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Norwegian University of Life Sciences
NO–1432 Ås, Norway
Phone +47 64 96 50 00
www.umb.no, e-mail: postmottak@umb.no

Ida Beitnes Johansen

Norwegian University of Life Sciences • Universitetet for miljø- og biovitenskap
Department of Animal and Aquacultural Sciences
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Stress responsiveness in salmonids: Effects on genes, organs, and organisms

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Preface

This thesis is based on studies planned and initiated at the Department of Animal and Aquacultural Sciences at the Norwegian University of Life Sciences, Ås under the supervision of Dr. Øyvind Øverli and Professor Morten Bakken. The experimental work was done at the Department of Molecular Biosciences, University of Oslo, Oslo, Norway in collaboration with Professor Göran E. Nilsson, at DTU Aqua, National Institute of Aquatic Resources, Technical University of Denmark, Hirtshals, Denmark in collaboration with Dr. Erik Höglund and at Boračko lake - Center for Fisheries, Konjic, Bosnia and Herzegovina in collaboration with Samir Muhamedagić and Professor Mensur Vegara. All lab analyses were carried out at the Department of Molecular Biosciences, University of Oslo and at the Institute for Experimental Medical Research, Oslo University Hospital, Oslo, Norway.

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Abbreviations

ACTH – adrenocorticotropic hormone

AFOG – acid-fuchsin-orange G

ANP – A-type natriuretic peptide

BDNF – brain-derived neurotrophic factor

BNP – B-type natriuretic peptide

BrdU - bromodeoxyuridine

CA – catecholamine

CC – corticosteroid

CNS – central nervous system

COL1a1 – collagen alpha 1(1)

COL1a2 – collagen alpha 2(1)

CR – cortisol receptor

Cp – crossing point

CSI – cardiosomatic index

CVS – cardiovascular system

DCX – doublecortin

DG – dentate gyrus

GC – glucocorticoid

GR – glucocorticoid receptor

HR – high responder

HPA – hypothalamic pituitary adrenal

HPI – hypothalamic pituitary interrenal

LR – low responder

LTS – long-term social

MCIP1 – modulatory calcineurin-interacting protein 1

MLP – muscle LIM protein

MR – mineralcorticoid receptor

NeuroD – neurogenic differentiation

NFAT – nuclear factor of activated T-cell

PCNA – proliferating cell nuclear antigen

qtPCR – quantitative real-time-polymerase chain reaction

RCAN1 – regulator of calcineurin 1

SMLC2 – slow myosin light chain 2

STC – short-term confinement

VMHC – ventricular myosin heavy chain

α-MSH – α-melanocyte stimulating hormone

List of papers

Paper I

Johansen, I. B., Sandvik, G. K., Nilsson, G. E., Bakken, M., Øverli, Ø. **Cortisol receptor expression differs in the brains of rainbow trout selected for divergent cortisol responses.** In press. Comparative Biochemistry and Physiology – part D.

Paper II

Johansen, I. B., Sandvik, G. K., Nilsson, G. E., Höglund, E., Bakken, M., Øverli, Ø. **Gene expression in the context of stress and stress coping style: Modulation of brain plasticity.** In revision. Brain Behavior and Evolution.

Paper III

Johansen, I. B., Lunde, I. G., Røsjø, H., Christensen, G. A., Nilsson, G. E., Bakken, M., Øverli, Ø. **Cortisol response to stress is associated with myocardial remodeling in salmonid fishes.** Accepted 11.01.2011. Journal of Experimental Biology.

Summary

Stress is an inescapable burden for fish like for all other animals. For individuals that frequently encounter stressful situations, repeated or prolonged physiological stress responses, can potentially compromise the physiological and psychological basis of that organism`s health and welfare. In particular, stress has been shown to adversely affect important biological systems such as the immune system, the central nervous system (CNS) and the cardiovascular system (CVS). The impact of stress largely depends on the inherent stress coping style of the affected individual. Stress coping style can be defined by the set of behavioral and physiological responses to stress that is consistently employed by one individual across unrelated and temporally separated situations. One such physiological stress response, which is highly heritable, yet subject to great individual variation, is the cortisol response. Cortisol, an interrenal/adrenal steroid stress hormone, is responsible for most stress-related diseases in man as well as in fish.

High heritability of the cortisol response has allowed for the generation of two strains of rainbow trout (*Oncorhynchus mykiss*) that differ consistently in stress-induced cortisol production and consequently in morphology, endocrinology, behavior and cognition. Low-responding (LR) fish were initially selected for low post-stress cortisol levels and subsequently display proactive behaviors and endocrine profiles. High-responding (HR) fish, on the other hand, were selected for high post-stress cortisol levels and display reactive behaviors and endocrine profiles.

The phenomenon of contrasting stress coping styles is attracting considerable scientific and public interest. Particularly, the recognition of individual variation in disease vulnerability has encouraged scientists to try to elucidate the biology behind stress coping. In this context, the LR-HR model serves as an excellent model to study the proximate mechanisms behind genetically linked behavioral and physiological trait characteristics. In this work we aimed at investigating CNS plasticity and cardiac remodeling by integrating studies at the molecular (genes), physiological-anatomical (organ) and behavioral (organism) levels in the context of divergent stress coping styles. In particular, we investigated putative CNS mechanisms controlling behavior and memory retention (i.e. mRNA expression of genes involved in the cortisol response, postembryonic neurogenesis and neuronal plasticity) in stressed and non-stressed LR and HR rainbow trout. Further, the association between cortisol responsiveness and cardiac morphology (i.e. size, composition and collagen depositions) and gene expression (i.e. mRNA expression of specific markers of cardiac remodeling) in LR and

HR rainbow trout was explored. Finally, we investigated if a trait correlation between cortisol responsiveness and heart size also existed in wild-type European brown trout (*Salmo trutta*).

We discovered that the expression of central cortisol receptors was affected by heritable variation in stress coping (i.e. LR vs. HR) and downregulated by stress in a brain region-specific manner. We also found that the expression of genes involved in CNS plasticity was affected by heritable variation in stress coping and also differentially affected by short – and long-term stress. Lastly, we show that high cortisol responsiveness was associated with cardiac remodeling (i.e. growth) in HR fish and in high cortisol-responding wild-type brown trout. Further, the cardiac growth in HR fish appeared to be caused mainly by hypertrophic growth of the compact myocardium. This growth was accompanied by focal collagen depositions and a high expression of genes involved in hypertrophy, development of fibrosis and the cortisol response. The latter indicates that cortisol is directly mediating the cardiac remodeling in HR trout.

Combined, the results showing that the expression of central cortisol receptors and genes involved in CNS plasticity differed between LR and HR trout, can contribute to increased knowledge about the proximate mechanisms behind genetically linked physiological and behavioral trait characteristics. Also, the strong association between cortisol responsiveness and cardiac remodeling in two salmonid species suggests that cortisol might be a general causative factor in salmonid cardiac disease. The presence of this anatomical-physiological trait correlation in a wild population of salmonids, also suggests an ecological-evolutionary role for individually variable heart function.

Sammendrag

Stress er et uunngåelig onde for fisk så vel som for alle andre dyregrupper. For individer som ofte opplever stressende episoder, kan gjentatte og/eller langvarige fysiologiske stressresponser potensielt kompromittere det fysiologiske og psykologiske grunnlaget for helse og velferd. Spesielt har stress vist seg å ha skadelige effekter på viktige biologiske systemer som sentralnervesystemet (SNS) og det kardiovaskulære systemet (KVS). Virkningen av stress avhenger i stor grad av det berørte individets iboende stressmestringsevne. En stressmestringsstrategi kan defineres ut ifra de atferdsmessige og fysiologiske stressresponsene som konsekvent anvendes av et individ på tvers av urelaterte og tidsavskilte situasjoner. Et eksempel på en slik fysiologisk respons, som er svært arvelig, dog gjenstand for betydelig individuell variasjon, er kortisol-responsen. Kortisol, et stress-steroidhormon produsert i interrenale celler/binyrene, er ansvarlig for de fleste stress-relaterte sykdommer hos fisk og hos menneske.

Det faktum at kortisol-responsivitet er svært arvelig, har gjort det mulig å utvikle to stammer av regnbueørret (*Oncorhynchus mykiss*) som konsekvent responderer på stress med forkjellige kortisol-nivåer. De to stammene viser også forkjeller i atferd, morfologi, endokrinologi og kognisjon. Lav-responsive (LR)-fisk ble opprinnelig selektert for en lav kortisol-respons etter stress og viser proaktiv atferd og fysiologi. Høy-responsive (HR)-fisk, derimot, ble selektert for en høy kortisol-respons etter stress og har en reaktiv atferd og fysiologi.

Fenomenet ”stressmestringsstrategi” vekker betydelig vitenskapelig og allmenn interesse. Spesielt har anerkjennelsen av individuell variasjon i sykdomssårbarhet oppmuntret forskere til å forøke å belyse biologien bak forkjeller i stressmestring. I denne sammenheng fungerer LR-HR-modellen som en utmerket modell for å studere proksimate mekanismer bak genetisk knyttede atferdsmessige og fysiologiske trekk. I dette arbeidet hadde vi som mål å undersøke SNS-plastisitet og remodellering av hjertet ved å integrere studier på det atferdsmessige (organismer), fysiologisk-anatomiske (organer) og molekylære (genregulering) plan i sammenheng med divergerende stressmestringsstrategier.

Mer presist undersøkte vi antatte SNS-mekanismer som kontrollerer atferd og minne-retensjon (dvs. mRNA-uttrykk av gener involvert i kortisol-responsen, postembryonal nevrogenese og nevronal plastisitet) i stressede og ustressede LR – og HR-regnbueørret. Vi har også undersøkt sammenhengen mellom kortisol-responsivitet og hjerte-morfologi (dvs. størrelse, komposisjon og kollagen-avsetninger) og gen-uttrykk av spesifikke markører for

hjerte-remodellering i LR – og HR-regnbueørret. Til sist undersøkte vi om det eksisterte en korrelasjon mellom kortisol-responsivitet og hjertestørrelse også i vill-type brunørret (*Salmo trutta*).

Vi viser at uttrykket av sentrale kortisol-reseptorer var påvirket av arvelig variasjon i stressmestring og ble nedregulert av stress avhengig av hjernedel. Vi viser også at uttrykket av gener involvert i SNS-plastisitet var påvirket av arvelig variasjon i stressmestring (LR vs. HR), men også ulikt berørt av korttids – og langtidsstress. Vi viser også at en høy kortisol-responsivitet var assosiert med remodellering av hjertet (dvs. vekst) i HR fisk og i vill-type brunørret med en høy kortisol-respons. Videre viser vi at hjerteveksten i HR-fisk hovedsakelig skyldtes hypertrofisk vekst av kompakt myokardium. Hjerteveksten var også ledsaget av fokale kollagenavsetninger og et høyt uttrykk av gener involvert i hypertrofi, utvikling av fibrose og kortisol-responsen. Sistnevnte indikerer at kortisol direkte medierer remodellering av hjertet i HR-ørret.

Våre resultater, som viser at uttrykket av sentrale kortisol-reseptorer og gener involvert i SNS-plastisitet varierer mellom LR – og HR- ørret, kan potensielt bidra til økt kunnskap om proksimate mekanismer bak genetisk knyttede fysiologiske og atferdsmessige trekk. Dessuten indikerer en sterk assosiasjon mellom kortisol-responsivitet og remodellering av hjertet, i to arter av laksefisk, at kortisol kan være en medvirkende faktor i utvikling av hjertesykdom hos salmonider. Tilstedeværelsen av denne anatomisk-fysiologiske sammenhengen i en vill populasjon av laksefisk, kan tyde på en økologisk-evolusjonær rolle for individuell variasjon i hjertefunksjon.

1. Introduction

"Stress" is a term that everyone speaks of and feels that they have experience with. Yet, defining stress has proven difficult and stress as a biological term has a long history of inconsistency (see Selye 1975). Nevertheless, one attempt to define stress that will also be used in this thesis, states that stress is "*a condition in which a threat to the biological functions of an organism is perceived by that organism and a set of physiological and behavioral responses are mounted to counteract this challenge"*(Øverli 2001). Fishes are frequently challenged by stress in nature as well as in artificial conditions such as in aquaculture. Potential stressors in nature include threat of predation, shortage of food, rapid and unpredictable changes in the environment, and establishment and maintenance of social hierarchies. In aquaculture, common stressors that fish encounter include netting, handling, veterinary examinations and treatments, parasite infections, poor water quality, as well as crowding and transportation.

In all contexts, such stressors can trigger adaptive as well as non-adaptive physiological stress responses. Although the initial phase of a physiological stress response is intended to counteract the stress challenge and restore physiological function, stressors that are severe, unpredictable and of long duration can eventually trigger and possibly maintain a pathophysiological response that causes impairments of important biological systems (i.e. immune system, central nervous system, osmoregulatory systems and the cardiovascular system) (Sapolsky 2004; Wendelaar Bonga 1997). In nature, the causes of stress seem unavoidable unless the stress is inflicted by humans. In aquaculture, however, stress can be reduced by modifying and optimizing practices. In all cases, employing a certain set of behavioral or physiological strategies aimed at minimizing the potentially deleterious effects of stress seems like a valuable strategy (Reale et al. 2010).

There is a large variation in how individuals respond to and cope with stress both in nature and under conditions of rearing in capture. In other words, the same stressors can have very different impacts on two individuals across closely related species, across populations of the same species, and even between individuals in the same population. How stress impacts on an individual depends largely on the behavioral and physiological responses employed by that individual in the face of a challenge. Some of these behavioral and physiological traits employed by one individual appear to be genetically linked. Thus, the same individual often employs similar responses across different stressful incidents. The consistency of such responses to stress has been termed stress coping style (Koolhaas et al. 1999). In nature,

contrasting stress coping styles can be favorable in contrasting environmental contexts (i.e. stable vs. unstable environments) (Sih et al. 2004). In a more stable aquaculture setting, however, selecting for good stress coping could be performed in order to avoid many of the detrimental effects associated with poor stress coping (Kittilsen et al. 2009).

Across most animal phyla, including humans, the phenomenon of contrasting stress coping styles is receiving considerable interest. Particularly, studies on individual variation in disease vulnerability can contribute to valuable information to the field of biomedicine (Koolhaas 2008). In order to elucidate the biology behind and the physiological consequences of stress coping, it is necessary to study the proximate mechanisms underlying such genetically linked behavioral and physiological trait characteristics. This can be approached from many angles, although combining behavioral (i.e. organism), physiological (i.e. organ) and molecular (i.e. genes) analyses is clearly an advantage. In this work we have tried to include these analyses to assess the effects of stress and stress-coping on two important biological systems; the central nervous system (CNS) and the cardiovascular system (CVS). For this task, we have employed two strains of rainbow trout, initially selected for high (HR) and low (LR) post-stress cortisol levels, and consequently employing different sets of physiological and behavioral responses (i.e. stress coping styles) when stressed (Schjolden and Winberg 2007; Øverli et al. 2007; Øverli et al. 2005).

When studying the functional principles, physiology and molecular biology of the CNS and the CVS, it can be of great value to use comparative models. In particular, the CNS and the CVS are considerably more plastic in fish (and other lower vertebrates) compared to mammals. For example, teleost fish show a pronounced capacity of postembryonic neurogenesis and an extraordinary potential for neuronal regeneration (Zupanc 2008). By contrast, postembryonic/adult neurogenesis is highly restricted in the mammalian brain which consequently possesses poor regenerative capacity. Comparative analysis of phenomena such as postembryonic neurogenesis could thus help in defining novel therapeutic approaches and more efficient strategies for wound healing and treatment of CNS pathology. Similarly, the hearts of some teleosts, including salmonids, demonstrate a high degree of plasticity, both anatomically and physiologically, in response to environmental changes (Gamperl and Farrell 2004). The cost of such high plasticity is that some teleosts are more prone to non-adaptive cardiac remodeling. Cardiac deformities and diseases are increasing problems in farmed animals, including salmonids (Brocklebank and Raverty 2002; Poppe et al. 2002; Poppe and

Taksdal 2000; Poppe et al. 2007; Takle et al. 2006). Still, the underlying causes of pathological cardiac remodeling in fish are largely unknown.

1.1 Stress physiology of fishes

The terms "stressor" and "stress" were first introduced by Hans Selye in the 1950`s. In his book "the physiology and pathology of exposure to stress", Selye suggested that however variable the nature of stressors, they always seem to elicit the same pattern of physiological responses. In particular, increased activity and enlargement of the adrenal gland was pointed out as a hallmark of the physiological stress response (Selye 1950).

In mammals, the adrenal gland produces and releases stress hormones like catecholamines (CAs) and corticosteroids (CCs) in response to stress. CA secretion (i.e. epinephrine and norepinephrine secretion) is governed by the hypothalamic-sympathetic-adrenal medulla-axis, whereas CC production and secretion is governed by the hypothalamic-pituitary-adrenal cortex (HPA)-axis. In fish, two types of specialized cells in the head kidney, serve as functional equivalents to the two organized layers of the mammalian adrenal gland. Firstly, chromaffin cells are functionally homologous to the mammalian adrenal medulla and are the main source of circulating CAs in fish. Secondly, interrenal cells are functionally homologous to part of the mammalian adrenal cortex (*zona fasciculata*) and the main source of CCs. The fish equivalent of the mammalian HPA-axis is therefore referred to as the hypothalamic-pituitary-interrenal (HPI)-axis.

The main CC in salmonid fish is cortisol (Chester Jones et al. 1980; Hargreaves et al. 1970; Nandi and Bern 1965; Patiño et al. 1987) and the adverse consequences of severe and chronic stress are mainly attributed to this hormone. The endocrine control of cortisol secretion in teleosts appears to be complex. Still, its regulation is suggested to be dominated primarily by proopiomelanocortin-derived pituitary hormones like adrenocorticotropic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH) and β-endorphin like in mammals (Chrousos and Gold 1992; Sumpter et al. 1986; Wendelaar Bonga 1997).

An elevation in plasma cortisol is the most commonly used stress-indicator in fish (Wendelaar Bonga 1997). In response to acute stress, plasma cortisol increases within a few minutes. However, it can take several hours or days for the plasma cortisol levels to return to normal. During chronic stress, plasma cortisol levels can remain well above control values for longer periods (for reviews, see Wendelaar Bonga 1997, Barton and Iwama 1991, Donaldson 1981).

The effects of cortisol in teleost fishes are broad. Since the mineralcorticoid aldosterone has only been demonstrated in minute amounts (if at all present) and without apparent physiological significance (Bern 1967; Chester Jones et al. 1980; Sangalang and Uthe 1994), the general belief is that cortisol acts both as a mineralcorticoid and a glucocorticoid in teleost fish (Bern and Madsen 1992; Stolte et al. 2008; Wendelaar Bonga 1997).

In addition to the essential functions of cortisol (i.e. hydromineral balance and energy metabolism) several deleterious effects of prolonged and high cortisol exposure have been reported in fish. For examples, cortisol has been shown to damage the skin (Iger et al. 1995), inhibit growth (Barton et al. 1987; McBride and Van Overbeeke 1971) and reproduction (Carragher et al. 1989) and to suppress several components of the immune system (Barton and Iwama 1991; Ellis 1981; Fevolden et al. 1993; Fevolden and Roed 1993; Pickering and Pottinger 1989). Since cortisol is a steroid hormone and hence lipophilic of nature, it readily passes the lipid bilayers in the body. Consequently, cortisol in mature female fish can also accumulate in fish eggs, prespawning, and perturb embryonic ontogeny (Eriksen et al. 2006).

Cortisol can also cross protective barriers such as the blood-brain barrier. In mammals, it has been shown that CCs inhibit brain cell proliferation (Ambrogini et al. 2002; Cameron and Gould 1994; Gould et al. 1991; Mayer et al. 2006; Montaron et al. 2006) and survival of newly formed cells (Ambrogini et al. 2002; Wong and Herbert 2004), suppress long-term potentiation (Pavlides et al. 1995b), cause retraction and simplification of dendrites (Woolley et al. 1990) and even kill neurons (Starkman et al. 1992). Despite the numerous reports of CC-induced remodeling of the mammalian brain, surprisingly few studies have investigated the effects of stress and cortisol on the fish brain (but see Sørensen et al., 2007 and 2010).

To conclude, severe and chronic stress is associated with elevated and prolonged levels of cortisol. Such an increase can potentially, and often does, compromise general health and the well-being of an organism. Still, the increase in post-stress cortisol levels is subject to great individual variation. Since cortisol has such broad somatic and central effects, it seems reasonable to expect that individual variation in cortisol responsiveness also results in individual variation in other traits, such as in behavior and cognition. Indeed, an increasing amount of evidence suggests that individual variation in physiology is also associated with behavioral, cognitive and emotional aspects of stress coping (for reviews, see Koolhaas et al. 2010; Korte et al. 2005; Øverli, 2007).

1.2 Stress coping styles

In human and animal populations there is variation in how stress impacts on individual behavior, physiology and general health. This variation is largely due to an inter-individual divergence in coping with stress. Coping has been defined as "*behavioral and physiological efforts to master the situation*" (Koolhaas et al. 1999). How one individual masters stress, does to a certain extent depend on the severeness of the stressor, although the same individual often employs similar physiological and behavioral responses across different stressful incidents. The consistency of behavioral and physiological responses to stress has been termed stress coping style and can be defined as "*A coherent set of behavioral and physiological stress responses which is consistent over time and which is characteristic to a certain group of individuals*" (Koolhaas et al. 1999). Various terms are used to categorize individuals employing different coping styles and animals are often classified as either "reactive" or "proactive" based on their distribution along a shy-bold continuum. Shy, reactive individuals are characterized by having a high HPA-axis reactivity and low activity of the sympathetic adrenal-medullar system. They display low levels of aggression, "freeze and hide" behavior, high behavioral flexibility and are in general low-risk taking. On the contrary, bold and proactive individuals are characterized by low HPA-axis reactivity and high sympathetic activity. They tend to employ the "fight or flight" strategy when stressed, are aggressive, rigid and routine forming and generally display high-risk behaviors (for reviews, see Korte et al. 2005, Coppens et al. 2010 and Koolhaas et al. 2010).

The evolution and biology of stress coping styles are receiving considerable scientific interest. Ultimately, questions are raised about why certain coping styles are evolutionary stable even if they represent an increased risk of maladaptive stress-induced disease (Koolhaas 2008). In ecological and evolutionary terms, the co-existence of such divergent coping styles can be explained by the different fitness consequences they convey. For example, it is suggested that proactive individuals display a set of behaviors that promotes survival and reproductive success in stable environments. On the contrary, reactive individuals are thought to perform better in changing environments (Sih et al. 2004).

To understand why these different coping styles persist, it is useful to elucidate the underlying physiological and molecular mechanisms behind individual variation in behavioral type. In the current work we have used two selection lines of rainbow trout (LR and HR) that have been classified as either proactive or reactive, based on certain behavioral, and physiological traits (Schjolden and Winberg 2007). These fishes therefore provide an

excellent model for studying behavior, physiology and molecular biology in the context of divergent stress coping styles.

1.3 Selection for stress responsiveness and resulting effects on stress coping style

Rainbow trout is one of the most extensively studied fish species in fields ranging from evolutionary ecology to behavior, physiology and genetics (Thorgaard et al. 2002). In 1997, Dr. Tom Pottinger at the Windermere Laboratory, Natural Environment Research Council Institute of Freshwater Technology, UK initiated a selection program creating two lines of rainbow trout differing in post-stress cortisol levels. Low-responding (LR) rainbow trout were selected for having a consistently low cortisol response to stress while high-responding (HR) rainbow trout were selected for having a high cortisol response to stress. Other than HPI-axis dynamics, the LR and HR lines have subsequently been shown to differ in a number of behavioral and neuroendocrine stress responses in a similar way as the proactive and reactive coping styles mentioned above. For example, LR fish respond to stress with a high sympathetic activity (blood epinephrine) compared to HR fish (Schjolden et al. 2006b). Also, LR fish have a tendency to become socially dominant whereas HR fish are more prone to become socially subordinate (Pottinger and Carrick 2001; Schjolden et al. 2006a; Schjolden and Winberg 2007; Øverli et al. 2005) (but see the context-dependent exception noted by Ruiz-Gomez et al. 2008). It has also been proposed that LR fish demonstrate higher levels of risk assessment than HR fish when transferred to a novel environment (Schjolden and Winberg 2007). This assumption was based on the observations that LR fish resume feeding before HR fish (Øverli et al. 2002a) and display greater initial efforts to escape the new environment (Schjolden et al. 2005).

Moreover, two recent studies on the LR-HR model show that the selection lines differ in extinction of previously learnt routines and conditioned fear responses. Ruiz-Gomez et al. (2011) showed that LR fish retained a learnt routine in a food foraging experiment whereas HR fish displayed a more flexible foraging behavior. Also, Moreira et al. (2004) reported that LR fish retained a conditioned fear response longer than HR fish, indicating differences in fear extinction. The main behavioral and neuroendocrine parameters characterizing proactive and reactive individuals across species are listed in Table 1 and compared with the behavioral and endocrine profiles of LR and HR fish.

