intestinal epithelium can result in integrity changes, i.e. effects on enterocytes, tight junction proteins, mucus composition and production and/or associated immune cells (Olsen et al., 2005; Saunders et al., 1994; Segner et al., 2012).

One possibility is that disturbances in the intestinal barrier are driven by autonomic and/or enteric nerves (see Fig. 14). The enterocytes are innervated by enteric nerves receiving input from the parasympathetic nervous system. Release of neurotransmitters such as acetylcholine and CRH from enteric nerves can induce changes in intracellular signaling cascades and increases in the cytosolic second messenger cAMP has been suggested to induce contraction of the actin-ring in the epithelial cytoskeleton, leading to changes in paracellular permeability (Bakker and Groot, 1989; Holmgren and Olsson, 2011; Tsigos and Chrousos, 2002).

A second possibility is that the lower intestinal integrity measured at a high rearing density (**paper II**) and after stress exposure in the HR group (**paper IV**) stem from effects driven by the immune system (see Fig. 14). Mast cells produce substances such as histamine and tryptase that are aimed at aiding the tissue in batteling foreign microbes but they can also cause an increased epithelial permeability, possibly linked to modulation

of the enterocyte acting ring and tight junction proteins (Jacob et al., 2005; Perdue et al., 1991; Santos et al., 1998; Santos et al., 2001). Release of histamine and tryptase may in turn have been triggered by enteric nerve activity or by the HPI-axis as both CRH administration (at least in mammals) and cortisol administration in salmonids has been shown to trigger mast cell degranulation (Reite, 1997; Santos et al., 2001). During stress, transient increases in CRH levels in the plasma originating from the hypothalamus have been measured in tilapia and it would be interesting to investigate the CRH plasma profiles during stress in LR and HR fish (Pepels et al., 2004).

It has also been shown that for mammalians, corticoids can suppress the secretion of antibodies, i.e. immunoglobulins (Ig). With decreased secretion of e.g. the secretory antibody IgA, luminal bacteria can more easily access the epithelium and start to influence the functionality of e.g. the tight junctions, leading to loss of intestinal integrity (Spitz et al., 1996). While the roles and function of secretory immunoglobulins are not as well studied in teleosts, one specialized mucosal antibody IgT, coating bacteria in the gut lumen has been found in rainbow trout (Zhang et al., 2010).

The lower intestinal integrity found in both the HR fish following stress and in fish living at high densities (**paper II**) could thus be either a direct or mixed effect of secondary responses possibly initiated by the HPI-axis (see Fig. 14).

However, linking the increased integrity displayed by the LR group during stress to any physiological effector is not as obvious. That stress can have a negative impact on intestinal integrity is well established (Meddings and Swain, 2000; Olsen et al., 2005; Segner et al., 2012; Sundh et al., 2010; Velin et al., 2004), whereas studies reporting signs of an enhanced barrier function following stress exposure is hard to find. Interestingly, **paper IV** is not the first study showing differences in how the intestinal barrier function responds to stress in connection to physiological stress reactivity. In mammals, individual differences in parasympathetic and corticoid hormone reactivity has been shown to affect the propensity for developing stress-induced intestinal dysfunction and gastric ulcers (Kiliaan et al., 1998; Paré and Redei, 1993; Saunders et al., 1997; Saunders et al., 1994). The Wistar-Kyoto rat, a strain with attenuated corticoid reactivity, displayed an increase in intestinal permeability and ulcers following stress exposure compared to a strain with normal corticoid reactivity. These stress-induced decreases in intestinal barrier function were found to be linked to enteric nerves