Table 1. General behavioral and endocrine differences used to characterize proactive or reactive individuals in the literature (Korte et al. 2005)*, compared to the behavioral and endocrine profiles of LR and HR trout (see text for references).

	Proactive*	Reactive*	LR	HR
Emotional state	Bold	Cautious	More bold in novel environments	Cautious in novel environments
Biological role	Establish and defend territory	Avoids danger within territory	Win fights for territorial dominance	Looses fights for territorial dominance
Behavioral flexibility	Rigid and routine like	Flexible	Rigid and routine like in food foraging	Flexible and thorough in food foraging
HPA(I)-output	Low	High	Low	High
Sympathetic reactivity	High	Low	High blood epinephrine	Low blood epinephrine

LR, low responder; HR, high responder; HPA(I), hypothalamic pituitary adrenal (interrenal)

1.4 Cortisol receptors

In addition to plasma cortisol concentrations, a limiting factor determining the effects of cortisol in a tissue, is the intracellular concentration of corticosteroid receptors (CRs) (Dong et al. 1989; Vanderbilt et al. 1987). Hence, altering the expression of these receptors renders a tissue capable of adjusting the biological response according to the requirements of the environment. Two receptors mediating the effects of CCs in vertebrates are mineralcorticoid receptors (MRs) and the glucocorticoid receptors (GRs). Both receptor types belong to the family of ligand-inducible transcription factors. In the absence of a ligand, most MRs and GRs reside in the cytosol. Upon ligand binding, the receptors translocate to the nucleus and form monomers or dimers (homodimers or heterodimers) to modulate gene transcription via transrepression or transactivation, respectively (Datson et al. 2008). Both cases represent the slow *genomic* actions of CCs. Genomic effects of CCs are likely the main mechanism by which CCs affect the brain (Datson et al. 2008). However, corticosteroid receptors have also been found in neuronal membranes (Orchinik et al. 1991), indicating that CCs can utilize alternative and more rapid transduction pathways via membrane-bound and for example G-protein coupled receptors (Makara and Haller 2001). Indeed, rapid non-genomic actions of CCs, which cannot be explained by activation or repression of gene transcription, have also been reported (Borski 2000; Mikics et al. 2004; Sandi et al. 1996).

The involvement of cortisol receptors in controlling physiological and behavioral responses is poorly investigated in fish. In mammals, however, there is an extensive literature on the role of corticosteroid receptors in the brain. Corticosterone, which is the main

glucocorticoid in rodents, binds to MRs predominantly localized in limbic brain structures (e.g. hippocampus) and with a 10-fold higher affinity than to GRs, which are widely distributed in the brain (De Kloet et al. 2005). This divergence in both ligand affinity and receptor distribution allows for fine adjustment of the central responses to CCs depending on the subregional MR/GR balance and circulating hormone levels (De Kloet et al. 2005).

The high expression of MRs and GRs in limbic brain structures (Gerlach and McEwen 1972; McEwen et al. 1968), reflects their involvement in memory, learning and general alertness. However, the actions of corticosteroids depend on which receptor they bind to and the hormones consequently display two modes of operation (De Kloet et al. 1998). Firstly, a "proactive" mode is mediated through MRs and involves maintenance of tonic HPA-axis reactivity and neuronal excitability. Secondly, increasing levels of corticosterone (as during stress) can act through GRs in a "reactive" mode. In this mode, corticosterone acts through a negative feedback mechanism to downregulate stress-induced HPA-axis activation and restore disturbances in homeostasis (De Kloet et al. 1998). In contrast to MR activation, extensive GR activation involves a decreased level of neuronal excitation. In this way corticosterone exerts biphasic effects on excitability of neurons (Diamond et al. 1992; Pavlides et al. 1995a). In parallel with this, biphasic effects of corticosterone upon memory are also observed (Pugh et al. 1997). The balance in hippocampal excitation and inhibition, accomplished through MRs and GRs, respectively, is therefore critical for attention and information processing during different doses of stress exposure (Chan-Palay and Kohler 1989).

A consequence of the difference in glucocorticoid affinity between MR and GR is that MRs are more than 80 % occupied by CC during basal resting conditions, whereas GR occupation varies with changes in plasma hormone levels (Reul and de Kloet 1985; Reul et al. 1987). This is probably also true for salmonid fishes, where the different cortisol receptor types also differ in glucocorticoid affinity and sensitivity. In rainbow trout, cortisol actions are mediated through GR1, GR2 and MR (Bury et al. 2003; Colombe et al. 2000) and rainbow trout MR has higher affinity for cortisol than do GRs (Bury and Sturm 2007). Furthermore, the two different GR paralogs also differ in glucocorticoid sensitivity (Stolte et al. 2006).

Like for the brain, little information is available regarding cardiac cortisol receptors in fish other than that they are abundantly expressed in this tissue (Greenwood et al. 2003; Sturm et al. 2005). In mammals, the cardiac MR has been clearly implicated in development of various pathologies including hypertrophy, inflammation, fibrosis and heart failure (Cittadini et al. 2003; Funder 2001; Qin et al. 2003; Ramires et al. 2000; Rocha et al. 2002). Since

teleosts lack the conventional MR ligand aldosterone (Bern 1967; Chester Jones et al. 1980; Sangalang and Uthe 1994; Stolte et al. 2008), cortisol is likely the most important MR ligand in fish (Bern and Madsen 1992; Wendelaar Bonga 1997), including rainbow trout (Colombe et al. 2000).

Since cortisol is acting as both a glucocorticoid and a mineralcorticoid in fish, it can be assumed that its effects are largely dependent on the regulation and abundance of the different cortisol receptors. Still cortisol receptor regulation is poorly investigated in fish. In Paper I and Paper III we have examined the expression pattern and/or regulation of MR, GR1 and GR2 in the brain and the heart of LR and HR rainbow trout, respectively.

1.5 Postembryonic neurogenesis in mammals

An important physiological phenomenon that is affected by stress and CCs, is postembryonic neurogenesis. Postembryonic neurogenesis, which is commonly referred to as adult neurogenesis in mammals, was discovered in the late 1990s (Eriksson et al. 1998) and confronted the long prevailing belief that no new neurons are formed in the adult human brain. As shown by Eriksson (1998) and others, adult neurogenesis does occur in two areas of the mammalian and human brain. Firstly, new neurons are generated in the anterior part of the subventricular zone from which they migrate into the olfactory bulb (Altman 1969; Bédard and Parent 2004; Curtis et al. 2007; Lois and Alvarez-Buylla 1994; Luskin 1993; Pencea et al. 2001). Secondly, newly generated neurons have been found in the dentate gyrus (DG) of the mammalian hippocampal formation (Altman and Das 1965; Eriksson et al. 1998; Gould et al. 1999; Kaplan and Bell 1984; Kornack and Rakic 1999; Seri et al. 2001). In the latter region, adult neurogenesis is suggested to provide an essential contribution to memory consolidation and the maintenance of normal cognitive function (Abrous and Wojtowicz 2008; Qiao et al. 2005; Shen et al. 2006).

The process of adult neurogenesis involves proliferation of neuronal stem cells and their progeny, a decision between cell survival and death, cell migration, cell differentiation, maturation, and finally integration into existing neuronal networks (Gage et al. 2008). Different experimental approaches have been used to confirm and study adult neurogenesis. Pioneering studies utilized injected thymidine analogs like [^{3}H]thymidine and bromodeoxyuridine (BrdU) to confirm the presence of and follow the fate of newborn brain cells (Altman and Das 1965; Miller and Nowakowski 1988; Sidman et al. 1959). Recently, several molecular approaches have been developed to study the same phenomenon, including

analysis of endogenous neurogenesis markers. The different stages in the process of adult neurogenesis are associated with activation of such markers which constitute stage-and cell-specific genes. Hence, the expression and activity of these genes is suggestive of new neuron formation (Kuhn and Peterson 2008). The advantage of using endogenous markers is that handling stress associated with injection of exogenous markers, such as [^{3}H]thymidine and BrdU, is avoided. For example, proliferating cell nuclear antigen (PCNA) is a nuclear protein serving as an auxiliary factor for DNA polymerase delta in the replication fork (Bravo et al. 1987; Prelich et al. 1987). It is required for DNA synthesis and thus expressed at high levels in dividing cells (Prelich and Stillman 1988).

The resulting total number of newly added neurons, not only depends on the rate of proliferation, but also on the probability of cell survival. Many newly generated cells die shortly after birth (Biebl et al. 2000; Kuhn et al. 2005). Upon reaching their target, newly generated neurons become dependent on neurotrophins for their survival (Oppenheim 1989). For adult neurogenesis, the neurotrophin brain-derived neurotrophic factor (BDNF) appears particularly important for differentiation and survival of newly generated neurons (Benraiss et al. 2001; Rossi et al. 2006; Scharfman and MacLusky 2006; Zigova et al. 1998).

Not all new brain cells become neurons. Therefore, markers of phenotypic identification of neuronal precursors and immature neurons are used to distinguish newborn neurons from for example newly generated glia cells. Two such commonly used markers are doublecortin (DCX) and neurogenic differentiation (NeuroD) (Kuhn and Peterson 2008). DCX encodes a microtubule-associated protein that is transiently and exclusively expressed in migrating neuroblasts (Brown et al. 2003b; Couillard-Despres et al. 2005; Francis et al. 1999; Gleeson et al. 1999; Rao and Shetty 2004). NeuroD is a helix-loop-helix transcription factor and a neuronal differentiation factor which is expressed in very young immature neurons and until they start to develop dendrites (Seki 2002b, a).

The regulation of adult neurogenesis is complex and a plethora of external and internal factors are suggested as either stimulating or inhibiting during the different stages of the adult neuronal development. For example, the effects of external and internal cues, such as stress and CCs, have been studied in great detail in mammals and are generally considered to inhibit adult neurogenesis (Gould et al. 1997; Mirescu and Gould 2006). Environmental regulation of neurogenesis is, however, scarcely studied in comparative models like teleost fish (but see Sørensen et al. (2007) and Von Krogh et al.(2010)).

1.6. Postembryonic neurogenesis in fish

The teleost brain is remarkably plastic and is, in contrast to the mammalian brain, able to generate a vast amount of new neurons in multiple proliferative zones in the brain, throughout life (Zupanc and Horschke 1995). Mapping studies have revealed dozens of proliferation zones (Ekström et al. 2001; Grandel et al. 2006; Kranz and Richter 1970; Richter and Kranz 1970; Zikopoulos et al. 2000; Zupanc et al. 2005; Zupanc and Horschke 1995). In addition to the many proliferation zones, the cell proliferation rate observed in teleost brains exceeds that of any mammalian brain studied, by at least one order of magnitude (Zupanc 2008). They also show an unsurpassed potential to replace new neurons lost to injury (for reviews, see Zupanc 1999, 2001 , 2006, 2008). These features make it particularly interesting to study postembryonic neurogenesis in fish – not only to explore the phenomenon in itself, but also to identify the factors that constrain the process of adult neurogenesis and hence neuronal regeneration in mammals.

Quantitative analysis has shown that cerebellum is the site of origin of the majority of all new brain cells in teleosts (Hinsch and Zupanc 2007; Zupanc and Horschke 1995). The teleost cerebellum is involved in control of coordination of movements, but also in sensory processing (Paulin 1993). Newly generated brain cells are also found in the optic tectum, which is homologous to the superior colliculus of mammals and to which retinal ganglion cells project (Nguyen et al. 1999; Raymond and Easter 1983). Postembryonic neurogenesis in the teleost cerebellum and optic tectum can be rationalized by the numerical matching hypothesis. This hypothesis suggests that an increase in the number of peripheral motor – and sensory elements leads to an increase in the corresponding central processing elements (Zupanc 2008).

The hypothalamus is the main site for HPI-axis regulation in fish as it is for HPA-axis regulation in mammals. Postembryonic generation of new neurons has been described also in this brain region (Ekström et al. 2001; Zikopoulos et al. 2001). Also, the distribution of cortisol receptors has been assessed in the hypothalamus of one teleost species, the common carp (*Cyprinus carpio L*). Stolte et al. (2008) showed that GR1, GR2 and MR were abundantly expressed in this brain region, likely reflecting their role in HPI-axis regulation.

One area of the teleostean brain, in which the existence of postembryonic neurogenesis is of particular interest from a comparative point of view, is the telencephalon. The dorsal part of this brain region is considered functionally homologous to the mammalian hippocampus (Bradford 1995; Northcutt 2008; Portavella et al. 2004; Portavella et al. 2002;

Rodriguez et al. 2002; Zupanc et al. 2005). A particularly high generation of new brain cells in the dorsal telencephalon of fish (Ekström et al. 2001; Zupanc et al. 2005; Zupanc and Horschke 1995) and in the hippocampus of birds (Barnea and Nottebohm 1994), reptiles (Lopez-Garcia et al. 1988) and mammals (Eriksson et al. 1998; Gould et al. 1999), suggests that the persistence of postnatal hippocampal neurogenesis is a conserved vertebrate trait (Zupanc 2008).

The same probably holds true for the expression of cortisol receptors, as both mammalian hippocampi (Gerlach and McEwen 1972; McEwen et al. 1968) and teleostean telencephali (Stolte et al. 2008) possess high levels of GRs and MRs. In mammals, limbic CRs are important mediators of the effects of stress and CCs on behavior and cognition (De Kloet et al. 1998). The effects of stress and CCs on adult neurogenesis are also most pronounced in the mammalian hippocampus. Accordingly, blocking of GRs has been shown to abolish CC-induced reduction of adult neurogenesis (Mayer et al. 2006) whereas genetic disruption of MR has been shown to impair hippocampal neurogenesis (Gass et al. 2000).

In conclusion, tight regulation of postembryonic neurogenesis and the expression of cortisol receptors are important for several aspects of behavior and cognition in mammals. Therefore, divergence in telencephalic postembryonic neurogenesis and/or cortisol receptor expression might well explain the divergence in behavior and cognition observed in LR and HR rainbow trout. Studies of the expression of cortisol receptors and genes involved in postembryonic neurogenesis in the telencephalon are part of the work presented in Papers I and Paper II, respectively.

1.7 Cardiac remodeling in salmonid fishes

Like the brain, the salmonid heart is remarkably plastic. It is composed of an inner spongious myocardium, supplied with oxygen from venous blood returning to the heart, and an outer compact myocardium, supplied with oxygen from coronary arteries delivering oxygenated blood from the gills. Having the outer compact tissue renders trout capable of producing a relatively high blood pressure compared to fish with only spongious myocardium (Pieperhoff et al. 2009). The salmonid heart can easily adapt both physiologically and anatomically in response to environmental cues (Gamperl and Farrell 2004). Such plastic changes can involve cardiomyocyte hypertrophy (growth of single cardiomyocytes) and hyperplasia (proliferation of cardiomyocytes) of both the compact and spongious tissue and can be adaptive responses to for example cold or sexual maturation (Farrell et al. 1988;

Franklin and Davie 1992; Vornanen et al. 2005). However, there are associated costs to such adaptations. A high potential for environmentally induced plastic changes readily predisposes fish to develop pathological cardiac remodeling; which is an increasing problem in farmed salmonids (Poppe et al. 2003; Poppe et al. 2002; Poppe and Taksdal 2000; Takle et al. 2006).

The underlying causes of pathological cardiac remodeling in fish remain largely unknown. Although stress is implicated as a risk factor for individuals already suffering from heart deformities (Brocklebank and Raverty 2002; Poppe et al. 2007), the link between individual variation in stress responsiveness and the development of cardiac disease is poorly investigated. In particular, this association has not been addressed in salmonid fish, of which approximately 2 million tons (2008) are processed yearly in the rapidly developing global aquaculture industry (FAO 2010).

Despite frequent reports on cardiac dysfunction and disease, few studies have aimed at identifying molecular markers of pathological cardiac remodeling in fish. However, it appears that some mammalian markers can be applied also in fish. For example, cold-induced hypertrophic rainbow trout hearts share several markers (VMHC, SMLC2 and MLP) with the hypertrophic mammalian heart (Vornanen et al. 2005). It is also likely that other commonly used markers of cardiac remodeling in mammals are conserved in fish. Examples of such markers in mammals are the nuclear factors of activated T-cells (NFAT) transcription factors, which are activated only in *pathological* cardiac hypertrophy (Wilkins et al. 2004). Other potential markers of pathological cardiac remodeling are markers of fibrosis. Fibrosis, which is caused by structural remodeling of the fibrillar collagen network (Abrahams et al. 1987; Caulfield and Bittner 1988), adversely increases tissue stiffness and impairs normal cardiac function (Weber 1993). Also, a hypertrophied and failing mammalian heart will start to secrete natriuretic peptides like type-A atrial natriuretic peptide (ANP) and type-B atrial natriuretic peptide (BNP) (Wei et al. 1993). mRNA transcripts encoding these peptides are commonly used as markers of heart failure (Gardner 2003). The expression of these genes was investigated in Paper III in the context of divergent stress responses.

1.8 Effects of coping style on gene expression

As animals respond to stressors, studying changes in gene expression is important for elucidating the mechanisms determining the impact of stress on the individual. However, studying gene expression without considering individual variation in stress responses can in some cases result in misinterpretation of the data. For example, MacKenzie et al. (2009)

demonstrated that common carp displaying behavioral predictors of divergent stress coping styles (bold vs. shy) also differed in baseline gene expression. In fact, in response to a challenge, the two different groups of fish showed diametrically opposite responses for 80% of the genes investigated. An uninformed conclusion would have been that the treatment was without effect on either up – or downregulation.

Hence, studying the effects of contrasting stress responses on the molecular level (i.e. effects on gene expression) might contribute to valuable information in the search for proximate mechanisms underlying not only divergent stress coping styles, but also overall stress effects. One aim, common for all experiments in this thesis, was therefore to investigate whether LR and HR fish show different transcriptional regulation.

1. 9 Aims

Paper I aimed at investigating the effects of short-term confinement stress and heritable variation in stress coping style (LR vs. HR) on the expression of cortisol receptors in different parts of the rainbow trout brain.

Paper II aimed at comparing the effects of short-term confinement stress, long-term social stress, and heritable variation in stress coping style (LR vs. HR) on the expression of genes involved in postembryonic neurogenesis and neuronal plasticity in different parts of the rainbow trout brain.

Paper III aimed at investigating the association between cortisol responsiveness to stress and cardiac size and morphology in two salmonid species. More precisely, we aimed at investigating the effect of heritable variation in stress coping style (and hence heritable variation in cortisol response to stress) on cardiac size and morphology and on gene expression of genes that are linked to vascularization, fibrosis, and cardiac hypertrophy in LR and HR rainbow trout. Secondly, we investigated if a trait correlation of post-stress cortisol response and heart size also existed in wild-type European brown trout.

2. Methods

2.1 Research animals

The generation of the LR and HR rainbow trout is described in detail elsewhere (Pottinger and Carrick 1999). In short, the parental generation of LR and HR fish (consisting of 2 year old rainbow trout of the Stirling strain) was established on the basis of consistent divergence in plasma-cortisol responses following repeated stress testing (3 hours of confinement stress once a month for five executive months). Crosses between the selected parents were carried out and the F1 generation hatched during 1997. The F5 generation of LR and HR rainbow trout was used in Paper I, the F5 and F6 generations were used in Paper II and adults of the F4 generation were used in Paper III.

In Papers I and II, juvenile rainbow trout of both genders were used. A previous comparison of post-stress cortisol levels in juvenile rainbow trout revealed no significant difference between males (135 ng/ml) and females (144 ng/ml) (Pottinger et al. 1995).

2.2 Short-term confinement stress

The confinement stress utilized in Papers I, II and III is commonly used as an acute short-term stressor for teleosts (Pottinger and Carrick 1999; Trenzado et al. 2003; Øverli et al. 2006). In Papers I and II, fish were placed in 1 l transparent containers filled with water and kept there for two hours. The water was bubbled with air. In Paper III, brown trout were confined in transparent boxes that were perforated and submerged in the aquaria as shown in Figure 1.

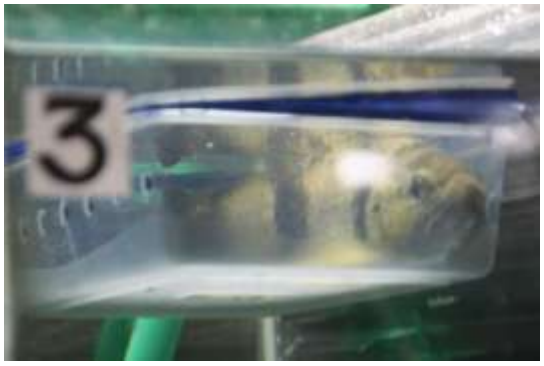

Figure 1. Confinement stress of brown trout (*Salmo trutta*)

2.3 Long-term social stress

Rainbow trout are highly territorial animals and two healthy rainbow trout will most often start fighting for territorial dominance if placed together in a confined area. If a large enough size difference exists between the two fish, the attacks are often unidirectional (from

the larger to the smaller fish) and the smaller individual is predicted to become socially subordinate. Being a subordinate individual in a social hierarchy has been shown to induce a prolonged stress response in several animal species (Blanchard et al. 1993; Eberhart et al. 1983; Haller et al. 1996; Raab et al. 1986; Sapolsky 1989), including in fish (Sloman et al. 2002; Winberg and Lepage 1998; Øverli et al. 1999). We exploited this phenomenon to induce long-term social stress in LR and HR rainbow trout in Paper II. Figure 2 shows a picture series of an example of a larger non-selected territorial rainbow trout attacking a smaller experimental fish, in this case an LR rainbow trout.

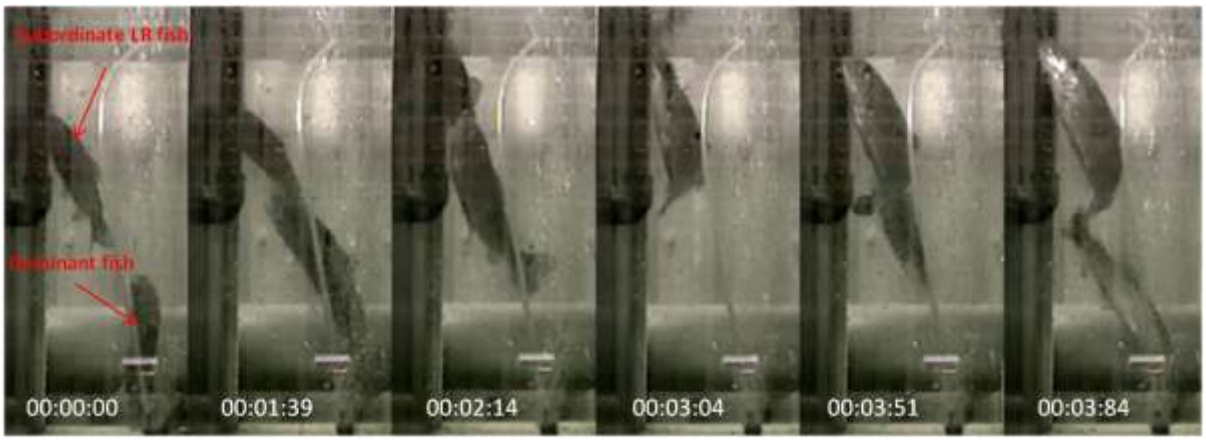

Figure 2. Picture series showing an example of an aggressive act during the long-term social stress experiment. A larger dominant territorial rainbow trout attacks a smaller subordinate rainbow trout. A timescale is provided (min:sec:ms) where the first picture shot is set to 00:00:00.

We assessed aggression levels of the larger territorial fishes in order to be able to predict whether LR and HR trout would experience similar exposure to the stressor in concern. Both attack latency and number of aggressive acts directed towards an intruder are frequently used to quantify aggression in fish (Carpenter et al. 2009; Höglund et al. 2001; Schjolden et al. 2009; Øverli et al. 2004; Øverli et al. 2002b) as in rodents (Haller et al. 1998; Hogg et al. 2000; Veenema and Neumann 2007). Therefore, we video recorded the first two hours after introducing the territorial fish to the intruder fish, registered latency to attack and calculated number of aggressive acts per minute during a 20 minutes period following the first attack.

2.4 Sampling of brains and hearts

The different brain parts investigated in Papers I and II are illustrated in Figure 3. The telencephalon consists of two small lobes at the anterior part of the brain. When isolating the telencephalon lobes, caution was made not to include the olfactory bulbs to which they are

weakly attached (not shown in the figure). The cerebellum consists of one large lobe localized dorsally on the posterior side of the brain. When isolating the hypothalamus, which is located on the ventral side of the brain, care was taken to include the same amount of tissue from every fish and the weakly attached hypophyseal gland was removed. The optic tectum consists of two dorsally situated lobes. The optic lobes were isolated by cutting the optic nerves.

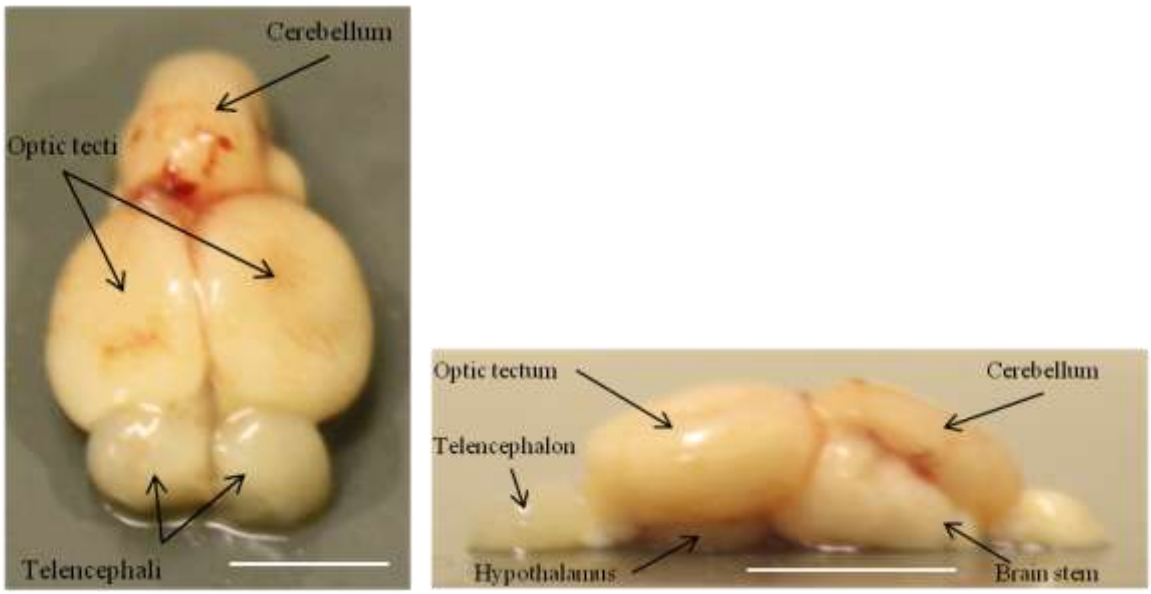

Figure 3. Dorsal view (left panel) and lateral view (right panel) of a freshly excised rainbow trout brain. Scale bars are 5 mm.

The salmonid heart consists of a sinus venosus, an atrium, a ventricle, and a bulbus arteriosis and is illustrated by a brown trout heart in Figure 4.

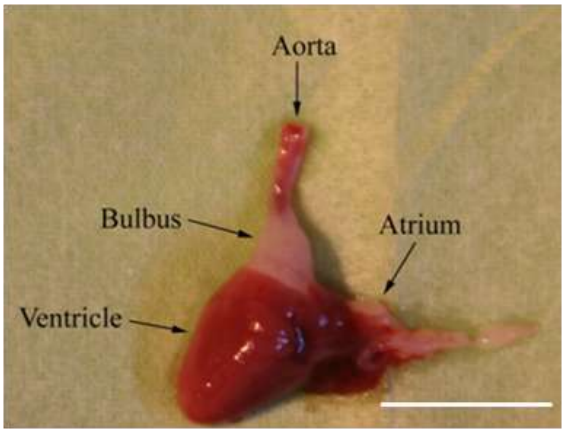

Figure 4. Photographic illustration of a brown trout heart.
From a 25 cm long brown trout. Scale bar is 1 cm.

Rainbow – and brown trout ventricles were isolated by carefully removing the atrium and bulbus. The ventricles were blotted dry before weighing. Of note, the apexes of ventricles

from both LR and HR rainbow trout were more rounded (Fig. 5) compared to those of the wild type brown trout ventricles which had a more triangular pyramid shape. Rounding of the ventricle apex is often observed in farmed salmonids. The functional significance and possible causes of these rounded ventricles are unknown (Poppe et al. 2003).

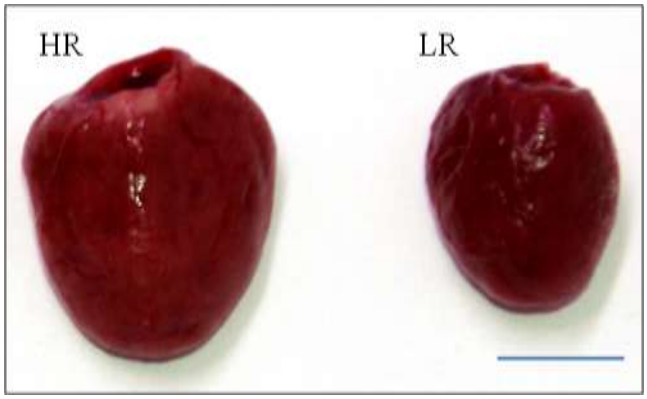

Figure 5. Illustrative examples of rounded ventricles from high-responding (HR) and low-responding (LR) rainbow trout
HR and LR fish were size matched (47 cm). Scale bar is 1 cm

2.5 Quantitative real-timePCR (qtPCT)

Cells in all organisms regulate the expression and turnover of gene transcripts. The number of mRNA copies in one cell or in a tissue is determined by both the rate of its expression and its degradation. qtPCR is an extremely sensitive method for quantifying gene transcripts and is commonly used for both diagnostic and basic research. In order to quantify mRNA transcripts using qtPCR, mRNA is converted to cDNA that is amplified through 20-45 series of temperature changes, called cycles. Each cycle allows for denaturation of double-stranded cDNA by heating of the sample and thereafter annealing of gene-specific primers to the single-stranded cDNA template by cooling of the sample. The amplification of cDNA by a heat stable DNA polymerase is followed by melting curve analysis. Specificity of the primers is verified from the melting curve analysis and by sequencing of the resulting cDNA product.

During the PCR reaction, a DNA-binding dye binds to double-stranded DNA, causing fluorescence of the dye. Therefore fluorescence intensity increases with increasing cDNA levels. The relative concentrations of cDNA present during the exponential phase of the reaction can thus be determined by plotting fluorescence intensity against cycle number on a logarithmic scale. Theoretically, but depending on the primer efficiency, the amount of cDNA doubles for every cycle. A detection threshold above background noise is determined and the cycle at which the fluorescence from a sample crosses the threshold, is called the cycle threshold (C_t) or crossing point (cp). Relative transcript abundance for each gene is

calculated using a formula that incorporates primer efficiency for the test gene and cp values for both test gene and reference gene. More than one reference genes can be used in every reaction to be able to determine which reference gene is best for normalization of the data. In the work presented in this thesis, the housekeeping genes β-actin and cyclophilin A were included in every PCR reaction. Although both reference genes were suitable and yielded identical results, we chose to use β-actin in all experiments because it is a commonly used houskeeping gene in rainbow trout.

3. Synopsis of results

3.1 Paper I

In Paper I we investigated the mRNA expression of the cortisol receptors GR1, GR2 and MR in different brain regions of short-term confinement-stressed and non-stressed LR and HR trout. Two-way ANOVA revealed that mRNA expression of MR was significantly higher in LR compared to HR fish in all brain parts investigated (telencephalon, hypothalamus, cerebellum and optic tectum), indicating that heritable variation in stress coping style affects cortisol receptor expression in trout. Further, the expression of MR was significantly reduced by stress in the telencephalon and the cerebellum, and this effect was most apparent in LR fish. GR1 was significantly reduced by confinement stress in the cerebellum, but not significantly affected in any other brain part. Finally, GR2 mRNA expression was higher in HR compared to LR cerebellum, but not affected by stress. Collectively, these results indicate that short-term confinement stress confers a downregulation of cortisol receptors in a brain region-specific manner in rainbow trout.

3.2 Paper II

In Paper II we investigated how heritable variation in stress responsiveness (LR vs. HR) and two different stressors interact to influence mRNA expression of genes involved in brain cell proliferation, neuronal differentiation, neuronal plasticity and survival. For this task, LR and HR rainbow trout were subjected to either short-term confinement (STC) stress, long-term social (LTS) stress or no stress (controls). In the STC stress paradigm, PCNA expression was significantly higher in HR compared to LR telencephali. The expression of this proliferation marker did, however, not differ between LR and HR fish in any other brain region in this experiment. Also, PCNA expression did not differ between LR and HR fish in any of the brain regions investigated in the LTS stress paradigm. Interestingly, though, two-way ANOVA revealed a positive effect of STC stress (in the telencephalon, hypothalamus and optic tectum) but a negative effect of LTS stress (in the hypothalamus, cerebellum and optic tectum) on PCNA expression in both LR and HR trout. Similarly, the expression of BDNF, which supports survival of newborn neurons and is involved in several aspects of neuronal plasticity, was increased by STC stress, but not affected by LTS stress. This indicates biphasic effects of stress on genes involved in brain cell proliferation, survival and neuronal plasticity.

The expression of DCX and NeuroD (differentiation factors that destine newly generated cells to become neurons) was, like telencephalic PCNA expression in the STC stress experiment, significantly higher in HR compared to LR brains in the LTS stress experiment. For DCX, this effect was seen in the telencephalon and hypothalamus, whereas NeuroD was significantly higher in HR compared to LR telencephalon and cerebellum. A higher expression of proliferation – and differentiation factors in HR compared to LR brains might indicate a higher rate of postembryonic neurogenesis in this fish line.

3.3 Paper III

In Paper III we investigated the size and morphology of LR and HR hearts. We also quantified the mRNA levels of genes involved in mediating the response to cortisol, as well as genes involved in cardiac hypertrophy, vascularization and fibrosis in LR and HR ventricles. Cardiosomatic index (CSI) (ventricle weight relative to body length) was on average 34 % higher in HR compared to LR fish. HR fish also showed larger ratios of compact versus spongious myocardium compared to LR fish. In addition, we found large differences in gene expression in the myocardium of HR fish and LR fish. Most consistently, there was a strong upregulation of genes involved in the hypertrophic gene program (i.e. VMHC, SMLC2 and RCAN1) and a trend towards increased cardiomyocyte hyperplasia (i.e. PCNA) in HR ventricles compared to LR ventricles. RCAN1 mRNA, a marker of pathological pro-hypertrophic NFAT activation in mammals, was significantly higher in HR compared to LR ventricles. Collectively, these findings indicate that the enlargement of the HR ventricles was mainly a result of hypertrophy of the compact myocardium, although some hyperplasia-related growth cannot be excluded.

AFOG-staining indicated extensive and non-homogenously distributed collagen depositions in HR compared to LR ventricles. Further, a higher COL1a2 expression and a trend towards higher COL1a1 expression was observed in the HR compared to LR hearts. Together, these findings indicate focal fibrosis. Commonly used mammalian markers of heart failure (ANP and BNP) did not differ between LR and HR ventricles.

The expression of all three cortisol receptors described in rainbow trout (GR1, GR2 and MR) was significantly higher in HR compared to LR ventricles, indicating that cortisol could be mediating the ventricular enlargement observed in HR fish.

Finally, we aimed at confirming a similar trait correlation between post-stress cortisol levels and heart size outside of the selected LR- and HR trout lines. Hence, cortisol

responsiveness after confinement and CSI was assessed in wild-type European brown trout. Cortisol levels after stress were indeed positively and significantly correlated with CSI in these fish. This finding suggests an evolutionary conserved correlation between cortisol responsiveness and heart size and that this trait correlation is not an incidental artifact of the LR-HR selection regime.

4. Discussion

In this thesis we investigated the effects of heritable variation in stress responsiveness (LR vs. HR) and different stressors (STC vs. LTS) on brain and cardiac plasticity. The work presented here shows that the expression of one cortisol receptor (MR), is higher in LR compared to HR rainbow trout in all brain parts investigated. Since MR is important for memory retention, cognition and several aspects of behavior, differences in MR expression between LR and HR can explain observed differences in fear extinction and anxiety-like behavior in these trout lines. Moreover, we show that the expression of cortisol receptors is downregulated in response to stress in a brain region-specific manner.

We also show that heritable variation in stress responsiveness is associated with altered expression of genes involved in postembryonic neurogenesis and neuronal plasticity in rainbow trout. Specifically, HR rainbow trout have a higher expression of genes involved in postembryonic neurogenesis than LR rainbow trout. In the following it will be discussed how this possibly can contribute to the different behavioral strategies observed in LR and HR fish in relation to stress and other typical traits that characterize stress coping styles (i.e. memory retention and routine formation). Also, it was shown that short-term and long-term stress differently affects expression of genes involved in brain cell proliferation, and, survival of newly generated cells and neuronal plasticity.

Finally, we have shown that cortisol response to stress is associated with cardiac remodeling in salmonids. Rainbow – and brown trout responding to stress with high cortisol levels have larger hearts than those responding to stress with low cortisol. Furthermore, a high expression of markers of hypertrophy in HR ventricles, indicates that the heart enlargement is a result of cardiac hypertrophy. Also, collagen staining and collagen expression indicate fibrosis and disruption of muscle structure in certain areas of the HR hearts. The expression of all cortisol receptors is also higher in HR compared to LR ventricles. Collectively, these results suggest that cortisol is directly mediating cardiac remodeling, and that the cardiac remodeling is likely of pathological character.

4.1 Are our results in accordance with existing literature on stress coping styles?

A portion of our results are listed in Table 2 and compared to relevant literature on rodents and birds. Some of the results in Table 2 are not directly comparable with current literature because no previous studies have investigated these particular systems in the context of stress coping styles. Regarding the expression of MR mRNA, two previous studies on mice

(Brinks et al. 2007) and zebra finches (Hodgson et al. 2007) show an association between low cortisol responsiveness post-stress and low basal levels of MR mRNA in the hippocampal area. Similarly, we show that LR fish display low levels of telencephalic MR mRNA (see Table 2). Possible links between MR mRNA expression and several aspects of cognition are discussed below in section 4.2.1.

Table 2. Summary of results comparable with relevant findings on proactive and reactive stress coping styles in the literature (for review, see Korte et al. 2005).

	Proactive*	Reactive*	LR	HR
MR mRNA expression in hippocampal area:	High*	Low*	High telencephalic expression (Paper I)	Low telencephalic expression (Paper I)
Hippocampal area plasticity and structure:				
	Small mossy fiber terminal fields	Large mossy fiber terminal fields	Low expression of telencephalic PCNA, DCX and NeuroD (Paper II)	High expression of telencephalic PCNA, DCX and NeuroD (Paper II)
	High expression of Growth arrest specific 5	Low expression of growth arrest specific 5		
	Low cytoskeleton gene expression	High cytoskeleton gene expression		
Cardiovascular pathology:				
	Hypertension, arteriosclerosis, myocardial fibrosis and cardiac tachyarrhythmia	Hypertension, arteriosclerosis and cardiac bradyarrhythmia	Smaller hearts with no apparent signs of pathology (Paper III)	Larger hearts with signs of pathology (Paper III)

* High versus low cortisol responsiveness in mice (Brinks et al. 2007) and zebra finches (Hodgson et al. 2007).

We are not aware of previous studies on genes involved in postembryonic neurogenesis in proactive versus reactive individuals. However, some studies have investigated morphology and gene expression of structural genes in proactive and reactive rodents. Collectively, these studies suggest that reactive individuals have better developed hippocampi (for review, see Korte el al. 2005). This suggestion is based on morphological measures of intra – and infra-pyramidal mossy fiber terminal fields (Schwegler et al. 1981;

Sluyter et al. 1994), expression of cytoskeleton gene transcripts and expression of growth arrest specific 5, a non-protein coding RNA that blocks GR-mediated transactivation (Feldker et al. 2003a; Feldker et al. 2003b; Kino et al. 2010). Our results show that the expression of proliferation (i.e. PCNA) – and differentiation markers (i.e. DCX and NeuroD) is higher in HR compared to LR telencephali. This indicates that postembryonic neurogenesis is more pronounced in HR fish. Possible links between hippocampal/telencephalic structure and behavioral flexibility in rodents and rainbow trout are discussed below in section 4.2.2.

Lastly, both proactive and reactive rodents appear to suffer from cardiovascular disease states such as hypertension and arteriosclerosis (see Table 2). In proactive individuals, these disease states are probably caused by high levels of sex steroids (i.e. testosterone) and/or CAs. In reactive individuals, on the other hand, the same disease states are thought to be caused by hypercortisolism (for review, see Korte et al. 2005). In our rainbow trout model, reactive HR trout display more signs of pathology than proactive LR trout. This is despite higher stress-induced CA levels in LR trout as previously shown by Schjolden et al. (2006b). In mammals, CAs are important mediators of stress-related cardiac remodeling as they induce myocardial hypertrophy and cardiomyocyte necrosis. In fish, though, the effects of CAs on the myocardium are less pronounced (Tota et al. 2010), and as the HR hearts display more signs of pathology than LR hearts despite lower post-stress plasma CA, we believe that cortisol constitutes the main stress-induced influence on the trout heart. Interestingly, a link between high cortisol responsiveness to stress and personality traits like social inhibition has been reported also in humans. This particular trait correlation, which is part of the so-called type-D personality construct, is associated with increased cardiovascular morbidity and mortality (Denollet 2000; Pedersen and Denollet 2003).

4.2 Can differences in gene expression explain behavioral and/or cognitive aspects of stress coping?

In Papers I and II we have investigated putative CNS mechanisms controlling behavior and cognition. In the following sections these mechanisms are discussed in relation to the behavioral and cognitive profiles of LR and HR fish.

4.2.1 Can central MR expression in LR and HR trout explain differences in cognition and behavior?

In rats, low levels of hippocampal MRs are associated with poor memory (Herrero et al. 2006) and overexpression of forebrain MR enhances memory (Lai et al. 2007), indicating that MR is important for learning. Also, high – responding zebra finches (*Taeniopygia*

guttata) with low levels of MR mRNA display impaired spatial learning (Hodgson et al. 2007). LR and HR fish have been shown to differ in retention of previously learnt fear responses, indicating differences in memory. However, they do not differ in the time to learn the association between an unconditioned stimulus and a conditioned fear stimulus (Moreira et al. 2004) nor in the time to learn where to find food in a T-maze (Ruiz-Gomez et al. 2011). Thus, a high MR expression in LR fish (see Table 2) does not appear to enhance these modes of learning.

MR could, however, be involved in other aspects of cognitive function, such as memory retention. In rodents, a high MR expression followed by a stress-induced downregulation of this receptor has been suggested to present a risk of reduced fear extinction (Korte 2001). This response pattern is indeed similar to what is seen in LR trout. This selection line show reduced/prolonged extinction of a conditioned fear response (Moreira et al. 2004). Further, in Paper I we show that the expression of MR mRNA was significantly higher in LR compared to HR in all brain regions investigated, but the expression was also significantly downregulated by STC stress in the telencephalon and the cerebellum. The downregulation of MR in the telencephalon was particularly evident in LR fish. Such a response pattern can explain why LR fish retain information about stressful events and previously learnt routines that have been created in moderately stressful settings.

In addition, the expression of MRs could possibly affect other aspects of behavior in rainbow trout. The level of locomotor activity in confinement has previously been used to reflect anxiety-like behavior in fish (Norton and Bally-Cuif 2010; Øverli et al. 2002a). In Paper I, HR fish show erratic swimming behavior and significantly more time spent moving compared to LR fish during confinement. Increased MR activation, reduces anxiety-like behavior in rats (Herrero et al. 2006; Rozeboom et al. 2007) and assuming that increased locomotor activity when stressed reflects anxiety in the current study, then differences in MR expression might explain differences in anxiety-like behavior observed between LR and HR fish.

4.2.2 Can expression of genes involved in postembryonic neurogenesis and neuronal plasticity explain differences in behavioral flexibility in LR and HR trout?

The different behavioral and physiological traits underpinning proactive and reactive animal profiles, like high versus low aggression, low versus high HPA-axis reactivity and routine-like versus flexible behavior, are likely genetically correlated and thought to evolve simultaneously by pleiotropy, gene-linkage or co-selection (Price and Langen 1992). One

such correlation links behavioral flexibility and aggression in mice. Proactive (aggressive) mice have a tendency to develop routines independently of new environmental stimuli whereas reactive (non-aggressive) mice are more flexible and perceptive in their behavior (Benus et al. 1991; Benus et al. 1990). With their morphologically better developed hippocampi (see Table 2), it has been suggested that non-aggressive reactive individuals might be better equipped to organize contextual information and novel sensory stimuli than aggressive proactive individuals (for review, see Korte et al. 2005). In Paper II we show that the expression of PCNA, NeuroD and DCX was higher in HR than in LR brains. The higher expression of these genes in HR compared to LR brains (including the telencephalon), indicate a higher postembryonic neurogenesis in this selection line. Although speculative, altered telencephalon gene expression and perhaps function might explain why HR fish show a more flexible behavior than do LR fish (Ruiz-Gomez et al. 2011).

4.3 To what extent are the effects of stress and stress coping style mediated by cortisol?

In this thesis we have investigated effects of stress and stress coping on the CNS and the heart. In both cases cortisol probably plays an important role since its secretion is induced by stress and, importantly, differs between LR and HR trout.

4.3.1 Effects of cortisol on cortisol receptor expression

Both MRs and GRs are subject to autoregulation by stress and CCs in several tissues both in mammals (Cidlowski and Cidlowski 1981; Okret et al. 1986; Pepin et al. 1990; Sapolsky et al. 1984; Svec and Rudis 1981) and in fish (Lee et al. 1992; Stolte et al. 2008). For example, in rainbow trout, the effects of stress and cortisol exposure on the expression of cortisol receptors have been investigated in a few tissues such as in the brain and in the liver. Dexamethasone (a synthetic glucocorticoid analog) was shown to downregulate GRs in whole rainbow trout brains (Lee et al. 1992). Paradoxically, in rainbow trout hepatocytes, both *in vivo* (Vijayan et al. 2003) and *in vitro* (Sathiyaa and Vijayan 2003), cortisol exposure resulted in an increase in the mRNA expression of GR. Interestingly, Stolte et al. (2008) showed that repeated cold stress but not 24 hours restraint stress reduced the expression of GR1, GR2 and MR in the whole brain, but not in the isolated hypothalamus. Both cold stress and restraint stress was, however, associated with increases in cortisol, indicating that cortisol was not the only mediator of the receptor downregulation in these experiments.

In Paper I we show that the effects of stress on brain cortisol receptors (i.e. MR and GR1) were region-specific. More precisely, stress downregulated the expression of MR in the

telencephalon and the cerebellum and GR1 was downregulated in the cerebellum. We also showed that the effects of stress coping style on basal cortisol receptor expression, was organ-specific. Whereas basal MR mRNA expression was significantly higher in LR compared to HR brains in Paper I, basal MR (as well as GR1 and GR2) mRNA expression was significantly higher in HR compared to LR ventricles in Paper III. The regulation of MRs and GRs at the level of gene expression has been shown to be important for negative feedback mechanisms exerted by CCs. However, likely depending on the various actions of CCs in different tissues, the regulation they confer on the expression of their own receptors might vary as well.

4.3.2 Effects of cortisol on PCNA expression and brain cell proliferation

In mammals, the inhibiting effects of stress on brain cell proliferation are largely mediated by CCs (Ambrogini et al. 2002; Cameron and Gould 1994; Gould et al. 1991; Mayer et al. 2006; Montaron et al. 2003; Wong and Herbert 2004). In Paper II we show that long-term social stress was associated with a decreased mRNA expression of the proliferation marker PCNA. In this study, plasma cortisol levels were significantly increased ($F_{(26)}$=9.97,p=0.004, data not included in Paper II) by LTS stress and also significantly higher in HR compared to LR fish ($F_{(26)}$=8.59,p=0.007, data not included in Paper II). There was also a significant interaction effect ($F_{(26)}$=6.53,p=0.017;Fig. 6, data not included in Paper II).

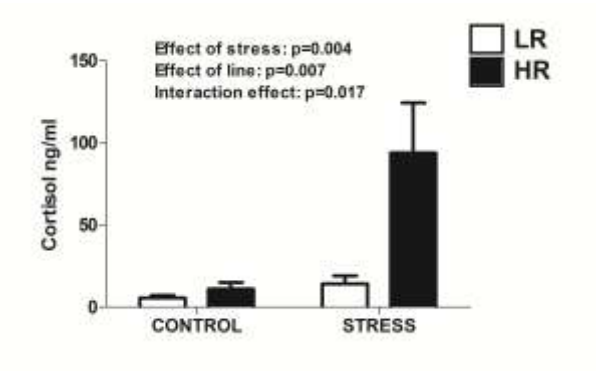

Figure 6. Cortisol levels in long-term stressed and control low-responding (LR) and high-responding (HR) rainbow trout
Values are given as means ± SEM. Statistical analyses were performed by Two-way ANOVA. For detailed ANOVA statistics see text.