receiving input from the parasympathetic nervous system, as described earlier (Kiliaan et al., 1998; Paré and Redei, 1993; Redei et al., 1994; Saunders et al., 1994; Saunders et al., 1997). HR/reactive individuals are often suggested to have a stronger parasympathetic reactivity compared to LR/proactive individuals (Koolhaas et al., 1999). The loss of intestinal integrity during stress in the HR fish could thus be an effect of enteric nerve activity, triggered by parasympathetic activity. That the LR and HR fish differ in their neural and CNS functionality is supported by the mRNA data from the telencephalon (**paper IV**).
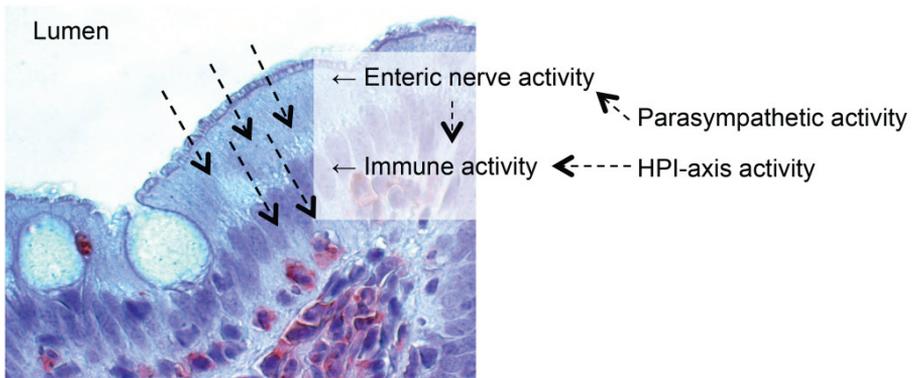
Fig. 14. Image showing possible mediators of reduced intestinal integrity, induced by primary stress responses.

In mice, interesting effects of intestinal microbiota modulating the hypothalamic-pituitary adrenal axis reactivity (mammalian equivalent to the HPI-axis) has also been found (Sudo et al., 2004). In germ-free mice the plasma corticoid response during stress was significantly higher and they also showed higher turn-over rates of serotonin in the CNS as well as differences in locomotor activity compared to mice with a normal gut microbiota (Heijtz et al., 2011; Sudo et al., 2004). In concurrence with this, the HR trout had higher levels of both serotonergic receptors and pumps in the telencephalon and there was a tendency for higher swimming activity in LR fish compared to HR fish (**paper IV**). Similar studies in teleosts could perhaps teach us more about the brain-gut axis and how it may modulate and interact with neurofunction and behaviour of fish.

SUMMARY & CONCLUSIONS

This thesis shows that changes to the captive environment affect both physiological and behavioural traits connected to welfare and post-release performance of Atlantic salmon and that both behavioural and physiological stress coping responses transfers into secondary effects on other physiological performance traits in salmonids.

Adding structural enrichment and lowering the rearing density during the juvenile parr stage for hatchery reared fish showed promising effects on fish welfare. Both a lowered density and structural enrichment succeeded in decreasing general aggression levels (**papers I, II**) and structural enrichment also showed positive effect on plasma cortisol levels (**papers I, II**). This suggests higher stress levels in barren tanks compared to tanks containing shelter. Decreasing the rearing density below standard also increased growth and had a positive effect on the intestinal barrier function (**paper II**). Links between the corticoid system and intestinal dysfunction in salmonids was also found (**paper IV**), where the intestinal barrier function of rainbow trout was highly influenced by HPI-axis reactivity. Low responding individuals (LR) displayed a lower intestinal integrity during basal conditions compared to high responding individuals (HR), while the HR group suffered a reduction in integrity following stress exposure. Negative correlations were also found between intestinal integrity and circulating levels of cortisol during basal conditions in the LR group (**paper IV**). These results, confirm that the HPI-axis is involved in the brain gut axis of fish and that cortisol may play a direct or indirect role in compromising intestinal integrity.

No signs of a desensitized HPI-axis (due to chronic stress) were found in fish living in barren tanks in **paper I**. However, when an acute stressor were applied inside the rearing tank in **paper II**, fish in the structurally enriched tanks showed markedly lower post-stress cortisol levels compared to the fish in the barren tanks (**paper II**). This is most likely not caused by differences in physiological HPI-axis reactivity but rather an effect of the sheltering quality of the structural enrichment, providing visual shielding and/or comfort.

The HPI-axis reactivity was also tested in the two sub-groups defined by a combination of both emergence and boldness behaviour, Early+Bold and Late+Shy (**paper III**). While no difference was found in post-stress cortisol levels, the Early+Bold group displayed signs of a higher reactivity in the brain chromaffin axis, as suggested by a higher level of swelling of

their red blood cell, probably a secondary effect of higher levels of circulating catecholamines.