This result shows that HR fish had considerably higher cortisol levels compared to LR fish at least at the day of sampling (at day 3 of subordination stress). PCNA mRNA expression, on the other hand, was similarly reduced in both LR and HR brains. The similar PCNA expression in LR and HR brains despite of considerably different cortisol levels,

indicates that cortisol was not alone mediating the effects of stress on PCNA expression in this study (although we cannot exclude the possibility that cortisol levels in LR fish were more increased at an earlier point in the experiment but returned to baseline levels before the time of blood sampling). Nonetheless, there was a significant negative correlation between cortisol levels and the expression of PCNA in LR fish (p=0.006) but not HR fish (p=0.27) in the cerebellum after LTS stress (Fig. 7A,B, data not included in Paper II).There was also a trend towards a negative correlation between cortisol and PCNA expression in the LR (p=0.09) but not the HR telencephalon (p=0.22; Fig. 7C,D, data not included in Paper II)

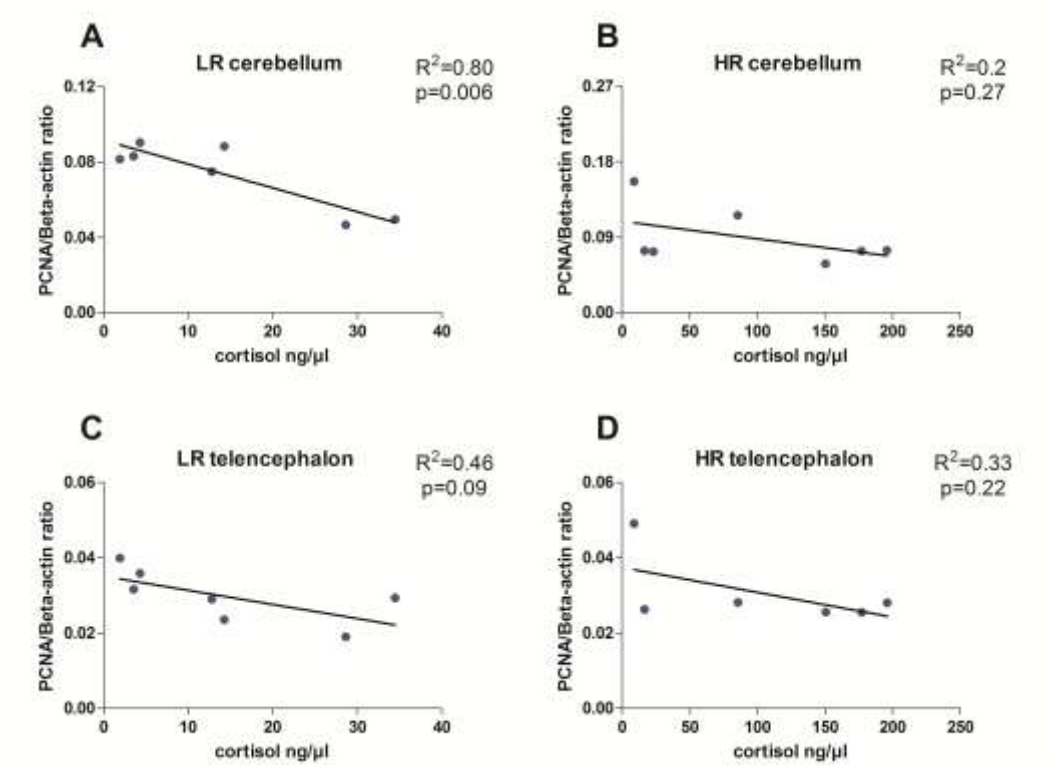

Figure 7. Correlations between cortisol and proliferating cell nuclear antigen (PCNA) mRNA expression in LR (A) and HR (B) cerebellum and LR (C) and HR (D) telencephalon
Regression line slope statistically different from zero is indicated by p-value. The statistical analysis was performed using linear regression analysis (least squares method) with Pearson's product moment correlation coefficient as a measure of the resulting linear relationship (R^2 and p values). HR, high-responding; LR, low-responding.

Recently a study has shown that rainbow trout fed pellets containing both a low or a high dose of cortisol, yielding plasma cortisol concentrations of 4.4 ng/ml and 67 ng/ml, respectively (compared to 1.3 ng/ml for the non-treated controls), displayed a 50 % reduction in PCNA immunostaining compared to the controls. There was no difference between the low – and high cortisol groups in PCNA immunolabeling despite large differences in plasma

cortisol (Sørensen et al. 2010). Collectively, these and our results indicate that cortisol is inhibiting PCNA mRNA and protein expression in these experiments with increasing cortisol concentrations (> control levels), but only up to a certain cortisol level. From our results, there appears to be a linear relationship between cortisol levels and PCNA mRNA in the LTS stress paradigm with relatively low cortisol levels but this relationship is lost at higher cortisol levels.

On the contrary to what we saw after LTS stress, STC stress increased the mRNA expression of the proliferation marker PCNA. Unfortunately, we were not able to measure cortisol after the short-term confinement stress due to a technical error. Thus, we were not able to correlate cortisol levels with PCNA expression in this experiment. However, confinement stress has in several studies been shown to induce a significant increase in plasma cortisol levels in trout (Pottinger and Carrick 1999, 2001; Schjolden et al. 2006a; Trenzado et al. 2003), including in Paper III. It is puzzling that the confinement stress used in Paper II, which was most likely associated with increased cortisol levels, positively rather than negatively affected the expression of PCNA. A likely explanation for this discrepancy is that the long-term social stress probably has a different neurobiological impact than short-term confinement stress. However, we cannot conclude whether this discrepancy is a result of fish perceiving the two stressors differently or whether it is an effect of variable duration and severity of the stress exposure. Nevertheless, it has previously been shown that duration is as important as the dose when it comes to the effect of cortisol on behavior (Øverli et al. 2002b).

Moreover, the regulation of brain cell proliferation in response to different stressors and CC exposure appears to be complex. For example, in the weakly electric fish *Apteronotus leptorhynchus*, administration of cortisol increased brain cell proliferation in the diencephalic ventricular zone but not in the surrounding areas (Dunlap et al. 2006).

When studying effects of stress, and not cortisol *per se*, a plethora of other external and internal cues appears to be involved in the regulation of brain cell proliferation. Therefore, it is likely that the effects of cortisol on brain cell proliferation and adult neurogenesis are highly context dependent. Certainly, not all physiological processes that elevate CCs result in an inhibition of brain cell proliferation and adult neurogenesis. For example, exercise or increased physical activity is associated with both increased levels of plasma corticosterone and increased levels of brain cell proliferation in mice (van Praag et al. 1999). Similarly, in rats and in zebrafish, environmental enrichment is associated with increased levels of CCs and increased brain cell proliferation (Brown et al. 2003a; Von Krogh

et al. 2010). In addition, several examples exist of inhibition of brain cell proliferation and adult neurogenesis in the absence of CC modulation. For example, early life stressors inhibit adult neurogenesis not only at the time of or immediately after the stressor (Tanapat et al. 1998; Zhang et al. 2002), but also into adulthood when CC levels are normalized (Lemaire et al. 2000; Mirescu et al. 2004). Moreover, rats exposed to inescapable foot shock stress displayed reduced DG proliferation despite of unchanged corticosterone levels (Malberg and Duman 2003). It has therefore been suggested that reduced levels of brain cell proliferation are not always directly linked to high levels of CCs.

4.3.3 Effects of cortisol on cardiac remodeling

In addition to the above mentioned link between high cortisol responsiveness and cardiac disease in reactive stress copers (see Table 2), oral administration of glucocorticoids has been recognized as a risk factor for heart disease in humans (Souverein et al. 2004). Also, *in vitro* stimulation with glucocorticoids has been shown to induce cardiomyocyte hypertrophy (Nichols et al. 1984). Moreover, 15 days of dexamethasone treatment resulted in cardiac remodeling in the form of increased heart weight and fibrosis in adult rats (Roy et al. 2009). Still, the role of CCs in cardiac hypertrophy in *adult* mammals remains controversial. In fact, some studies show that glucocorticoids inhibit cardiac hypertrophy because they are able to antagonize aldosterone (Hafezi-Moghadam et al. 2002; Halonen et al. 2007; Tsai et al. 2007). Nonetheless, for *fetal* mammalian hearts there are several reports showing that CCs can induce cardiomyocyte hypertrophy (Lumbers et al. 2005; Reini et al. 2008; Rudolph et al. 1999; Slotkin et al. 1991). Characterization of cardiac morphogenesis in some teleost species, including rainbow trout, indicates that these fish hearts closely resembles what is seen in early embryonic stages in mammals. They do for example posses both a spongious and compact myocardium (Oštádal 1999). Thus, it is possible that the effects of cortisol on the mammalian heart are dependent on the developmental stage of the animal. Since the teleost heart resembles the fetal mammalian heart, cortisol might have a permanent hypertrophic effect on the rainbow trout myocardium.

The observed associations between cortisol responsiveness and heart size in two different salmonid species studied in Paper III, strongly suggest that cortisol is causing cardiac growth in salmonids. Additionally, the high expression of cortisol receptors in the HR compared to LR ventricles strongly suggests a direct role for cortisol in the development of pathological cardiac remodeling. Furthermore, cortisol is thought to be the main ligand for

both GRs and MR in fish (Bern and Madsen 1992; Wendelaar Bonga 1997). In mammals, MR activation is associated with numerous pathological conditions, including hypertrophy and fibrosis (Funder 2001; Takeda et al. 2002).

4.4 Is heart growth pathological?

Hypertrophic growth of the mammalian heart can occur in response to diverse pathophysiological stimuli (i.e. hypertension and ischemia) as a compensatory mechanism that serves to preserve pump function. Nonetheless, a prolonged and irreversible hypertrophic state is generally a leading predictor of cardiac events (i.e. death or hospitalization) (Lloyd-Jones et al. 2002). On the other hand, certain non-pathophysiological factors (i.e. athletic conditioning and pregnancy) can stimulate adaptive and non-pathological reversible heart growth (Oakley 2001). The enlargement of an elite athlete`s heart is obviously associated with increased cardiac performance. Despite rare cases of cardiovascular-related sudden deaths in association with sporting activity, most exercise-induced cardiac hypertrophy is not pathological (Fagard 1996; Oakley 2001). Thus, a bigger heart is not necessarily detrimental.

Salmonid hearts can undergo hypertrophy (and hyperplasia) as a routine remodeling mechanism (Farrell et al. 1988). For example, rainbow trout experience cardiac hypertrophy during cold acclimation (Vornanen et al. 2005). Such an increase in ventricular mass is thought to offset the reduction in cardiac power output observed with acute decreases in temperature. It also enhances stroke volume (Graham and Farrell 1989). Also, cardiac enlargement, observed in male salmonids upon reaching sexual maturity (Armstrong and West 1994; Clark et al. 2009; Franklin and Davie 1992), is hypothesized to support increased functional demands placed on the hearts during the spawning period (Gamperl and Farrell 2004). Thus, there are indications that a bigger fish heart has an improved performance.

Nevertheless, the relatively large hearts observed in female HR trout in Paper III were likely induced by cortisol and not by exercise, sexual maturation or cold. Also, the enlargement was accompanied by increased NFAT-activity (i.e. RCAN1 expression) and by focal fibrosis (i.e. AFOG-staining and COL1a2 expression). Therefore, we doubt the larger HR hearts perform better than the smaller LR hearts, although further studies are needed to assess the effect of the observed cardiac remodeling on HR cardiac function and performance.

4.5 Do GR1 and GR2 differ in function and are they similarly regulated?

Following the identification of two GR paralogs in rainbow trout (Bury et al. 2003), some efforts have been made to separate the two receptors with regard to function and

regulation. Bury et al. (2003) showed that GR1 and GR2 have similar cortisol affinity, but that the GR2 transactivational activity is induced at far lower concentrations of cortisol than GR1. Their different potency as transcription factors, implies different physiological regulation as GR2 will be able to induce transcriptional activity at cortisol levels induced by mild stress (< 10 ng/ml), whereas GR1 will be active only with higher cortisol levels associated with more severe stress (>100 ng/ml) (Bern and Madsen 1992; Bury et al. 2003; Wendelaar Bonga 1997). Differences in receptor function are also indicated by different expression levels of GR1 and GR2 genes in different tissues in the cichlid *Haplochromis burtoni* (Greenwood et al. 2003) and also by differences in subcellular localization. Whereas unliganded GR1 tends to reside in the cytosol, more GR2 is found in the nucleus in the absence of ligand (Becker et al. 2008).

We show very few changes in the cortisol receptors GR1 and GR2 in response to short-term confinement stress in Paper I (although GR1 was significantly reduced in the cerebellum). Nevertheless, when correlating the relative expression levels of GR1 and GR2 in all brain regions and in all groups after the STC stress, we found a significant correlation between the expression of GR1 and GR2 (Fig 8A; data not included in Paper I). Also, a strong positive correlation was found between GR1 and GR2 expression in the cardiac ventricle samples used in Paper III (Fig 8B; data not included in Paper III). GR1 and GR2 expression did not correlate well with the expression of other genes (e.g. PCNA, NeuroD, DCX) from the same stress paradigm, except from MR (data not shown).

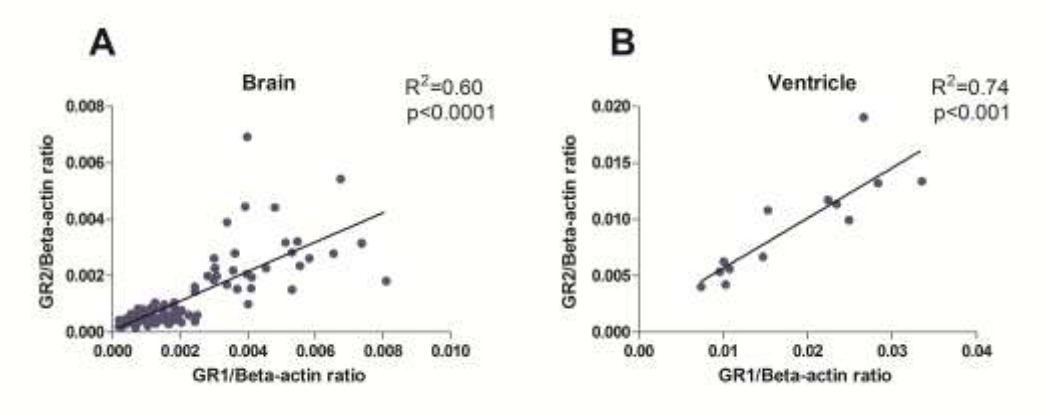

Figure 8. Correlation between mRNA expression of glucocorticoid receptor (GR) 1 and 2 in the brain (A) and cardiac ventricle (B) of rainbow trout
Regression line slope statistically different from zero is indicated by p-value. The statistical analysis was performed using linear regression analysis (least squares method) with Pearson's product moment correlation coefficient as a measure of the resulting linear relationship (R^2 and p values).

Collectively, these results indicate that GR1 and GR2 (and MR), in broad terms, are similarly regulated in rainbow trout.

Intriguingly, two GR2 variants were found by sequencing of the real-time products from brain tissue (Papers I and II) and heart tissue (Paper III) in the current work. One variant has not been described before, but aligned well with parts of a sequence from the *Oncorhynchus mykiss* transcriptome project (GenBank accession number EZ911215.1). Similarly, two MR variants (MRa and MRb) have also been described in rainbow trout (Sturm et al. 2005). There is a high degree of nucleotide identity between the two MR variants, which differ only marginally in the non-conserved A/B domain (transactivation region of receptor). According to Sturm et al. (2005), the two MR variants likely represent paralogs reflecting the tetraploid ancestry of salmonids (Bailey et al. 1978). Like proposed for the two MR variants, it is possible that the two GR2 variants that were found in the current work, are paralogs created by a salmonid-specific genome duplication. Like Sturm et al. (2005), we did not design specific primers to distinguish between the different MR variants. For simplicity, MRa and MRb are referred to as MR in Papers I and III. The new GR2 variant (EZ911215.1) is aligned with parts of the known GR2 variant (NM001124482.1) in Figure 9. The qtPCR primers used in Papers I and III that do not distinguish between the two variants are also shown in Figure 9. Although the two variants differ slightly in nucleotide sequence, the amino acid sequences are identical in the area shown in Figure 9 (data not shown). The two GR2 variants are collectively referred to as GR2 throughout this thesis.

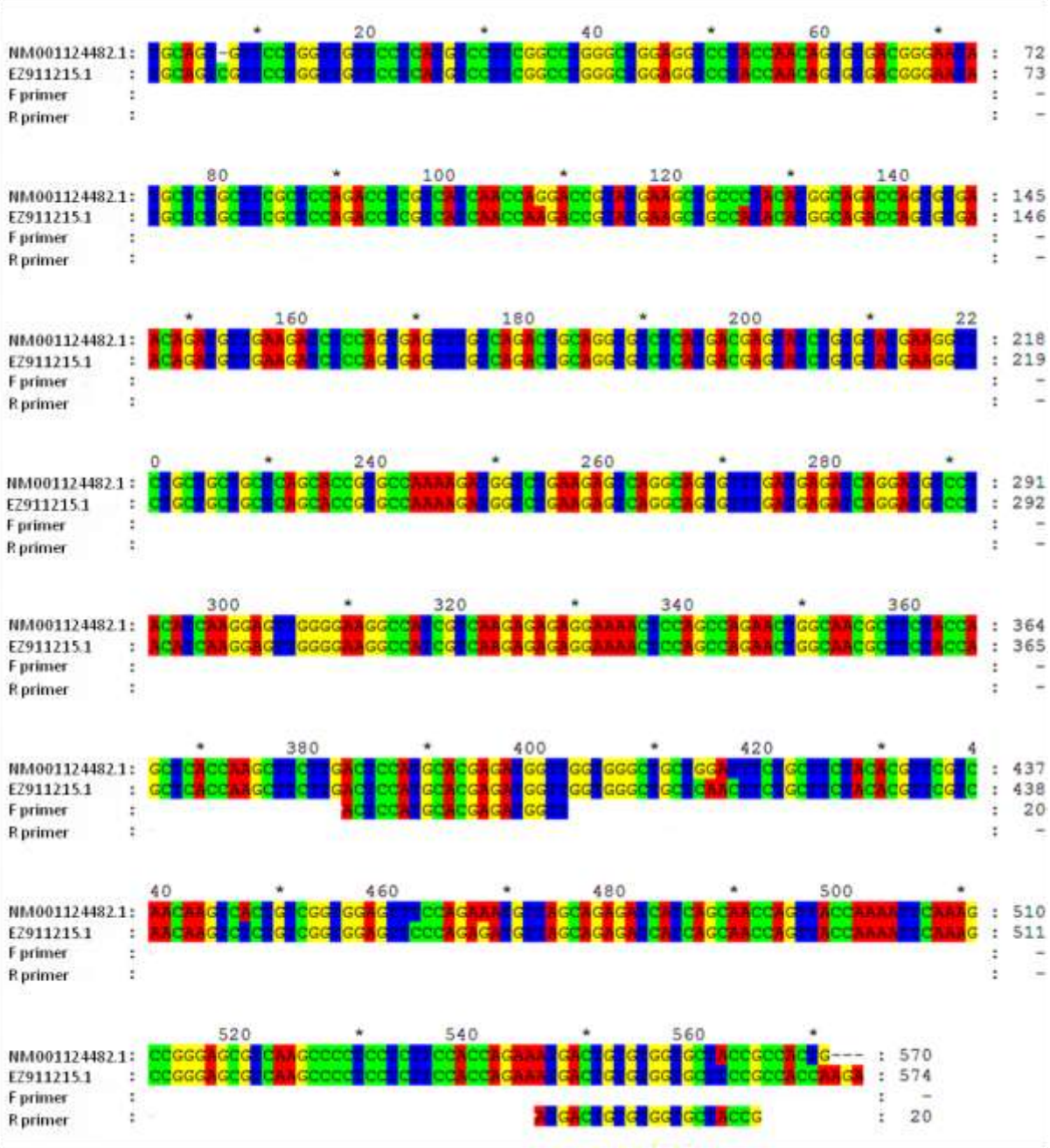

Figure 9. Alignment of parts of nucleotide sequences of previously described GR2 variant with a new variant discovered in brain and cardiac tissue of LR and HR rainbow trout
The start of the GR2 sequences corresponds to nucleotide number 1636 in the GR2 sequence (NM00112482.1) retrieved from NCBI. F primer, forward primer; R primer, reverse primer.

4.6 Methodological considerations: What can mRNA data tell us?

The use of molecular tools to characterize changes in gene expression in response to environmental changes has become extensive, also in fishes (Aksakal et al. 2010; Macqueen et al. 2010; Terova et al. 2011). One important advantage of using qtPCR to analyze gene dynamics in the fish brain, is that it allows accurate quantification of mRNA in small tissue samples (i.e. telencephalon, hypothalamus) from individual fish. Indeed, one reason for

developing the qtPCR technique was because of the need to accurately quantify changes in nucleic acids in small pieces of tissue with only small amounts of nucleic acids present (Saiki et al. 1988). The limitation of quantifying mRNA is that translational and post-translational regulation is not accounted for. In the current work, the number of analysis that could be performed was largely restricted by the availability of LR and HR fish. Also, small-sized tissue samples made additional analyses on the same samples difficult. Moreover, specific antibodies for rainbow trout (or closely related species) are generally not commercially available. Although custom made antibodies can be made for protein analyses purposes (i.e. western blot analyses and immunohistochemistry), it is either considerably time consuming and/or expensive.

Furthermore, mRNA levels are frequently reported to closely correlate with protein levels. One example of this is the cortisol receptor GR for which mRNA and protein levels have been shown to be equally regulated in numerous studies (Breslin et al. 2001; Burnstein et al. 1991; Dong et al. 1988; Ramdas et al. 1999; Vedeckis et al. 1989).

In some cases the detection of changes in mRNA expression is in fact the direct aim of the study, rather than changes in protein levels. For example, when studying the NFAT transcription factors and their activation, changes in mRNA levels of a direct target gene serves as a direct measure of NFAT activity. RCAN1 (also known as MCIP1), whose mRNA levels were quantified in Paper III, is one such direct target gene of NFAT (Oh et al. 2010; Rothermel et al. 2003). A second example is the natriuretic peptides and heart failure markers ANP and BNP studied in Paper III. mRNA levels of ANP and BNP in blood or tissue are, as the main method, quantified by qtPCR (Roncon-Albuquerque et al. 2004; Vanderheyden et al. 2008) both in the clinic and in experimental models associated with hypertrophy (Gardner 2003).

Also, since collagen turnover is a dynamic process (Laurent 1987) that contributes to iterations in cardiac tissue structure seen in various disease states, including in the hypertrophied HR hearts in Paper III (Weber et al. 1994), mRNA expression of fibrosis-promoting collagen genes can give important information about the generation of fibrotic tissue. Similarly, as a result of the long turnover rate of the PCNA protein (proliferation marker used in Papers I and III) (>20 hours) (Kurki et al. 1988) compared to PCNA mRNA (1-2.5 hours), analysis of PCNA mRNA transcripts allows reliable and sensitive detection of proliferating cells (Köhler et al. 2005). Nevertheless, future work should aim at investigating gene activity both at the transcriptomic and proteomic levels.

4.7 Concluding remarks and future perspectives

From the results of Paper I we propose that differential expression of MR in the LR and HR fish is at least partly responsible for differences in memory retention and appraisal of novel stimuli among other aspects of the different behaviors observed between these selection lines. Also, the receptors appear to be subject to negative regulation by stress in a brain region-specific manner. Notably, however, the study does not demonstrate a cause-effect relationship, but directs the avenue for further studies testing e.g. the involvement of MR and GR in cognitive and behavioral aspects of stress coping style. To further study the involvement of cortisol receptors in fish cognition and behavior, specific antagonists for MR (spironolactone) and GR (mifepristone) could be used in behavioral studies to look for changes in behavior and memory retention.

In Paper II we show that short-term confinement and long-term social stress differently affect the expression of genes involved in the process of postembryonic neurogenesis. The fact that postembryonic neurogenesis in fish seems to be less sensitive to acute stress than adult neurogenesis in mammals, emphasizes the importance of studying this phenomenon in comparative models. The high potential for generating new neurons even in stressful environments also implies a great capacity for replacing neurons lost to injury. Studying postembryonic neurogenesis in fish is therefore important from a bio-medical aspect as well as for understanding the evolutionary constraints that limit adult neurogenesis in the mammalian brain (Zupanc 2009).

In Papers I and II we used molecular methods to reveal the effects of stress and stress responsiveness on the expression of genes involved in the cortisol response and CNS plasticity. Again, the functional relationship between gene regulation and stress coping style was not conclusively demonstrated, but merely suggested by the present data together with theoretical considerations. Future approaches to investigate these genes further in the context of stress and stress copings styles in fish, could include qualitative studies investigating more precisely where the resulting proteins are located and where the stress effect is most evident. Immunohistochemistry and *in situ* hybridization are two suitable methods to study the distribution of proteins or mRNA in well defined brain areas, respectively. Also, a study investigating the time line in which changes in mRNA or protein expression occur could be of great interest.

In Paper III we show that high post-stress cortisol levels seem to be associated with cardiac remodeling and pathological cardiac gene expression in salmonid fish. While the

significance of cortisol-related cardiac remodeling for the fish remains speculative, both hypertrophy (Lloyd-Jones et al. 2002) and high serum cortisol (Yamaji et al. 2009) have been identified as independent risk factors for adverse cardiac events in humans. Thus, a follow-up study should involve experiments to determine if HR fish have impaired cardiac function and are more prone to stress-induced mortality. One approach to assess whether the HR hearts are impaired, could be to investigate maximum performance in a perfused heart preparation.

In aquaculture, there is increasing worry for stress-induced mortality of fish carrying cardiac anomalies (Brocklebank and Raverty 2002; Poppe et al. 2007). Here we suggest that cortisol-induced myocardial remodeling may be one of the explanatory factors. One approach to investigate the direct involvement of cortisol on cardiac hypertrophy and fibrosis, could be to treat intact fish hearts *in vivo* or cardiomyocytes *in vitro* with cortisol. To look at receptor-specific effects, MR and GR antagonists could be used as well.

Overall our findings show that stress responsiveness and stress coping style affects gene-expression in the CNS and the heart of rainbow trout. When using molecular tools to characterize changes in gene expression in response to a challenge or clinical treatment, coping style should therefore be included as an explanatory variable when interpreting the results.

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Paper I

LR rainbow trout isolated in aquaria (Photo: Ida Beitnes Johansen)

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Cortisol receptor expression differs in the brains of rainbow trout selected for divergent cortisol responses

Ida B. Johansen [a,*], Guro K. Sandvik [b], Göran E. Nilsson [b], Morten Bakken [a], Øyvind Øverli [a]

[a] Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, PO Box 5003, N-1432 Aas, Norway
[b] Institute of Molecular Biosciences, University of Oslo, PO Box 1041, Blindern, N-0316 Oslo, Norway

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ABSTRACT

In rainbow trout (*Oncorhynchus mykiss*), selection for divergent post-stress plasma cortisol levels has yielded low (LR)- and high (HR) responsive lines, differing in behavioural and physiological aspects of stress coping. For instance, LR fish display prolonged retention of a fear response and of previously learnt routines, compared to HR fish. This study aims at investigating putative central nervous system mechanisms controlling behaviour and memory retention. The stress hormone cortisol is known to affect several aspects of cognition, including memory retention. Cortisol acts through glucocorticoid receptors 1 and 2 (GR1 and 2) and a mineralcorticoid receptor (MR), all of which are abundantly expressed in the salmonid brain. We hypothesized that different expressions of MR and GRs in LR and HR trout brains could be involved in the observed differences in cognition. We quantified the mRNA expression of GR1, GR2 and MR in different brain regions of stressed and non-stressed LR and HR trout. The expression of MR was higher in LR than in HR fish in all brain parts investigated. This could be associated with reduced anxiety and enhanced memory retention in LR fish. MR and GR1 expression was also subject to negative regulation by stress in a site-specific manner.

1. Introduction

In human and animal populations there is an individual variation in how stress impacts behaviour, physiology and general health. This variation is largely due to an inter-individual divergence in coping with stress. The consistency of behavioural and physiological responses to stress has been termed stress coping style and can be defined as "A coherent set of behavioural and physiological stress responses which is consistent over time and which is characteristic to a certain group of individuals" (Koolhaas et al., 1999). To understand why different coping styles exist, it is useful to elucidate the underlying physiological and molecular mechanisms behind individual variation in behavioural type. Such insight might help to understand why the presence of more than one individual coping style is evolutionarily stable. On an applied level, understanding proximate mechanisms behind individual variation in the vulnerability to stress-related disease, can also give valuable contribution to the biomedical field (e.g. the development of personalized medicine).

The current study aims at investigating putative central nervous system mechanisms controlling behavioural and cognitive components of stress coping in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout is one of the most intensive studied fish species in fields ranging from evolutionary ecology to behaviour, physiology and genetics (Thorgaard et al., 2002). We have studied two genetically distinct lines of rainbow trout differing consistently in stress coping style after being selected for low (LR)- and high (HR) cortisol responsiveness post stress (Pottinger and Carrick, 1999). These selection lines have been established as a comparative model for studying heritable variation in stress coping style. Other than hormone dynamics, the trout lines differ in behaviour and personality traits. LR fish are typically more proactive and have a tendency to become socially dominant whereas HR fish are reactive and prone to become socially subordinate (Pottinger and Carrick, 2001; Schjolden et al., 2006; Schjolden and Winberg, 2007; Øverli et al., 2005). Recently, two studies on the LR–HR model show that the selection lines differ in extinction of previously learnt routines and conditioned fear responses. Ruiz-Gomez et al. (2010) showed that LR fish retain a learnt routine in a food foraging experiment whereas HR fish display a more flexible foraging behaviour. Also, Moreira et al. (2004) reported that LR fish retain a conditioned fear response longer than HR fish, indicating differences in fear extinction. Previous studies have, however, not specifically addressed the transcription of genes involved in neural control of the stress response and in the above behavioural and cognitive aspects of stress coping.

Cortisol is the main glucocorticoid in salmonid fish (Hargreaves et al., 1970; Nandi and Bern, 1965; Patiño et al., 1987) and has major effects on several tissues in the fish body, including the brain. This steroid hormone mediates the stress response and has, in mammals, been shown to play important roles in memory and learning (Kirschbaum et al., 1996; Lupien and McEwen, 1997; Shors et al., 1992). In salmonid fish, the actions of cortisol are mediated through

* Corresponding author. Department of Animal and Aquacultural Sciences, PO Box 5003, N-1432 Aas, Norway. Fax: +47 64965101.
E-mail address: ida.johansen@umb.no (I.B. Johansen).

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the glucocorticoid receptors 1 and 2 (GR1 and GR2) and a mineralcorticoid receptor (MR) (Bury et al., 2003; Colombe et al., 2000). These act as ligand-inducible transcription factors (Arriza et al., 1987; Hollenberg et al., 1985) and all are abundantly expressed in the salmonid brain (Lee et al., 1992; Sturm et al., 2005). Since teleost lack the conventional MR ligand aldosterone (Bern, 1967; Chester Jones et al., 1980; Sangalang and Uthe, 1994), cortisol is likely the most important MR ligand in fish (Bern and Madsen, 1992; Stolte et al., 2008; Wendelaar Bonga, 1997), including rainbow trout (Colombe et al., 2000). The rainbow trout MR is activated at lower cortisol levels than the GRs and is expressed at higher levels in the brain than in other tissues investigated. This indicates that the trout MR has a similar role as the mammalian MR in the brain (Sturm et al., 2005). In mammals, brain MR activation has been shown to maintain neuronal excitability, and enhance and prolong long-term potentiation (LTP) (Pavlides et al., 1996). By contrast, extensive GR activation appears to be involved in decreased neuronal excitation and suppression of LTP (Pavlides et al., 1995). Thus, the GR:MR ratio is important in controlling behavioural reactivity and memory storage, making cortisol receptors promising candidates for explaining differences in memory retention and expression of previously learnt routines observed within the LR–HR model.

We hypothesized that GRs and MRs are differently expressed in LR and HR brains for two reasons. Firstly, LR and HR fish differ in behaviour and memory and MR and GRs are implicated as mediators of different aspects of cognition. Secondly, LR and HR consistently respond to stress with low and high cortisol, respectively. From a negative-feedback paradigm, they might differ in basal receptor expression. Additionally, the effects of stress on the expression of the different cortisol receptors in different regions of the fish brain are unknown and stress might affect the receptor expression differently in the two lines. To test the hypotheses, we utilized the selected trout lines in a simple two-factor experimental design; with a control condition and a short-term confinement (STC) stress paradigm, and compared the mRNA expression of cortisol receptors in stressed and non-stressed LR and HR fish.

2. Materials and methods

2.1. Animals

The selection regime generating the two lines of rainbow trout (*O. mykiss,* Salmonidae) (LR and HR) was initiated in 1997 at Windermere Laboratory, NERC Institute of Freshwater Technology, United Kingdom and is described in detail elsewhere (Pottinger and Carrick, 1999). In short, the parental generation of LR and HR fish was established on the basis of consistent divergence in plasma cortisol responses following repeated stress testing (3 h of confinement stress once a month for five executive months).

The study procedures of the experimental animals were reviewed and approved by the Norwegian Animal Research Authority (http://www.fdu.no/fdu/). The experimental fish used in the study were juveniles derived from the F5 generation of rainbow trout from the selection regime. The fish were hatched and reared at the University of Oslo (Blindern, Norway) from eggs transported from the original NERC Windermere facility, and were 68.0 ± 1.28 (mean ± SEM) g of body mass when the study commenced. The two selection lines (LR and HR) were kept in separate holding tanks (750 L) which were continuously supplied with aerated tap water at 8 °C. Light was regulated at 12 h cycles.

2.2. Stress testing, behavioural observations and sampling

At the beginning of the experiment, fish were taken from the holding tanks, weighed and transferred to 250 L glass aquaria. Observation aquaria were divided in four compartments by opaque PVC plates, and LR and HR fish were placed in alternating compartments (one fish per 50 L). During acclimation, fish were fed commercial food pellets once daily.

Locomotor activity during confinement stress is one of the most reliable behavioural indicators of stress coping style in salmonids (Kittilsen et al., 2009; Øverli et al., 2002a, 2006). On days 4 and 5 following transfer, 8 LRs and 8 HRs were subjected to confinement stress involving transfer to 1 L transparent containers, where the fish were kept individually for 2 h. On each test, the first 20 min after transfer to confinement was video recorded. Locomotor activity was quantified as time spent moving (TSM) following Øverli et al. (2006). 8 LRs and 8 HRs were not subjected to confinement stress but kept undisturbed in their respective aquaria to serve as non-stressed controls. At day 5, control fish were netted and rapidly anaesthetized in a benzocaine solution (1 g/L water), while stressed fish were sampled directly following the last period of confinement. Brains were excised and four different brain parts (telencephalon, hypothalamus, optic tectum and cerebellum) were frozen on liquid nitrogen for later analysis. Blood samples were collected for plasma cortisol analysis. Unfortunately, these plasma samples were lost due to a technical error. Nevertheless, several previous studies on the LR–HR model have confirmed that a consistent difference in post-stress plasma cortisol levels is present between the selection lines (Pottinger and Carrick, 2001; Schjolden et al., 2006; Trenzado et al., 2003).

2.3. Quantitative real-time PCR

mRNA abundance was measured using quantitative real-time PCR (qPCR) and will for simplicity here be referred to as mRNA expression. RNA was extracted from brain tissue (telencephalon, hypothalamus, optic tectum and cerebellum) using TRIzol® reagent. RNA concentrations were assessed using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA quality was determined from RNA integrity numbers (RINs) calculated by a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and all samples with RIN values below 5.0 were excluded from further analysis. First strand cDNA was synthesized from 300 to 500 ng (according to size of brain part) DNase I (DNA-free™ Kit, Ambion Applied Biosystems)-treated total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo dT_{12-18} primers synthesized by Invitrogen.

Our genes of interest have previously been sequenced in rainbow trout and were retrieved using NCBI (www.ncbi.nlm.nih.gov/; accession numbers are given in Table 1). Gene specific primers for rainbow trout β-actin, MR, GR1 and GR2 were designed using the web-based Primer3 program (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi),

Table 1
Primer sequences used for quantitative real-time PCR.

Gene name:	Forward primer (5′→3′):	Reverse primer (5′→3′):	Genbank accession numbers
β-actin	AGCCCTCCTTCCTCGGTAT	AGAGGTGATCTCCTTGTGCATC	NM001124235.1
GR1	AGGTTGTCTCAGCCGTCAAA	GCAGCTTCATCCTCTCATCAT	NM001124730
GR2	ACTCCATGCACGAGATGGTT	CGGTAGCACCACACAGTCAT	NM001124482.1, EZ911215.1
MR	CAGCGTTTGAGGAGATGAGA	CCACCTTCAGAGCCTGAGAC	AY495581.1 (a), AY495585.1 (b)

GR1, glucocorticoid receptor 1; GR2, glucocorticoid receptor 2; MR, mineralcorticoid receptor.

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and synthesized by Invitrogen. β-actin, which was stable between the experimental groups and not affected by the confinement stress (data not shown), served as internal control gene. A minimum of five primer pairs were designed at exon junctions for each gene and the primers showing the lowest Cp values and a single peak melting curve were chosen and are listed in Table 1. The qPCR products were also sequenced to verify that the primers amplified the right cDNA. Two MR variants (MRa and MRb) have been described in rainbow trout (Sturm et al., 2005). There is a high degree of nucleotide identity between the two variants, which differ only marginally in the non-conserved A/B domain. Therefore, like Sturm et al. (2005), we did not design specific primers to distinguish between the two isoforms. For simplicity, MRa and MRb will be referred to as MR. Two GR2 variants were also found by sequencing of the qPCR products. One variant has not been described before, but aligned well with parts of a sequence from the *O. mykiss* transcriptome project (GenBank accession number EZ911215.1). The GR2 primers used in the current study did not distinguish between the two variants and the variants will collectively be referred to as GR2.

qPCRs were carried out using a Roche LC480 light cycler® (Roche Diagnostics, Penzberg, Germany). Reactions were 10 μL and included Light cycler® 480 SYBR Green I Master (Roche diagnostics GmbH, Mannheim, Germany), primers (5 μM) and cDNA. Cycling conditions were as follows: 10 min at 95 °C, then 42 cycles of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C followed by melt curve analysis. All reactions were run in duplicates and controls without DNA template were included to verify the absence of cDNA contamination.

Relative gene expression data was calculated from qPCR raw data with Eq. (1): ${}_{IC}E^{Cp}/{}_{GOI}E^{Cp}$ = Expression of GOI in ratio to IC where IC is internal control (β-actin), GOI is gene of interest, E is priming efficiency, and Cp is crossing point. E values were calculated for each qPCR reaction using LinRegPCR software (version 11.30.0) (Ruijter et al., 2009).

2.4. Statistical analysis

Values are given as means ± SEM. All statistical analyses were performed with GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com). Locomotor activity during acute stress was compared between the lines using t-test, and two-way ANOVA was used for determining the effect of the two independent variables (genetic line and experimental treatment) on the dependent variables (gene expression).

3. Results

3.1. Confinement stress test

The average mean TSM, in percent of the first 20 min of confinement, for both days was significantly higher for HR fish compared to LR fish (5.3 ± 2.2 for LR fish and 21.3 ± 4.0 for HR fish, $t_{(14)} = 3.52$, $p < 0.01$). Moreover, HR fish showed more erratic swimming behaviour during confinement compared to LR fish.

3.2. Gene expression analysis

The expression of MR, GR1 and GR2 mRNA was quantified in the telencephalon, hypothalamus, cerebellum and optic tectum of confinement-stressed and control LR and HR fish.

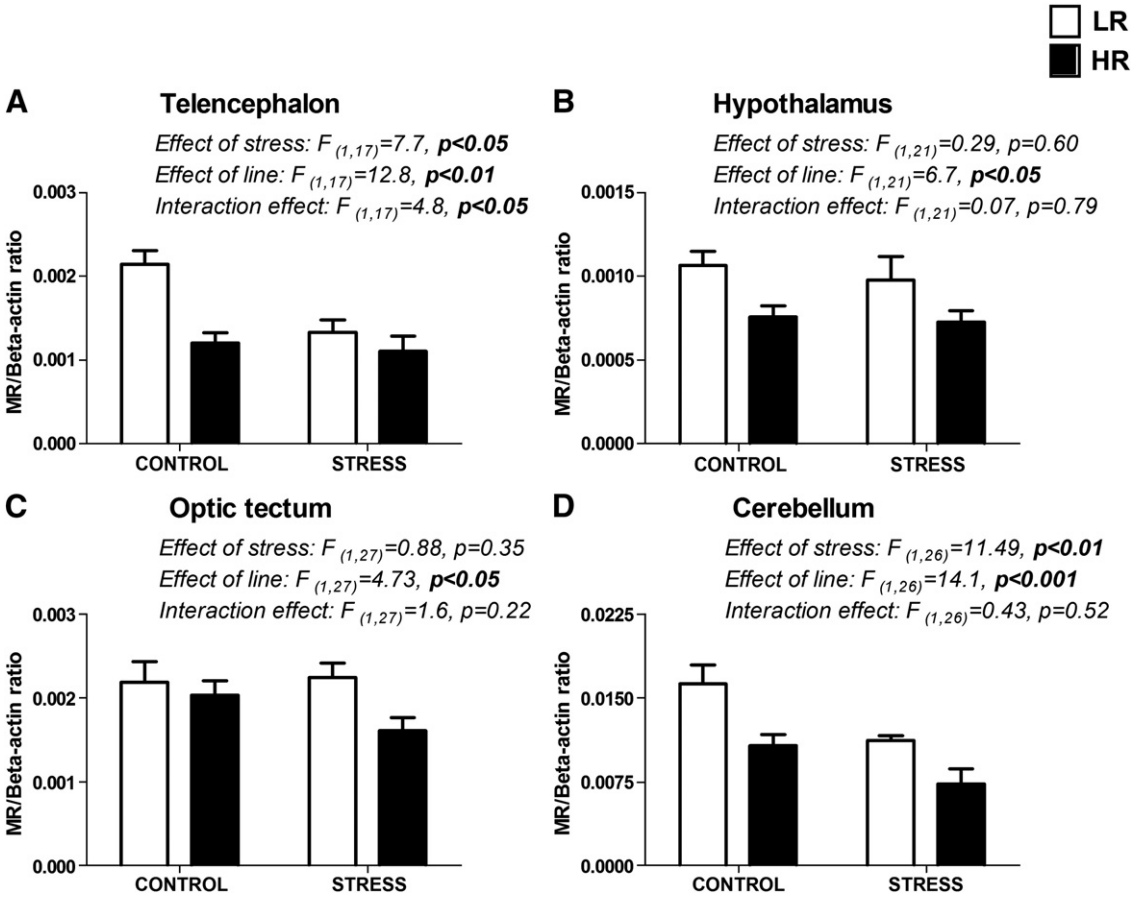

Fig. 1. MR expression in LR and HR brains. mRNA expression of mineralcorticoid receptor (MR) in the telencephalon (A), hypothalamus (B), optic tectum (C) and cerebellum (D) of control and short-term confinement-stressed LR and HR rainbow trout. Values represent means ± SEM. Statistical analyses were performed by two-way ANOVA. Statistically significant p values appear in bold.

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The expression of MR was significantly higher in LR than in HR telencephalon ($p<0.01$), hypothalamus ($p<0.05$), optic tectum ($p<0.05$) and cerebellum ($p<0.001$) as shown in Fig. 1A–D. The expression of MR was reduced by stress in the telencephalon ($p<0.05$) (Fig. 1A) and cerebellum ($p<0.01$) (Fig. 1D). Thus, the large difference in MR expression present in the control condition appeared to be abolished by stress.

The expression of GR1 did not differ between the selection lines in any of the brain parts investigated (see Fig. 2A–D), but was significantly reduced in the cerebellum in fish exposed to confinement stress ($p<0.01$), as shown in Fig. 2D. There was, however, a tendency towards higher GR1 expression in LR fish compared to HR fish in the optic tectum ($p=0.07$).

The expression of GR2 was not reduced by confinement stress in any of the brain parts investigated (see Fig. 3A–D) but its expression was significantly higher in the cerebellum of HR fish compared to LR fish ($p<0.05$), as shown in Fig. 3D.

4. Discussion

The results show that the expression of MR mRNA, but not GR mRNA, is significantly higher in LR trout compared to HR trout in all brain regions investigated in this study (telencephalon, hypothalamus, optic tectum and cerebellum). This suggests that selection for stress responsiveness alters MR expression in the rainbow trout brain. Similarly, high or low corticosterone responsiveness to stress in mice (Brinks et al., 2007) and birds (Hodgson et al., 2007) is also associated with high or low MR mRNA expression, respectively. Since the difference in MR expression between LR and HR fish is present under control conditions, at least in the telencephalon, it is unlikely to be an effect of the stress exposure, but rather reflecting a chronic state that potentially can confer permanent changes in cognition and behaviour.

The level of locomotor activity in confinement has previously been used as an indicator of anxiety-like behaviour in fish (Norton and Bally-Cuif, 2010; Øverli et al., 2002b). Øverli et al. (2002b) showed that HR fish spent significantly more time moving in the face of an intruder than LR fish and interpreted the higher locomotor activity in HR fish as anxiety-like erratic behaviour. Similarly, in the current study HR fish showed a more erratic swimming behaviour and a significantly higher locomotor activity compared to LR fish during confinement. Increased MR activation reduces anxiety-like behaviour in rats (Herrero et al., 2006; Rozeboom et al., 2007). Assuming that increased locomotor activity, as observed during confinement, reflects anxiety in the current study, then differences in MR expression might explain differences in anxiety-like behaviour observed between LR and HR fish.

Moreover, LR and HR trout display differences in the retention of a previously learnt fear response (Moreira et al., 2004) and of routines (Ruiz-Gomez et al., 2010). In rats, low levels of hippocampal MRs are associated with poor memory (Herrero et al., 2006) and over-expression of forebrain MR enhances memory (Lai et al., 2007). Also, high corticosterone-responding zebra finches (*Taeniopygia guttata*) with low levels of MR mRNA display impaired learning (Hodgson et al., 2007). The authors of the latter study suggested that such performance impairment could be attributable to the differences in adrenal steroid receptor densities in the bird brain. Thus, one may speculate that LR fish retain previously learnt responses better than HR fish since they have a higher expression of MRs. However, the

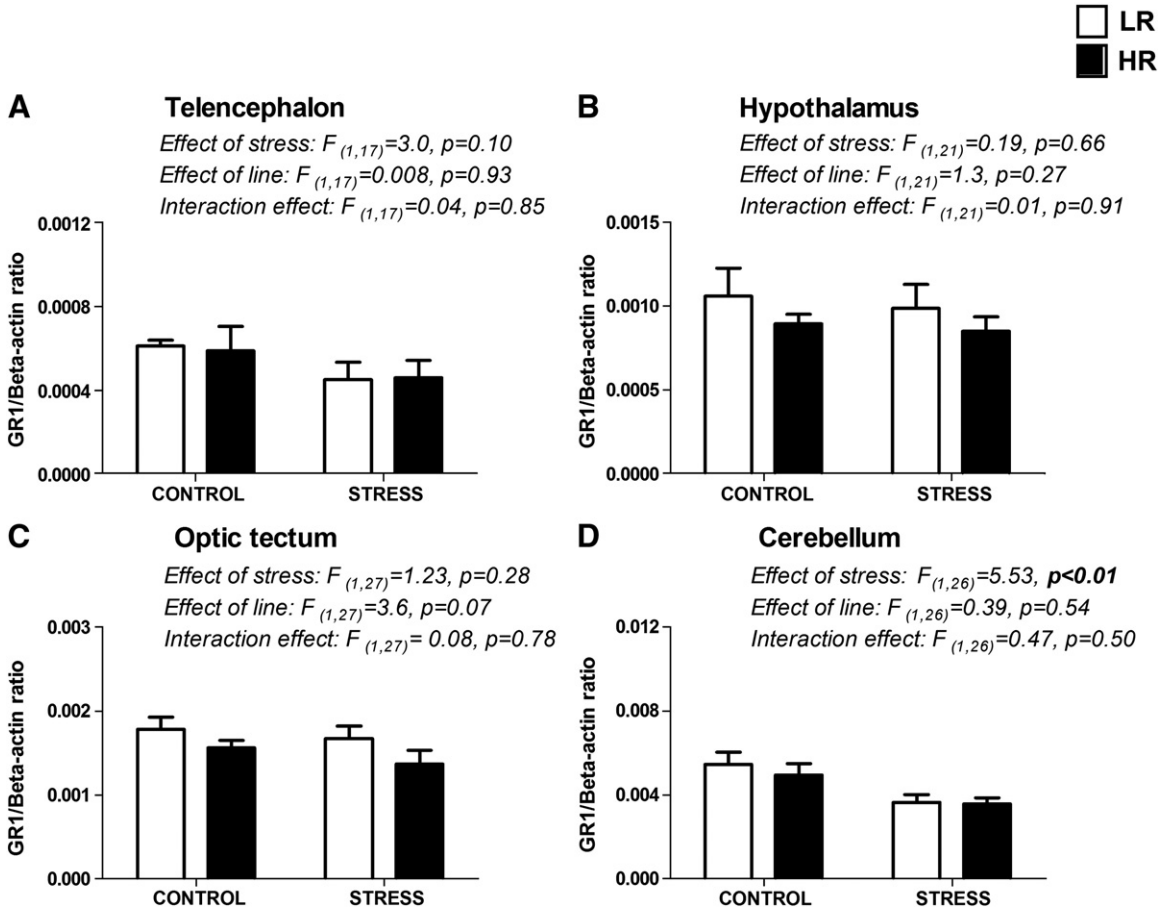

Fig. 2. GR1 expression in LR and HR brains. mRNA expression of glucocorticoid receptor 1 (GR1) in the telencephalon (A), hypothalamus (B), optic tectum (C) and cerebellum (D) of control and short-term confinement-stressed LR and HR rainbow trout. Values represent means ± SEM. Statistical analyses were performed by two-way ANOVA. Statistically significant p values appear in bold.