The Early+Bold group was also smaller compared to the Late+Shy group and as they developed into smolts they exhibited a lower maximum metabolic rate and aerobic scope following strenuous swimming (**paper III**). After release, a higher risk-taking linked to a lower aerobic capacity to escape from a predator, could clearly be disadvantageous. This result is also interesting to link with the general size effect found on smolt migration from **paper II**. In that study, smaller individuals suffered from lower migration success across all treatments, possibly caused by a higher predation pressure.

HPI-axis reactivity has previously been found to influence both emergence timing and boldness in rainbow trout, suggesting a higher prevalence of Early+Bold individuals within a LR group. Therefore linking to the results from **paper III**, one might expect to find a lower aerobic capacity in LR rainbow trout. This was not supported in **paper V**, where LR fish showed a lower composite metabolic rate but no differences in MMR or AS between the two groups.

A possible higher boldness and activity score was, however, found in LR fish as they showed a tendency for higher activity levels in an open field trial compared to HR fish.

Unlike hypothesized, early and late emerging individuals in **paper III** showed no differences in boldness behaviour when measured neither at the parr nor the pre-smolt stage. In comparison to previously published papers this suggests a possible uncoupling between behavioural traits and emergence time during development of the fry into parr and migratory smolts.

While boldness was not tested directly in the hatchery fish in **papers I** and **II,** structural enrichment did have a negative effect on growth in **paper II**. This decreased growth may be caused by risk-sensitive behaviour, depressing the feeding rate as the fish prefer to stay sheltered. In-tank structure did also decrease the growth rate of the larger individuals in relationship to the smaller, possibly caused by an increased difficulty in monopolizing the food supply in a more complex environment. Previous familiarity to shelter also increased shelter seeking propensity in a novel environment, which could be a positive behavioural attribute after release (**papers I, II**). However, in-tank sheltering structures were not unequivocally positive, as the combination of sheltering structures with high rearing density had a clear negative effect on smolt migration success in paper II. This clearly exhibits the

importance of evaluating interactive effects of different hatchery alterations before implementation.

CONCLUDING REMARKS & FUTURE PERSPECTIVES

Due to the many positive effects linked to both decreased density and in-tank shelter, I suggest future studies, optimizing at which rearing density structural enrichment is beneficial. I also suggest adjusting the design of the hatchery tanks to different life stage requirements.
One possible approach could be to offer structural enrichment for the fish during their naturally bottom-dwelling life stages, the fry and parr stage. During parr-smolt transformation, the fish would probably benefit more from enrichment factors more linked to sea survival such as getting accustomed to eating live-prey and alterations in water current strength, which have shown promising effects in previous studies (Brown et al., 2003; Hyvärinen and Rodewald, 2013; Sneddon, 2003).

"The tyranny around the golden mean" describes the risks of focusing on central tendencies and discarding the variation around the mean (Bennett, 1987). This is a trap especially important to avoid when working with conservational releases, where individual variation as such is vital to preserve. While the captive environment is a highly competitive environment with high densities of fish and no shelter, it also provides abundant food and protection against predators. The expected survival of a juvenile while in captivity is therefore much higher compared to its wild counterpart in the river, while after release both the survival and fitness is instead many times lower (Einum and Fleming, 2000; Fleming and Gross, 1993; ICES, 2015). Due to the higher survival probability for the juvenile while in the hatchery, a lower survival rate following release is not only expected but necessarily not a bad thing as one would want to strive for the strongest selection pressure being of natural origin. However both a directed selection pressure and/or a weaker selection pressure in the hatchery can lead to a decrease in phenotypic and genetic variation over time. The challenge therefore lies in providing an environment that ensures a high welfare, produces a high quality and wild-like smolt but that also neither disfavors nor promotes certain physiological or behavioural extremes. While a hatchery environment will never fully live up to the complexity of a natural stream there is certainly room for improvement in terms of how standard hatchery tanks are designed today.

Furthermore, while there is much to learn concerning optimizing the practice and design of hatcheries rearing salmon for release, data collected from such hatcheries could help in developing future conservational efforts and release program also for other species. In many locations, salmon hatchery releases has been running for decades or even for more than a century, providing an unprecedented knowledge-base concerning how fish population structure and its individual's physiological and behavioural traits are molded by the captive environment.

ACKNOWLEDGEMENTS

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