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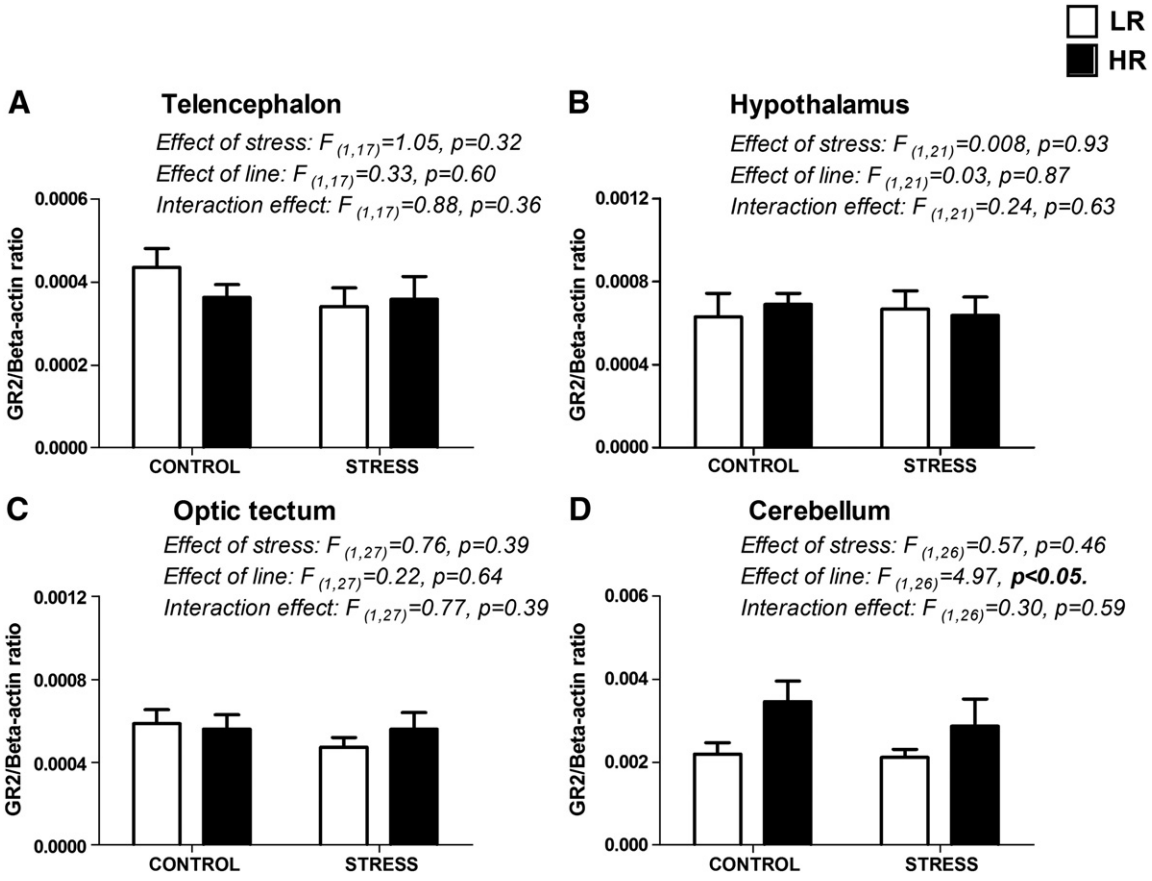

Fig. 3. GR2 expression in LR and HR brains. mRNA expression of glucocorticoid receptor 2 (GR2) in the telencephalon (A), hypothalamus (B), optic tectum (C) and cerebellum (D) of control and short-term confinement-stressed LR and HR rainbow trout. Values represent means ± SEM. Statistical analyses were performed by two-way ANOVA. Statistically significant p values appear in bold.

physiological consequences of MR activation in the fish brain have yet to be identified. Most studies on MR expression and function in fish have focused on gill tissue and osmoregulation (Scott et al., 2005; Sloman et al., 2001). However, fish MRs are broadly expressed beyond gill tissue. In fact, compared with the 11 other rainbow trout tissues examined by Sturm et al. (2005), brain tissue exhibited the highest expression levels of MR mRNA, indicating an important role of this receptor in fish brain. In further support of this, blockade of both MR and GR by specific antagonists resulted in inhibition of aggression in rainbow trout (Schjolden et al., 2009). Furthermore, high expression levels of MR in the fish telencephalon also implies a role in memory and cognition since this brain region is thought to contain areas functionally homologous to the mammalian hippocampus (Bradford, 1995; Northcutt, 2008; Portavella et al., 2004; Portavella et al., 2002; Rodriguez et al., 2002; Zupanc et al., 2005).

We show that stress resulted in a down-regulation of MR expression in the telencephalon, and this effect was particularly evident in the LR fish. Down-regulation of MR expression after stress has been linked to reduced fear extinction (Korte, 2001). The MR expression pattern thus provides a likely explanation to why LR fish retain previously learnt routines and information about stressful events longer than HR fish (Moreira et al., 2004; Ruiz-Gomez et al., 2010).

The effect of stress on the expression of cortisol receptors in specific regions of the salmonid brain has not been previously investigated. However, one study showed that the synthetic glucocorticoid dexamethasone induced down-regulation of GRs in whole rainbow trout brains (Lee et al., 1992). Also, a study on common carp (*Cyprinus carpio*) showed a down-regulation of GR1, GR2 and MR mRNA in the whole brain but not in the isolated hypothalamus after cold transfer stress (Stolte et al., 2008). We show that the expression of MR was significantly reduced by stress in the telencephalon. MR and GR1 were also significantly reduced by stress in the cerebellum. The first observation is likely due to autoregulation of this receptor, since the teleost telencephalon is believed to contain the equivalent of the mammalian hippocampus, which is targeted for negative feedback regulation by glucocorticoids (Chao et al., 1998; Reul et al., 1987; Spencer et al., 1990). The latter indicates that the cerebellum is also sensitive to negative feedback signalling via stress/cortisol. None of the receptors was down-regulated in the hypothalamus or optic tectum, indicating site-specific down-regulation of cortisol receptors in the rainbow trout brain. GR2 expression was not affected by stress in any of the brain parts, suggesting a different regulation of GR2 and GR1 expression as an effect of stress.

However, in the cerebellum, GR2 mRNA expression was significantly higher in HR fish compared to LR fish.

We can only speculate on the physiological consequences of the above differences in receptor expression. Regarding possible differential functions of GR1 and GR2, Bury et al. (2003) showed that GR1 and GR2 have similar cortisol affinity, but that the GR2 transactivational activity is induced at far lower concentrations of cortisol than GR1. Their different potency as transcription factors, imply different physiological regulation as GR2 will be able to induce transcriptional activity at cortisol levels induced by mild stress (<10 ng/ml), whereas GR1 will be active only with higher cortisol levels associated with more severe stress (>100 ng/ml) (Bern and Madsen, 1992; Bury et al., 2003; Wendelaar Bonga, 1997). A 9 amino acid insert in the C-terminal (DNA-binding domain) of GR1 is thought to affect its transactivation potency and make it more different from mammalian GRs than GR2 (Stolte et al., 2006). Differences in receptor function are also indicated by differential

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expression of GR1 and GR2 genes in different tissues in the cichlid *Haplochromis burtoni* (Greenwood et al., 2003). Although expected, separate biological functions of GR1 and GR2 have yet to be experimentally defined. Nevertheless, a higher expression of GR2 in HR fish cerebelli compared to LR fish indicates that HR fish might be more sensitive to the actions of cortisol in this brain part than LR fish.

MRs and GRs are both involved in the homeostatic control of the hypothalamic-pituitary-adrenal (HPA)-axis in mammals. The specific roles of teleost GRs and MRs in the functional equivalent of the mammalian HPA-axis, the hypothalamic-pituitary-interrenal (HPI)-axis, have not been investigated. However, cortisol has been shown to exert a negative feedback function at all three levels of the HPI-axis (Bradford et al., 1992; Fryer and Lederis, 1986). In mammals, limbic MRs is thought to maintain basal activity of the HPA system, presumably by tonic influence on the production and release of corticotrophin in the hypothalamus–hypophyseal system (Reul and de Kloet, 1985). Brain and hypophyseal GRs, on the other hand, are thought to mediate the feedback signal of elevated adrenal hormone secretion (De Kloet, 1991; De Kloet and Reul, 1987; Reul and de Kloet, 1985). However, blockade of brain MR in mammals results in a stronger increase in the glucocorticoid stress response during a mild stressor (Pace and Spencer, 2005; Ratka et al., 1989). Higher MR mRNA expression in LR fish might therefore be at least partly responsible for the lower cortisol response consistently observed in this selection line.

5. Conclusions

To our knowledge, this is the first study to report mRNA expression of GRs and MR in different salmonid brain regions following stress. Since the behavioural functions of cortisol receptors in fish brains are unknown at the moment, the current discussion is mainly based on mammalian studies. We cannot exclude the possibility that this receptor system serves other functions or is subject to other means of regulation in teleosts compared to mammals. Also, the comparison between mammalian and teleost cortisol receptors is complicated by the fact that teleost fish express multiple cortisol receptor subtypes. However, considering the large degree of conservation inherent in this receptor system, both in physiological and molecular components (Stolte et al., 2006; Sturm et al., 2005; Wendelaar Bonga, 1997), it seems relevant to compare findings in teleosts and mammals. Based on this comparison, we propose that differential MR expression is at least partly responsible for differences in memory retention and appraisal of novel stimuli observed between LR and HR selection lines. Also, the receptors appear to be subject to negative regulation by stress in a site-specific manner. Additional experiments are needed to fully elucidate the functional implications of diverging cortisol receptor expression in animals showing alternative stress coping styles.

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Paper II

LR and HR rainbow trout (Photo: Ida Beitnes Johansen)

Gene expression in the context of stress and stress coping style: Modulation of brain plasticity

Ida B. Johansen[1], Guro K. Sandvik[2], Göran E. Nilsson[2], Erik Höglund[3], Morten Bakken[1] and Øyvind Øverli[1].

1. Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Aas, Norway.
2. Physiology Program, Department of Molecular Biosciences, University of Oslo, Norway.
3. Danish Institute for Fisheries Research, Department of Marine Ecology and Aquaculture, North Sea Center, Hirtshals, Denmark.

Abstract

Understanding individual variation and gene-environment interactions is imperative for the interpretation of transcriptomic data in adaptable biological systems. Selection for divergent post-stress plasma cortisol levels in rainbow trout (*Oncorhynchus mykiss*) has yielded low (LR) - and high-responsive (HR) lines differing in behavioral and physiological aspects of stress coping. Using this model, we here investigate how heritable variation in stress coping style interacts with different stressors to affect the expression of genes involved in postembryonic plasticity and neurogenesis. mRNA levels of candidate genes were quantified by qtRT-PCR in brain tissue from LR and HR subjected to short-term confinement (STC) and long-term social (LTS) stress. STC led to increased expression of proliferating cell nuclear antigen (PCNA) in both lines, and the expression was higher in HR compared to LR telencephali. LTS reduced PCNA expression in both LR and HR brains. Brain-derived neurotrophic factor (BDNF) was increased by STC stress, but not affected by LTS stress. Neurogenic differentiation (NeuroD) and doublecortin (DCX) were higher in HR compared to LR fish after LTS stress. Our data indicate that genes involved in postembryonic plasticity and neurogenesis are affected by STC and LTS stress in a biphasic manner, and also differently affected by heritable differences in stress coping style.

Introduction

Individual variation in personality and stress coping have had large consequences for diverse disciplines ranging from evolutionary ecology, to biological psychiatry and physiology. In particular, research on individual variation in disease vulnerability is receiving increased attention [Buske-Kirschbaum et al. 2001; Cohen and Hamrick 2003; Kavelaars et al. 1999; Koolhaas 2008; Koolhaas and Bohus 1991; Segerstrom 2003]. In this context, MacKenzie et al. [2009] recently published an eye opening paper on the relationship between personality type and the expression of immune response-related genes. These authors showed that information on coping style (see Koolhaas et al., 1999 for a definition of terminology) is essential for the interpretation of transcriptomic data. If all individuals in the tested population were analyzed simultaneously as one homogenous group, the treatment (inflammatory challenge) had no detectable effect except increasing the amount of unexplained variability in the data. If, however, individuals were screened behaviorally and grouped into bold and shy categories prior to the challenge test, it appeared that the two groups differed in baseline expression and even showed diametrically opposite responses to the challenge. This suggests a fundamental difference in how the immune system works at the molecular level in the context of stress coping styles. Studying changes in gene expression as individual animals respond to challenge is an invaluable tool for elucidating, in broad terms, the mechanisms that determine the impacts such challenges have on the individual. Hence, since stress coping style appears to have a significant impact on the effects of treatment on the immune system, it would be of interest to study also to what degree the principle applies to other plastic biological systems.

The central nervous system, as exemplified by the role of postembryonic neurogenesis and neuronal plasticity in behavior, emotion, memory and cognition [Qiao et al. 2005; Sahay et al. 2008; Shen et al. 2006], is another such readily adaptable biological entity. Although the regulation of postembryonic neurogenesis (commonly referred to as adult neurogenesis in mammals) by internal and external cues such as stress and glucocorticoids has been studied in great detail in mammalian species [Gould et al. 1997; Mirescu and Gould 2006], environmental regulation of postembryonic neurogenesis and neuronal plasticity is scarcely studied in comparative models (but see Sørensen et al. 2007 and Von Krogh et al. 2010). Compared to mammals, the teleost brain is remarkably plastic and is able to generate a vast amount of new neurons in multiple proliferative zones in the brain throughout life [Zupanc and Horschke 1995].

Quantitative analysis has shown that cerebellum, which is involved in control of coordination of movements and in sensory processing [Paulin 1993], is the site of origin of the majority of all new brain cells in teleosts [Hinsch and Zupanc 2007; Zupanc and Horschke 1995]. Another area of the teleostean brain, in which the existence of postembryonic neurogenesis is of particular interest from a comparative point of view, is the telencephalon. The dorsal part of this brain region is considered functionally homologous to the mammalian hippocampus [Bradford 1995; Northcutt 2008; Portavella

et al. 2004; Portavella et al. 2002; Rodriguez et al. 2002; Zupanc et al. 2005]. A particularly high generation of new brain cells in the dorsal telencephalon of fish [Ekström et al. 2001; Zupanc et al. 2005; Zupanc and Horschke 1995] and in the hippocampus of birds [Barnea and Nottebohm 1994], reptiles [Lopez-Garcia et al. 1988] and mammals [Eriksson et al. 1998; Gould et al. 1999], suggests that the persistence of postnatal hippocampal neurogenesis is a conserved vertebrate trait [Zupanc 2008]. Other brain regions containing proliferative zones include the optic tectum, which is homologous to the superior colliculus of mammals and to which retinal ganglion cells project [Nguyen et al. 1999; Raymond and Easter 1983], and the hypothalamus, which is the main site for HPI-axis regulation in fish.

Additionally, the cell proliferation rate observed in teleost brains exceeds that observed in any mammalian brain studied, by at least one order of magnitude [Zupanc 2008]. Fishes also show an unsurpassed potential to replace new neurons lost to injury (for reviews, see Zupanc 1999, 2001a, 2001b, 2006, 2008). Taken together, these features make it particularly interesting to study postembryonic neurogenesis and plasticity in fish, an endeavor which currently is hampered by the lack of a thorough understanding of gene-environment interactions, which may affect these processes. In this context, the current study was designed to investigate the effect of different stressors (long-term social - vs. short-term confinement) on the expression of genes involved in postembryonic neuronal plasticity and neurogenesis in a teleost model of heritable variation in stress coping style. To this task we have used high (HR) – and low (LR) -responsive strains of rainbow trout (*Oncorhynchus mykiss*) selected for divergent post-stress cortisol levels [Pottinger and Carrick 1999]. These strains have been established as a comparative model for contrasting stress coping styles, differing in hormone dynamics, memory retention, routine formation and several aspects of behavior [Moreira et al. 2004; Schjolden and Winberg 2007; Øverli et al. 2005]. In general, LR fish are characterized by a more proactive (aggressive and bold) and less flexible behavioral profile than HR fish [Moreira et al. 2004; Pottinger and Carrick 2001; Ruiz-Gomez et al. 2011; Schjolden et al. 2006].

The different stages in the process of postembryonic neurogenesis is associated with the activation of stage-and cell specific genes [Kuhn and Peterson 2008]. Hence, the activation of such genes is suggestive of new neuron formation. Consequently, we quantified the expression of proliferating cell nuclear antigen (PCNA), brain-derived neurotrophic factor (BDNF), neurogenic differentiation (NeuroD) and doublecortin (DCX) to obtain indices of neuroplastic changes in the telencephalon, optic tectum, cerebellum, and hypothalamus of LR and HR rainbow trout. The chosen genes are markers of cell proliferation (i.e. PCNA) [Celis and Celis 1985; Köhler et al. 2005; Prelich et al. 1987a; Prelich and Stillman 1988; Takahashi and Caviness 1993], early neuronal differentiation of new-born precursor cells (i.e. NeuroD and DCX) [Brown et al. 2003; Couillard-Despres et al. 2005; Rao and Shetty 2004; Steiner et al. 2006], and neuronal plasticity and survival of new neurons (i.e. BDNF) [Benraiss et al. 2001; Rossi et al. 2006; Thoenen 1995; Zigova et al. 1998].

Materials and methods

Subjects and housing

The selection regime generating the two lines of rainbow trout (LR and HR) was initiated in 1997 at the Windermere Laboratory, Natural Environment Research Council Institute of Freshwater Technology, UK and is described in detail elsewhere [Pottinger and Carrick 1999]. In short, the parental generation of HR and LR fish was established on the basis of consistent divergence in plasma cortisol responses following repeated stress testing (3 h of confinement stress on five occasions). Acute confinement stress has thereafter been used both to select breeding material for further generations and to investigate behavioral and neuroendocrine responses to stress in the HR-LR model [Pottinger and Carrick 2001; Ruiz-Gomez et al. 2008; Schjolden and Winberg 2007]. In the current study, we used juveniles of the 5th and 6th generations of offspring. The study procedures of the experimental animals were reviewed and approved by the Norwegian Animal Research Authority (http://www.fdu.no/fdu/).

Short-term confinement (STC) stress

The experimental fish for the STC study were 16 LR and 16 HR juveniles derived from the F5 generation of rainbow trout from the selection regime, with an average body weight of 71.5 ± 5.9 g (mean ± SD). LR and HR fish were kept in separate holding tanks (250 l) that were continuously supplied with aerated tap water at 8°C and with light kept at 12 h cycles. The experiment, carried out during April 2007, was conducted in glass aquaria (100 x 50 x 50 cm) continuously supplied with aerated tap water (10 l/min, 8°C). Each aquarium was divided into four compartments of 50 l each, by PVC walls.

At the beginning of the experiment, fish were taken from the holding tanks, weighed and transferred to the aquaria, where they were kept for 5 days. LR and HR fish were placed in alternating compartments (one fish per 50 l). During acclimation, fish were hand fed commercial food pellets once daily.

Long-term social (LTS) stress

The experimental animals for this study were derived from the F6 generation (16 LRs and 16 HRs; weighing 117.0 ± 28.6 g of body weight; mean ± SD). Larger non-selected rainbow trout (243.8 ± 37.3 g of body weight; mean ± SD) were obtained from a commercial fish farm, and the larger fish were employed as a social stressor on the smaller HR and LR juveniles.

The two different lines (LR fish and HR fish) and the non-selected rainbow trout were kept in separate holding tanks (250 l) which were continuously supplied with aerated tap water at 12°C and with light regulated at 12 h cycles. The experiment, which was carried out during May 2009, was conducted under similar conditions as the STC study, i.e. in glass aquaria (100 x 50 x 50 cm) continuously supplied with aerated tap water (10 l/min, 8°C), again with each aquarium divided into

four compartments of 50 l each. At the beginning of the experiment, fish were taken from the holding tanks, weighed and transferred to the aquaria where they were isolated for 5 days. LR, HR and non-selected trout were placed in alternating compartments (one fish per 50 l). During acclimation, fish were hand fed commercial food pellets once daily.

Behavioral observations and sampling

STC stress

On days 4 and 5 after transfer to isolation, 8 LR and 8 HR fish were subjected to a daily confinement test involving transfer to 1 l transparent containers for 2 hours. 8 LRs and 8 HRs were not subjected to confinement stress and served as controls. After the confinement test on day 5, the fish were anaesthetized in a benzocaine solution (1 g/l water). Brains were excised and divided into four different brain parts (telencephalon, optic tectum, cerebellum, and hypothalamus). Separate brain parts were analyzed by quantitative real time PCR (qtRT-PCR) for gene expression.

LTS stress

After 5 days of isolation 8 LR and 8 HR fish were transferred to aquarium compartments with a resident non-selected fish 1.45 ± 0.13 (mean ± SEM) times their own size. Rainbow trout are territorial fish and the bigger non-selected fish were used to stress the experimental intruder fish by acting aggressively and becoming dominant. To control whether LR and HR fish received similar amounts of stress, the first 20 minutes following this encounter was video recorded to register latency to attack and quantify the number of aggressive acts directed towards them. The latency to first attack (in seconds) was not statistically different for LR and HR fish (688.9 ± 196.5 (mean ± SEM) for the LR fish and 843.6 ± 372.5 (mean ± SEM) for the HR fish, $t_{(13)}$=0.32, P=0.76) nor was number of aggressive attacks/per min directed towards them (4.4 ± 2.6 (mean ± SEM) for LR fish and 2.0 ± 0.9 for HR fish, $t_{(13)}$=0.91, P=0.40). After three days with a bigger resident fish, the LR and HR fish were anaesthetized in a benzocain solution (1 g/l water). Brains were excised and divided into four different brain parts (telencephalon, optic tectum, cerebellum, and hypothalamus). Separate brain parts were analyzed by qtRT-PCR for gene expression.

qtRT- PCR

mRNA abundances were measured using qtRT-PCR. These data will for simplicity here be referred to as gene expression. RNA was extracted from brain tissue (telencephalon, optic tectum, cerebellum, and hypothalamus) using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer`s protocol. Total RNA concentrations were assessed using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA quality was determined from RNA integrity numbers (RINs) obtained by a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and all samples with RIN values below 5.0 were excluded from further analysis. First

strand cDNA was synthesized from 300-500 ng (according to size of brain part) DNase I (DNA-free™ Kit, Ambion Applied Biosystems)-treated total RNA using Superscript III reverse transcriptase (Invitrogen) with oligo dT $_{12\text{-}18}$ primers.

Specific primers for rainbow trout β-actin, PCNA, BDNF, NeuroD and DCX were designed using the web-based Primer3 program (http://frodo.wi.mit.edu/***cgi bin***/primer3/primer3_www.cgi), and synthesized by Invitrogen (Invitrogen Dynal AS, Oslo, Norway). β-actin, which was stable between the experimental groups and not affected by either of the stress paradigms, served as an internal control gene. β-actin (GenBank accession no. NM001124235.1), BDNF (GenBank accession no. GU108576.1) and PCNA (GenBank accession no. EZ763721.1) sequences were retrieved using NCBI (www.ncbi.nlm.nih.gov/). Rainbow trout DCX and NeuroD sequences were retrieved at dfci (http://compbio.dfci.harvard.edu) database based on BLASTs with *Taeniopygia guttata* DCX (GenBank accession no. XM002196104.1) and *Salmo salar* NeuroD (GenBank accession no. BT058820.1) respectively, found in the NCBI database. A minimum of five primer pairs was designed at exon junctions for each gene and the primers showing the lowest Cp values, a single peak melting curve and amplification of the right amplicon were chosen and are listed in Table 1. Primer specificity tests, including melting curve analysis and sequencing of the amplicons, were performed to ensure quantification of the correct sequences.

Table 1. Primer sequences used for qtRT-PCR

Gene name:	Forward primer (5`→ 3`):	Reverse primer (5`→ 3`):
β-actin	AGCCCTCCTTCCTCGGTAT	AGAGGTGATCTCCTTGTGCATC
PCNA	AGCAATGTGGACAAGGAGGA	GGGCTATCTTGTACTCCACCA
BDNF	GACCAAGGATGTCGACCTGT	GCTGTCACCCACTGGCTAAT
NeuroD	CCTTTAGGAGAAGTGCGGATA	ATTGGCCCAAGTATTCGTTT
DCX	TCCGCTTCATCTTCTCCATC	CAGTTGGGGTTGACGTTCTT

PCNA, proliferating cell nuclear antigen; BDNF, brain-derived neurotrophic factor; NeuroD, neurogenic differentiation; DCX, doublecortin.

qtRT-PCRs were carried out using a Roche LC480 light cycler (Roche Diagnostics, Penzberg, Germany). Reaction volumes were 10 µl and included Light cycler® 480 SYBR Green I Master (Roche diagnostics GmbH, Mannheim, Germany), primers (5 µM each) and cDNA. Cycling conditions were as follows: 10 min at 95°C, 42 cycles of 10 sec at 95°C, 10 sec at 60°C and 10 sec at 72°C followed by melt curve analysis. All reactions were run in duplicates and controls without DNA template were included to verify the absence of cDNA contamination.

Relative gene expression data was calculated from qtRT-PCR raw data using the formula:

$_{IC}E^{Cp}/_{GOI}E^{Cp}$= Expression of GOI in ratio to IC

where IC is internal control (β-actin), GOI is gene of interest, E is priming efficiency, and Cp is crossing point. E values were calculated for each qtRT-PCR reaction using LinRegPCR software (version 11.30.0) [Ruijter et al. 2009].

Statistical analysis

All statistical analyses were performed with GraphPad Prism (version 5.00 for Windows, www.graphpad.com). Gene expression was analyzed using a Two-way ANOVA with genetic line and experimental treatment as independent variables. Other analysis of stressed LR and HR fish (i.e. received aggression) were performed using t-tests. Normality and homogeneity of variance was ascertained by Kolmogorov Smirnov test and Levene's test.

Results

Gene expression analyses

The abundances of BDNF, PCNA, NeuroD and DCX mRNA were quantified in the telencephalon, cerebellum, optic tectum and hypothalamus of STC - and LTS-stressed and control LR and HR fish. Table 2 shows the effects of STC stress and selection line and Table 3 shows the effects of LTS stress and selection line on gene expression of all genes and in all brain parts investigated. Results are summarized below and significant effects are graphed for illustration.

Table 2. Changes in gene expression after short-term confinement stress
Table includes effect of stress and line. Significant interaction effects were not present (results not shown).

Brain part:	Gene:	Effect of stress:	Effect of line:	Direction of effects:
Telencephalon				
	PCNA	$F_{(1,17)}$=4.36 , P=0.05	$F_{(1,17)}$=4.49 , P=0.05	stress↑, HR>LR
	BDNF	$F_{(1,17)}$=0.93 , P=0.35	$F_{(1,17)}$=0.47 , P=0.52	
	NeuroD	$F_{(1,18)}$=0.001 , P=0.97	$F_{(1,18)}$=0.01 , P=0.91	
	DCX	$F_{(1,18)}$=0.00002 , P=0.99	$F_{(1,18)}$=2,60 , P=0.12	
Hypothalamus				
	PCNA	$F_{(1,21)}$=7.56 , P=0.01	$F_{(1,21)}$=1.25 , P=0.28	stress↑
	BDNF	$F_{(1,21)}$=4.26 , P=0.05	$F_{(1,21)}$=0.64 , P=0.43	stress↑
	NeuroD	$F_{(1,21)}$=3.60 , P=0.07	$F_{(1,21)}$=1.89 , P=0.18	
	DCX	$F_{(1,21)}$=0.09 , P=0.77	$F_{(1,21)}$=0.21 , P=0.65	
Cerebellum				
	PCNA	$F_{(1,26)}$=2.39 , P=0.13	$F_{(1,26)}$=0.89 , P=0.36	
	BDNF	$F_{(1,26)}$=3.42 , P=0.08	$F_{(1,26)}$=1.46 , P=0.24	
	NeuroD	$F_{(1,26)}$=3.57 , P=0.07	$F_{(1,26)}$=0.02 , P=0.88	
	DCX	$F_{(1,26)}$=1.38 , P=0.86	$F_{(1,26)}$= 2.29, P=0.33	
Optic tectum				
	PCNA	$F_{(1,27)}$=6.87 , P=0.01	$F_{(1,27)}$=3.56 , P=0.07	stress↑
	BDNF	$F_{(1,27)}$=21.36 , P<0.0001	$F_{(1,27)}$=0.85 , P=0.36	stress↑
	NeuroD	$F_{(1,27)}$=2.18 , P=0.36	$F_{(1,27)}$=0.87 , P=0.15	
	DCX	$F_{(1,27)}$=0.03 , P=0.56	$F_{(1,27)}$=0.34 , P=0.86	

PCNA, proliferating cell nuclear antigen; BDNF, brain-derived neurotrophic factor; NeuroD, neurogenic differentiation; DCX, doublecortin; HR, high-responsive; LR, low-responsive.

Effects of stress-responsiveness, STC and LTS stress on PCNA expression

The expression of PCNA was significantly higher in HR compared to LR telencephali (P =0.05) in the STC experiment. There was also a tendency towards higher expression of PCNA in HR compared to LR optic tecti (P=0.07) after STC stress. The expression of PCNA did, however, not differ between

LR and HR fish in the other brain parts in the STC experiment nor in any of the brain parts following LTS stress. Interestingly, the expression of PCNA was significantly increased in the telencephalon (P=0.05), hypothalamus (P=0.01) and the optic tectum (P=0.01) of STC stressed (Fig. 1A) LR and HR fish compared to LR and HR controls. In contrast to the stimulatory effect of STC stress, the expression of PCNA was significantly decreased by LTS stress (Fig. 1B) in the hypothalamus (P=0.0004), optic tectum (P=0.004) and in the cerebellum (P<0.0001). There was also a tendency towards decreased expression of PCNA in the telencephalon (P=0.08) as an effect of LTS stress.

Table 3. Changes in gene expression after long-term social stress

Table includes effect of stress and line. Significant interaction effects were not present (results not shown).

Brain part:	Gene:	Effect of stress:	Effect of line:	Direction of effects:
Telencephalon				
	PCNA	$F_{(1,23)}$=3.40 , P=0.08	$F_{(1,23)}$=0.36 , P=0.56	
	BDNF	$F_{(1,23)}$=0.77 , P=0.39	$F_{(1,23)}$=0.06 , P=0.80	
	NeuroD	$F_{(1,23)}$= 0.14, P=0.71	$F_{(1,23)}$=0.52 , P=0.004	HR>LR
	DCX	$F_{(1,23)}$=0.02 , P=0.89	$F_{(1,23)}$=7.7 , P=0.01	HR>LR
Hypothalamus				
	PCNA	$F_{(1,22)}$=17.01 , P=0.0004	$F_{(1,22)}$=0.06 , P=0.80	stress↓
	BDNF	$F_{(1,22)}$=0.004 , P=0.95	$F_{(1,22)}$=0.004 , P=0.95	
	NeuroD	$F_{(1,22)}$=0.27 , P=0.60	$F_{(1,22)}$=1.78 , P=0.20	
	DCX	$F_{(1,22)}$=0.14 , P=0.72	$F_{(1,22)}$=4.36 , P=0.05	HR>LR
Cerebellum				
	PCNA	$F_{(1,27)}$=22.82 , P<0.0001	$F_{(1,27)}$=1.90 , P=0.18	stress↓
	BDNF	$F_{(1,27)}$=0.02 , P=0.89	$F_{(1,27)}$=1.13 , P=0.30	
	NeuroD	$F_{(1,27)}$=5.56 , P=0.03	$F_{(1,27)}$=6.77 , P=0.01	stress↓, HR>LR
	DCX	$F_{(1,27)}$=0.50 , P=0.48	$F_{(1,27)}$=1.19 , P=0.28	
Optic tectum				
	PCNA	$F_{(1,27)}$=10.7 , P=0.004	$F_{(1,27)}$=1.35 , P=0.26	stress↓
	BDNF	$F_{(1,27)}$=2.90 , P=0.10	$F_{(1,27)}$=0.55 , P=0.47	
	NeuroD	$F_{(1,27)}$=0.15 , P=0.70	$F_{(1,27)}$=1.28 , P=0.27	
	DCX	$F_{(1,27)}$=0.10 , P=0.76	$F_{(1,27)}$=1.93 , P=0.18	

PCNA, proliferating cell nuclear antigen; BDNF, brain-derived neurotrophic factor; NeuroD, neurogenic differentiation; DCX, doublecortin; HR, high-responsive; LR, low-responsive.

Effects of stress-responsiveness, STC and LTS stress on BDNF expression.

The expression of BDNF did not differ between LR and HR fish in any brain part investigated in any of the experiments. The expression was, however, significantly increased by STC stress in the hypothalamus (P=0.05) and the optic tectum (P<0.0001) (Fig. 2). There was also a tendency towards increased BDNF expression in the cerebellum (P=0.08). The expression of BDNF was not affected by LTS stress in any of the brain parts investigated.

Effects of stress-responsiveness, STC and LTS stress on NeuroD and DCX expression.

There was no significant effect of selection line on NeuroD or DCX expression in the STC experiment. The mRNA expression of DCX was, however, significantly higher in HR fish compared to LR fish in the telencephalon (P=0.01) and in the hypothalamus (P=0.05) (Fig. 3A) in the LTS experiment. Similarly, the mRNA expression of NeuroD was significantly higher in HR compared to LR brains, although in the telencephalon (P=0.004) and in the cerebellum (P=0.01) in the same experiment. Finally, the expression of NeuroD (Fig. 3B) was significantly reduced by LTS stress only in the cerebellum (P=0.03). Neither NeuroD nor DCX expression was significantly affected by the STC stress although there was a tendency towards increased expression of NeuroD in the hypothalamus (P=0.07) and the cerebellum (P=0.07).

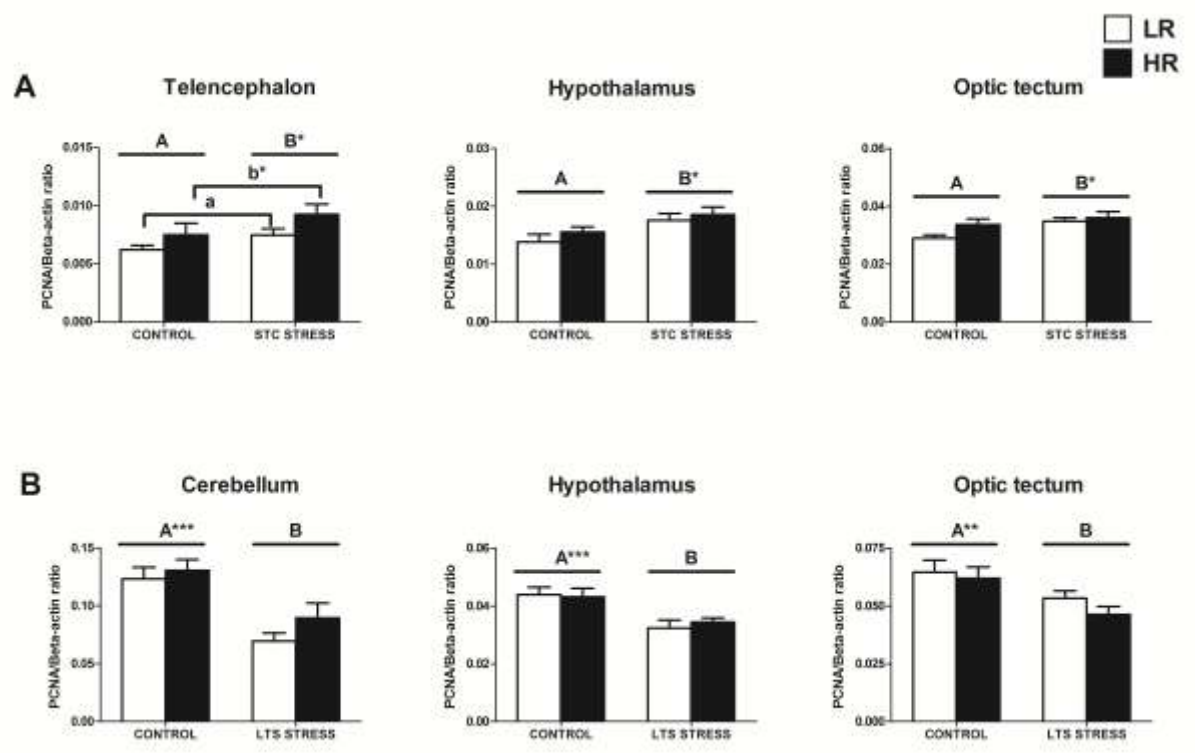

Figure 1. Expression of the proliferating cell nuclear antigen (PCNA) after (A) short-term confinement (STC) and (B) long-term social (LTS) stress

Values represent means ± SEM. Different capital letters (A/B) indicate an effect of stress. Different small caps (a/b) indicate an effect of line. Statistical analyses were performed by Two-way ANOVA. * = P<0.05, ** = P<0.01 and *** = P<0.001. For detailed ANOVA statistics see Table 2 or Table 3.

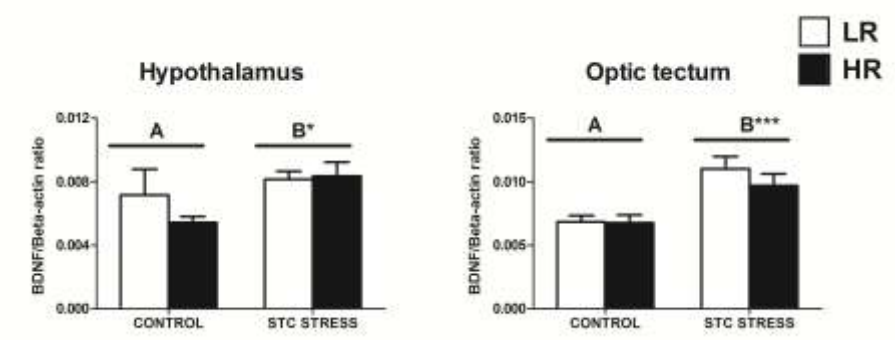

Figure 2. Expression of brain-derived neurotrophic factor (BDNF) after short-term confinement (STC) stress
Values represent means ± SEM. Statistical analyses were performed by Two-way ANOVA. Different capital letters (A/B) indicate an effect of stress. * = P<0.05 and *** = P<0.001. For detailed ANOVA statistics see Table 2.

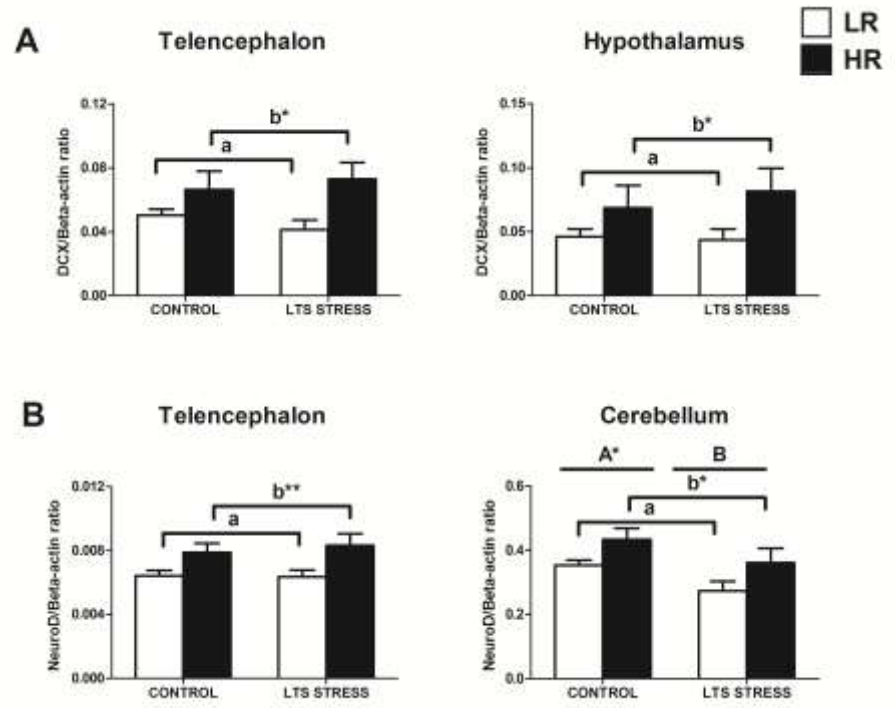

Figure 3. Expression of (A) doublecortin (DCX) and (B) neurogenic differentiation (NeuroD) after long-term social (LTS) stress
Values represent means ± SEM. Different capital letters (A/B) indicate an effect of stress. Different small caps (a/b) indicate an effect of line. Statistical analyses were performed by Two-way ANOVA. * = P<0.05 and ** = P<0.01. For detailed ANOVA statistics see Table 3.

Discussion

Differences in gene expression between the selection lines

We found that HR fish have significantly higher expression levels of PCNA in the telencephalon compared to LR fish in the STC stress experiment. HR fish also showed higher expression levels of NeuroD in the telencephalon and cerebellum and a higher expression of DCX in the telencephalon and hypothalamus compared to LR fish in the LTS stress experiment. As there were no significant

interaction effects, the ANOVA results can be interpreted as indicating a general increase, i.e. not specifically occurring in either the control situation or after stress.

NeuroD is a bHLH transcription factor involved in neuronal differentiation and survival. In the adult hippocampus of mice, immature new-born neurons express NeuroD and the differentiation factor is thought to be essential for postembryonic hippocampal neurogenesis [Steiner et al. 2006]. DCX is a widely used marker for phenotypic identification of neuronal precursors and immature neurons. It is a microtubule-associated protein involved in neuronal migration and neurite outgrowth and it is transiently and exclusively expressed in neuronal precursors and new-born neurons in the hippocampus of mice [Brown et al. 2003; Couillard-Despres et al. 2005]. Thus, differences in expression of genes involved in postembryonic neurogenesis, particularly in the telencephalon, appear to be involved in some of the central processes differentiating stress coping styles (i.e. behavior flexibility). However, further studies are needed to clarify the association between the expression of these genes and stress coping styles.

PCNA expression following STC and LTS stress

STC stress and LTS stress was found to affect the expression of PCNA differently. Specifically, after STC stress, the expression of PCNA was significantly increased in the telencephalon, hypothalamus and optic tectum of both LR and HR fish. On the contrary, LTS stress had the opposite effect and significantly reduced the expression of PCNA in the hypothalamus, optic tectum and the cerebellum, while no change was observed in the telencephalon. These findings indicate that regulation of expression of these genes in the fish brain is different following STC and LTS stress, implying a dose-dependent effect of stress on postembryonic brain cell proliferation, provided that changes in PCNA mRNA reflect changes in the proliferative activity of brain cells [Köhler et al. 2005] (see discussion below).

In mammals, both acute stress and long-term social stress has been shown to reduce proliferation of progenitor cells [Gould et al. 1997; Gould et al. 1998; Heine et al. 2004] and the general picture has for a long time been that this phenomenon is not dependent on species or type of stressor, but rather that the effect appears to generalize across stressors. However, recent studies also in rat [Parihar et al. 2009] and monkey [Lyons et al. 2010] show that mild stress can enhance brain cell proliferation and adult hippocampal neurogenesis. We here show that STC stress in fish can induce expression of the proliferation marker PCNA. Whether this corresponds to increased cell proliferation remains to be investigated. On the other hand, also coherent with mammalian studies, it appears that LTS stress decreased the PCNA expression in the present study. This is in line with the finding that social stress reduces BrdU-labeling in subordinate rainbow trout telencephali [Sørensen et al. 2007].

The stress hormone cortisol (the main corticosteroid hormone of salmonid fish, like in humans) is a likely candidate mediating the different effects of short - and long-term stress on brain

function [de Kloet et al. 1999; Diamond et al. 1992; McEwen 1999] . It is tempting to speculate that the inverted U-shaped dose- and duration-response curve of cortisol might be responsible for the opposing effect of the two different stressors employed in this study. In support of this stance, von Krogh et al. [2010] recently reported that environmental enrichment was associated with both an increase in forebrain PCNA immunostaining and a modest, but statistically significant, increase in cortisol production in zebrafish (*Danio rerio*). With reference to cognition and underlying structural processes, an increase in brain cell proliferation after a short and relatively mild stressor might serve to prepare the brain for new incoming information associated with the stressor. If a stressor turns out to be more severe and of longer duration, perhaps this increased potential for information processing is dispensable.

One could argue that other parameters than the duration and type of stressor differed between the STC and LTS treatments, and that these factors could affect the experimental outcome. However, the confinement test [Øverli et al. 2006] and intruder test [Höglund et al. 2001; Winberg et al. 2001] are both established methods that have been shown to give a highly predicative and reproducible effect on the stress – and cortisol response in salmonid fish. Therefore, we find it likely that the effects of these two different experimental paradigms are a result of the different stressors rather than other parameters.

The interpretation of the results of the current study also depends on the implications that can be ascribed to the different candidate genes that were chosen as markers for brain structural processes. There exist several approaches for labeling dividing cells in the brain, the most common involving injections of thymidine analogs such as BrdU and ^{3}[H] thymidine. To avoid handling stress associated with such injections, the use of endogenous markers like PCNA to identify proliferating cells in the adult brain can be used [Belvindrah et al. 2002; Curtis et al. 2003; Freundlieb et al. 2006; Gould and Tanapat 1997; Ino and Chiba 2000; Jin et al. 2001; Kitayama et al. 2003], including in teleost fish [Ekström et al. 2001; Grandel et al. 2006; Wullimann and Puelles 1999]. Furthermore, this proliferation marker has been shown to correlate with BrdU staining in proliferation zones in the teleost brain [Ekström et al. 2001; Grandel et al. 2006] and its cytological presence is therefore regarded as representing a reliable marker of proliferative activity. PCNA is a small nuclear protein that acts as a cofactor for DNA polymerase delta [Bravo et al. 1987; Prelich et al. 1987b] and is required for DNA synthesis [Prelich et al. 1987a; Prelich and Stillman 1988]. It is expressed throughout the entire cell cycle but the protein levels are thought to be highest during the S-phase and lowest in the G_2/M-phases [Celis and Celis 1985; Kawabe et al. 2002; Takahashi and Caviness 1993]. Most studies utilize immuno-labeling of the PCNA protein which will start to accumulate already in G_1-phase. Furthermore, the half-life of a PCNA protein exceeds 20 h [Kurki et al. 1988], possibly leading to detection of cells that are no longer cycling. PCNA mRNA, on the other hand, has a turnover of about 1- 2.5 h and detection of this short-lived transcript therefore provides a better

estimation of the S-phase cell pool and is better suited for looking at dynamic changes in the proliferative activity of the cell [Chang et al. 1990; Köhler et al. 2005].

An increase in cell proliferation markers such as PCNA can not be directly correlated with an increase in a differentiated population of neurons. A proportion of the neuronal progenitors does not become fully differentiated and dies by apoptosis. Also, the progenitor cells can be differentiated into neurons as well as glial cells, so not all newly generated cells give rise to neurons. However, it has been shown that many of the PCNA-positive cells found in the brain do differentiate into neurons [Valente et al. 2009]. Furthermore, in fish, the survival rate of newly born neurons has been shown to be particularly high [Zupanc et al. 2005]. Since the expansion of progenitor populations is fundamental for neurogenesis, the analysis of cell cycle components must be a critical component in studies of neurogenesis and can provide useful information about quantitative changes in proliferative activity.

Short-term confinement stress increases BDNF expression

Important mediators of stress and corticosteroid action in the brain might be neurotrophins such as BDNF. This neurotrophin is a member of the nerve growth factor family and has been shown to promote survival of new-born neurons [Benraiss et al. 2001; Rossi et al. 2006; Zigova et al. 1998], regulate synaptic efficacy [Thoenen 1995] and be involved in memory processes such as LTP [Bramham and Messaoudi 2005; Figurov et al. 1996; Korte et al. 1995; Patterson et al. 1992].
We observed an increase in BDNF mRNA in the hypothalamus and optic tectum after repeated STC (2 h) stress. Similarly, Smith et al. [1995a; 1995b] showed an increase in mRNA levels of BDNF in the hypothalamus after 2 h acute restraint stress in rats. As opposed to the expression of PCNA (se separate discussion) the significant increase after STC was abolished, but not reversed to a decrease, by LTS. The expression of BDNF did not differ between LR and HR fish in any of the stress paradigms.

Conclusions

We show here that short-term confinement and long-term social stress in rainbow trout differently affect the expression of genes involved in the process of postembryonic neuronal plasticity and neurogenesis. The fact that postembryonic neurogenesis in fish seems to be less sensitive to acute stress than in mammals, emphasizes the importance of studying this phenomenon in comparative models. A high potential for generating new neurons even in stressful environments also implies a great capacity for replacing neurons lost to injury, so studying neurogenesis in fish is important from a bio-medical aspect as well as for understanding the evolutionary constraints that limit postembryonic neurogenesis in the mammalian brain [Zupanc 2009]. In this context, it must be kept in mind that both inter- and intra-specific variation can reveal important information about the regulation and functional significance of neurogenesis and neuronal plasticity.

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Paper III

Boračko Jezero, Konjic, Bosnia and Herzegovina (Photo: Ida Gjervold Lunde)

Cortisol response to stress is associated with myocardial remodeling in salmonid fishes

Ida B. Johansen[1], Ida G. Lunde[2, 3], Helge Røsjø[3,4,5], Geir Christensen[2, 3], Göran E. Nilsson[6], Morten Bakken[1], Øyvind Øverli[1]

[1] Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences (UMB), Ås, Norway.

[2] Institute for Experimental Medical Research, Oslo University Hospital, Ullevaal, Oslo, Norway.

[3] Center for Heart Failure Research, University of Oslo, Oslo, Norway.

[4] Medical Department, Akershus University Hospital, Lørenskog, Norway.

[5] Institute for Clinical Medicine, University of Oslo, Oslo, Norway.

[6] Physiology Programme, Department of Molecular Biosciences, University of Oslo, Oslo, Norway.

Summary

Cardiac disease is frequently reported in farmed animals, and stress has been implicated as a factor for myocardial dysfunction in commercial fish rearing. Cortisol is a major stress hormone in teleosts, and this hormone has adverse effects in the myocardium. Strains of rainbow trout (*Oncorhynchus mykiss*) selected for divergent post-stress cortisol levels (high responsive: HR, and low responsive: LR) have been established as a comparative model to examine how contrasting stress-coping styles differ in physiological and behavioral profiles. We show that the mean cardiosomatic index (CSI) of adult HR fish is 34% higher than in LR fish, mainly due to hypertrophy of the compact myocardium. To characterize the hypertrophy as physiological or pathological, we investigated specific cardiac markers at the transcriptional level. HR hearts had higher mRNA levels of cortisol receptors (MR, GR1, and GR2), increased RCAN1 levels suggesting enhanced pro-hypertrophic NFAT signaling, and increased VEGF gene expression reflecting increased angiogenesis. Elevated collagen (Col 1a2) expression and deposition in HR hearts supported enhanced fibrosis, while the heart failure markers ANP and BNP were not upregulated in HR hearts. To confirm our results outside the selection model, we investigated the effect of acute confinement stress in wild-type European brown trout (*Salmo trutta*). A positive correlation between post-stress cortisol levels and CSI was observed, supporting an association between enhanced cortisol response and myocardial remodeling. In conclusion, post-stress cortisol production correlates with myocardial remodeling, and coincides with several indicators of heart pathology, well-known from mammalian cardiology.

Introduction

The salmonid heart demonstrates a high degree of plasticity, both anatomically and physiologically, in response to environmental changes (Gamperl and Farrell, 2004). It is composed of an inner spongious myocardium which is supplied with oxygen from venous blood returning to the heart, and an outer compact myocardium that contains coronary blood vessels delivering oxygenated blood from the gills (Pieperhoff et al., 2009). Plastic changes can involve cardiomyocyte hypertrophy and hyperplasia of both compartments (Gamperl and Farrell, 2004), and this is associated with an increased risk of myocardial remodeling and dysfunction, which is an increasing problem in farmed salmonids (Poppe and Taksdal, 2000; Poppe et al., 2002; Poppe et al., 2003; Takle et al., 2006). Myocardial dysfunction is also a problem in other farmed animals, including broiler chickens, where myocardial remodeling

and failure is a leading cause of death (Olkowski et al., 1996). The underlying causes of pathological remodeling in fish have not been determined.

Stress can be defined as a condition in which a threat to the biological functions of an organism is perceived by that organism and a set of physiological and behavioral responses are mounted to counteract this challenge. Severe stress is clearly associated with a poor prognosis in individuals with established cardiac pathology and disease, including in humans (Engel, 1971; Meerson, 1994; Maxwell and Robertson, 1998; Brocklebank and Raverty, 2002; Poppe et al., 2007). Still, the mechanism linking stress-responsiveness to the development of cardiac disease is poorly understood in fish. In particular, the association between stress and cardiac remodeling has previously not been addressed in salmonid fish, of which approximately 2 million tons (2008) are processed per year in the rapidly developing global aquaculture industry (Food and Agriculture Organization, 2010).

Cortisol is the major steroid stress hormone in salmonid fishes and humans. It has diverse effects on several tissues, including the myocardium. The effects of cortisol in salmonid fish are mediated through both the mineralcorticoid receptor (MR) and the glucocorticoid receptors 1 and 2 (GR1 and GR2) (Colombe et al., 2000; Bury et al., 2003). Previous work has shown these receptors to be abundantly expressed in the myocardium of teleosts, including the rainbow trout (Greenwood et al., 2003; Sturm et al., 2005). However, a direct relationship between cortisol stress response and myocardial morphology and function has previously not been addressed. Of note, in contrast to mammals, where aldosterone is an important hormone in myocardial remodeling (Funder, 2001; Rocha et al., 2002; Qin et al., 2003), most teleosts, including salmonids, do not produce aldosterone (Bern, 1967; Sangalang and Uthe, 1994), and mineralcorticoid functions are instead mediated by cortisol (Bern and Madsen, 1992; Wendelaar Bonga, 1997). Furthermore, in mammals, glucocorticoids like cortisol directly induce protein synthesis and hypertrophy of cardiomyocytes *in vivo* and *in vitro* (Nichols et al., 1984; Lumbers et al., 2005), and plasma cortisol levels have been found to represent an independent risk factor of cardiac events and death (Yamaji et al., 2009).

Accordingly, to examine the effect of stress and cortisol on myocardial morphology and function, we have examined cardiac structure and gene expression in the hearts of two genetically distinct strains of rainbow trout (*Oncorhynchus mykiss)* that respond to stress with either a high (high responsive, HR) or low (low responsive, LR) cortisol production (Pottinger and Carrick, 1999). We hypothesized that adult HR fish, consistently responding to stress with high serum cortisol, would have bigger hearts than LR fish. However, as cardiac

remodeling may either be physiological or pathological in mammals, we also aimed to assess if stress-responsiveness and hence consistently different cortisol exposure throughout life, would induce signs of pathology in fish. We were especially interested in genes mediating the response to cortisol, particularly those that are linked to vascularization, fibrosis, and cardiac hypertrophy. Finally, we investigated if a trait correlation of post-stress cortisol response and heart size also existed outside of the selected LR- and HR trout lines. To this end, we examined heart size and studied cortisol-responsiveness in wild-type European brown trout (*Salmo trutta*).

Materials and methods

High and low responsive strains of rainbow trout

The selection regime initiated at Windermere Laboratory, NERC Institute of Freshwater Technology, UK, generating the two lines of rainbow trout (LR and HR), has been described in detail (Pottinger and Carrick, 1999). In short, the parental generation of HR and LR fish was established on the basis of consistent divergence in plasma cortisol responses following repeated stress testing (3 hours of confinement stress once a month for five executive months). The crosses between the selected parents were carried out and the F1 generation hatched during 1997. The F4 generation of LR and HR rainbow trout was hatched in spring 2006, and transported to the Norwegian Institute for Water Research (NIVA), Marine Research Station (Solbergstrand, Norway) in December 2007. The fish were then mixed and reared in two 1000 L fiberglass tanks until sampled as fully adult sexually mature individuals two years later (January 2010). At the time of sampling all LR and HR trout were 40 months old and had an average body length of 47.5 ± 5.3 and 49.2 ± 4.8 cm (mean ± s.d.), respectively. Only mature females were available for this study. The transport and study procedures of the experimental animals were reviewed and approved by the Norwegian Food Safety Authority (www.mattilsynet.no) and the Norwegian Animal Research Authority (http://www.fdu.no/fdu/), respectively.

Confinement stress

Female European brown trout hatched at Aqua Center Boračko Lake, Konjic, Bosnia & Herzegovina were used for these experiments. The parent generation was wild adult endemic brown trout caught in the river Neretva drainage. At the onset of the experiment, the female fish were 21 months old and had an average body length of 25.9 ± 1.7 cm (mean ± s.d.). Brown trout were transferred to 50 L aquaria and light was provided by overhead windows.

Light tubes situated 40 cm above each aquarium were turned on 15 minutes after sunrise every day to provide additional light for behavioral observations. The artificial light was turned off 15 minutes prior to sunset. During the experimental period (March 10–June 25) photoperiod increased by 10 minutes per day at the experimental location (Boračko lake). The fish were hand fed commercial food pellets daily. On day 16 the experimental fish were subjected to standardized confinement stress essentially as described by Øverli et al. (2006). In brief, fish were placed in pierced 1.5 L or 1.9 L plastic boxes (Gefrier Box Frosty, PLAST TEAM GmbH, Flensburg, Germany) adjusted to the size of the fish (1.5 L boxes for fish ranging from 20 – 25 cm and 1.9 L boxes for fish ranging from 25 – 30 cm). The boxes were submerged in water in the aquarium for 2 hours. The experimental model was approved by the State Veterinary Office of Bosnia and Herzegovina (http://www.vet.gov.ba).

Sampling and imaging of trout hearts

HR and LR trout were anaesthetized in benzocaine (Benzoak®, A.C.D. SA, Braine-L′allerud, Belgium) (1.5 ml/L) and body length was measured (cm). Three LR and three HR hearts were surgically excised from the fish, the bulbus and atrium removed, and the ventricles placed in 4% PFA (Electron Microscopy Sciences, Hatfield, PA, USA) for histochemistry analysis. Six LR and six HR hearts were sampled for gene expression analysis and the ventricle was weighed before being cut into smaller pieces, that were put on RNA later® (Ambion, Austin, TX, USA) and stored at -20°C.

Following confinement stress, 27 brown trout were anaesthetized in benzocaine (1.5 ml/L) and length was measured (cm). A blood sample was taken and the blood was immediately spun (3 minutes at 14000 rpm). Plasma was frozen and stored at -20°C for subsequent cortisol analysis. The hearts were excised and the atrium and bulbus removed before the ventricles were blotted dry and weighed (g). The cardiosomatic index (CSI) was determined by calculating the ratio of ventricle weight to fish length (g/cm). Body length, rather than body weight, was used to calculate CSI since several fish had running eggs upon netting from the holding tank. Images were taken of the ventricles using a Canon EOS350 digital camera (Canon, Tokyo, Japan) and processed in Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA).

Cortisol analysis

Plasma cortisol levels from the brown trout were analyzed using a specific radioimmunoassay (RIA) essentially as described earlier by Pottinger and Carrick (2001). In short, ethyl acetate (Merck Chemicals, Darmstadt, Germany) was used for cortisol extraction. 60 Ci/mmol [1,2,6,7 – ^{3}H] cortisol (Amersham Pharmacia Biotech, Little Chalfront, UK) was added to all samples, standards and controls before the donkey anti-cortisol antibody was added (1:6000 dilution, AbD Serotec, Dusseldorf , Germany). Samples were tested against a standard curve made with inert cortisol (Sigma Aldrich, St. Louis, MO, USA) in scintillation fluid (Ultima Gold, Perkin Elmer, Waltham, MA, USA) on a Packard Tri-Carb A1900 TR liquid scintillation analyzer (Packard Instrument, Meriden, CT, USA). Cortisol concentrations were calculated from the equation of a 3-parameter hyperbolic function fitted to a plot of the percentage of [^{3}H] cortisol bound against inert cortisol using Sigmaplot 11 (SPSS Science, Systat Software Inc, San Jose, CA, USA).

Histochemical AFOG-staining and imaging

Rainbow trout hearts stored in 4% PFA were cut into thick slices using a razor blade. These slices were further fixed in fresh 4% PFA at 4°C before embedding in paraffin. Dewaxed sections of 5 µm thickness were subjected to acid-fuchsin-orange G (AFOG) staining. AFOG sections were scanned or micrographed using Axioscope with a 5X objective (Zeiss, Germany), and images were processed in Adobe Photoshop CS3.

Calculation of compact myocardium area

Scanned AFOG-stained ventricular sections from rainbow trout hearts were processed using ImageJ (NIH, Bethesda, MD, USA). In brief, color images were converted into 8-bit grayscale, pixels scaled to mm, and brightness and contrast adjusted before edge detection was applied, allowing area calculation in mm^2 of the two muscular layers; the compact and spongious myocardium. Area of compact myocardium was divided by total area, giving the relative area of compact myocardium in percentage.

RNA extraction and qRT-PCR analysis

The hearts stored in RNA later were thawed and refrozen in liquid nitrogen before freeze-fractured in a BioPulverizer (Biospec products, inc., Bartlessville, OK, USA). The pulverized heart was thoroughly mixed before 280 mg were put into a 15 ml plastic tube and stored at -80°C for a maximum of four days. RNA was extracted from heart tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer`s protocol. The

quality and quantity of the RNA was assessed using 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), respectively. RNA quality was determined from RNA integrity numbers (RINs) calculated by the 2100 Bioanalyzer (range: 1-10). RINs for the heart samples ranged from 9.50 to 9.90 with an average of 9.7 ± 0.03 (mean ± s.e.m.), confirming excellent RNA quality. First strand cDNA was synthesized from 2 ng DNase I (DNA-free™ Kit, Ambion Applied Biosystems)-treated total RNA using Superscript III reverse transcriptase (Invitrogen) with oligo dT_{12-18} primers synthesized by Invitrogen.

The selected cardiac marker genes used in the current study are presented in Table 1. Gene-specific primers for rainbow trout β-actin, proliferating cell nuclear antigen (PCNA), ventricular myosin heavy chain (VMHC), slow myosin light chain 2 (SMLC2), muscle LIM protein (MLP, also called CRP), regulator of calcineurin 1 (RCAN1, also called MCIP), mineralcorticoid receptor (MR), glucocorticoid receptor 1 and 2 (GR1 and GR2), vascular endothelial growth factor (VEGF), collagen alpha 2 (1) (COL1a2), collagen alpha 1 (1) (COL1a1), A-type natriuretic peptide (ANP), and B-type natriuretic peptide (BNP), were designed using the web-based Primer3 program (http://frodo.wi.mit.edu/***cgi bin***/primer3/primer3_www.cgi), and synthesized by Invitrogen. The housekeeping gene β-actin was chosen as the internal control gene. GenBank accession numbers for the genes whose sequences were retrieved from NCBI (www.ncbi.nlm.nih.gov/) are listed in Table 1. Rainbow trout RCAN1 was retrieved at the dfci database (http://compbio.dfci.harvard.edu) based on a BLAST with *Danio rerio* RCAN1 found in the NCBI database (GenBank accession no. BC076439.1). A minimum of five primer pairs was designed at exon junctions for each gene and the primers showing the lowest Cp values, a single peak melting curve and amplification of the right amplicon were chosen and are listed in Table 1. The real-time PCR products were also sequenced to verify that the primers amplified the right cDNA.

Statistics

Data are expressed at single observations points or group mean ± s.e.m. Average CSI of LR fish was normalized to 100%. mRNA levels and differences in CSI and area of compact myocardium between the HR and LR groups were examined by the Student's *t* test, while the association between CSI and post-stress plasma cortisol was assessed by linear regression analysis (least squares method) with Pearson's product moment correlation coefficient as a measure of the resulting linear relationship (R^2 and p). mRNA expression levels are presented

as normalized values to LR average (fold change), and differences were tested using an unpaired t-test. P-values <0.05 were considered statistically significant. All statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Table 1. Specific marker genes with primers used for qt-RT-PCR

Gene	Primer pairs:	GenBank accession numbers:	Function/Marker of:
β-actin	F:AGCCCTCCTTCCTCGGTAT R:AGAGGTGATCTCCTTGTGCATC	NM001124235.1	Housekeeping gene
PCNA	F:AGCAATGTGGACAAGGAGGA R:GGGCTATCTTGTACTCCACCA	EZ763721.1	Cardiomyocyte hyperplasia
VMHC	F:TGCTGATGCAATCAAAGGAA R:GGAACTTGCCCAGATGGTT	AY009126.1	Cardiomyocyte hypertrophy
SMLC2	F:TCTCAGGCGGACAAGTTCA R:CGTAGCACAGGTTCTTGTAGTCC	NM001124678.1	Cardiomyocyte hypertrophy
MLP	F:AGTTCGGGGACTCGGATAAG R:CGCCATCTTTCTCTGTCTGG	NM001124725.1	Cardiomyocyte hypertrophy
RCAN1	F:AGTTTCCGGCGTGTGAGA R:GGGGACTGCCTATGAGGAC	BC076439.1 (*D. Rerio*)*	NFAT-activity/pathological cardiomyocyte hypertrophy
MR	F:CAGCGTTTGAGGAGATGAGA R:CCACCTTCAGAGCCTGAGAC	AY495581.1	Cortisol sensitivity
GR1	F:AGGTTGTCTCAGCCGTCAAA R:GCAGCTTCATCCTCTCATCAT	NM001124730.1	Cortisol sensitivity
GR2	F:ACTCCATGCACGAGATGGTT R:CGGTAGCACCACACAGTCAT	NM001124482.1	Cortisol sensitivity
VEGF	F:AGTGTGTCCCCACGGAAA R:TGCTTTAACTTCTGGCTTTGG	AJ717301.1	Angiogenesis
COL1a2	F:GGTTCGGCGAGACCATTA R:GTTGTGTGGCCATGCTCTG	NM001124207.1	Fibrosis
COL1a1	F:CGCTTCACATACAGCGTCAC R:AATGCCAAATTCCTGATTGG	NM001124177.1	Fibrosis
ANP	F:CCACAGAGGCTCTCAGACG R:ATGCGGTCCATCCTAGCTC	NM001124211.1	Heart failure
BNP	F:TGGCCTTGTTCTCCTGTTCT R:GGAGACTCGCTCAACCTCAC	NM001124226.1	Heart failure

F: Forward primer 5`→3`; R: Reverse primer 5`→3`. For full gene names, see List of symbols and abbreviations. * The rainbow trout RCAN1 sequence was found by BLAST with *Danio rerio* RCAN1 (see Materials and methods for details).

Results

HR fish have larger ventricles and compact myocardium than LR fish

To examine the relationship between selection for post-stress plasma cortisol levels and heart size, freshly excised ventricles from adult HR and LR fish were weighed and CSI calculated. The CSI of HR fish was 33.70±6.26% higher than that of LR fish (100.00±7.67%; p=0.0035; Fig.1A, B). To evaluate the structural basis for the increased relative heart size of HR fish, sections of the ventricles were converted into grey-tone images to distinguish the compact and spongious muscular layers. The relative area of compact myocardium in HR ventricles was 48.40±2.95% while that of LR fish was 33.40±2.44% (p=0.0173; Fig.1C-D).

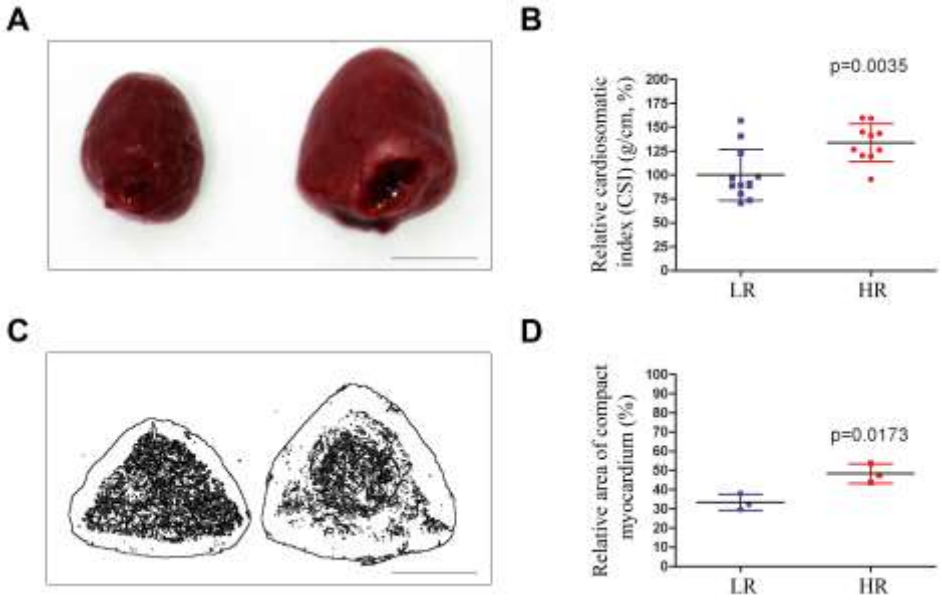

Fig. 1 HR fish have bigger ventricles and more compact myocardium than LR fish
Representative image of ventricles of size-matched (47 cm long) LR (left) and HR (right) fish. Scale bar = 1 cm (**A**). Relative cardiosomatic index (CSI) (g/cm) of LR and HR fish (n=12/blue and 10/red, respectively) shown as one graphical point per fish and as mean ± s.e.m in % relative to LR (**B**). Representative image of ventricular sections of size-matched (47 cm long) LR (left) and HR (right) fish converted to black-and-white, showing the outer compact and the inner spongious myocardium. Scale bar = 1 cm (**C**). Area of compact myocardium relative to spongious myocardium (%) in LR (blue) and HR (red) ventricle sections shown as one graphical point per fish and as mean ± s.e.m, n=3 (**D**). Statistical differences were tested using an unpaired t-test and p-values are indicated.

Markers of cardiomyocyte hypertrophy are upregulated in ventricles of HR fish

To evaluate if the increased heart size in the HR fish was a result of myocyte hypertrophy or hyperplasia, markers of the two processes were measured by qRT-PCR. There was a 1.39±0.15-fold higher PCNA expression in HR ventricles compared to LR (1.00±0.12), however this result was not statistically significant (p=0.07; Fig.2A). In contrast, there was 2.40±0.29-fold increased expression of VMHC in HR compared to LR ventricles (1.00±0.14; p=0.0015) and a 3.31±0.73-fold increase of SMLC2 in HR compared to LR ventricles (1.00±0.40; p=0.0194; Fig.2B-C). There was also a 1.93±0.41-fold higher MLP expression in HR ventricles than in LR ventricles (1.00±0.23), however this finding was not significant (p=0.0763; Fig.2D).

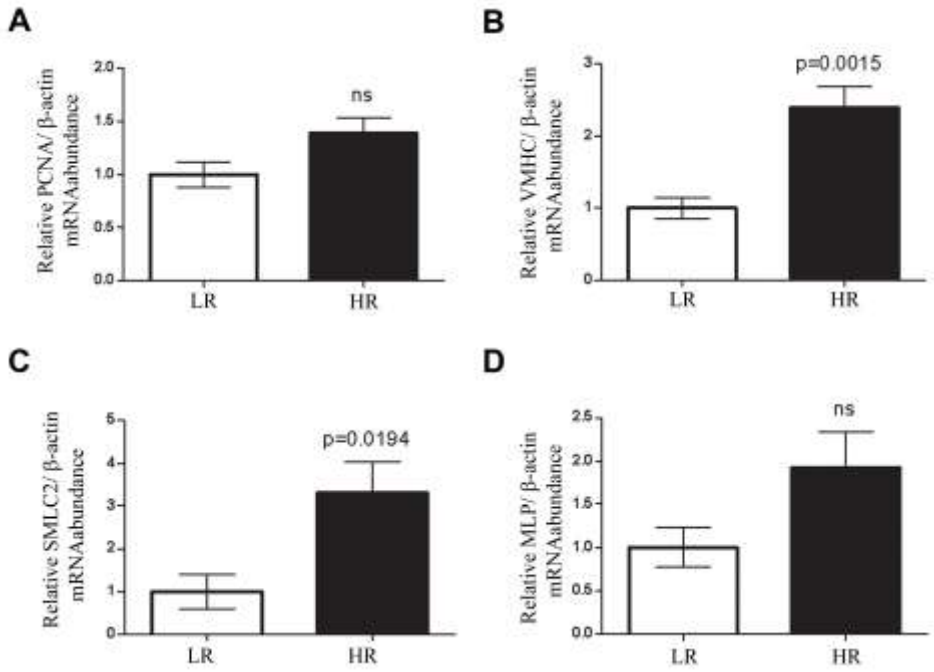

Fig. 2 Markers of hypertrophy, but not of hyperplasia, are upregulated in ventricles of HR fish
Relative mRNA expression levels of proliferating cell nuclear antigen (PCNA) (**A**) and of the hypertrophic markers ventricular myosin heavy chain (VMHC) (**B**), slow myosin light chain 2 (SMLC2) (**C**) and muscle LIM protein (MLP) (**D**) relative to the standard gene β-actin in HR and LR ventricles, presented as mean ± s.e.m. relative to LR expression, n=6. Statistical differences were tested using an unpaired t-test and p-values are indicated. ns = non significant.

Pro-hypertrophic NFAT signaling is upregulated in the HR fish ventricle

To further examine the cardiac hypertrophy of the HR fish, we assessed if the calcineurin-NFAT pathway was activated, as this signaling pathway is one of the major pathways involved in mammalian pathological hypertrophy. mRNA expression level of the NFAT target gene RCAN1 was 2.96±0.38-fold upregulated in HR ventricles compared to LR (1.00±0.14; p=0.0007; Fig.3).

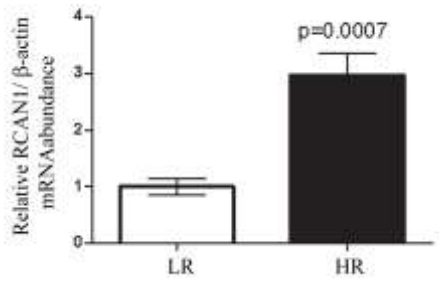

Fig. 3 Pro-hypertrophic NFAT signaling is upregulated in the HR fish ventricle
mRNA expression level of the nuclear factor of activated T-cell (NFAT)-regulated regulator of calcineurin 1 (RCAN1) gene relative to the standard gene β-actin in HR and LR ventricles, presented as mean ± s.e.m. relative to LR expression, n=6. Statistical difference was tested using an unpaired t-test and the p-value is indicated.

Vascularization is increased in the HR fish ventricle

Visual investigation of surface vessels of HR and LR ventricles clearly revealed a higher degree of vascularization of HR ventricles (representative image in Fig. 4A), but this

difference was not quantified. In line with this observation, VEGF expression was 2.66±0.19-fold greater in HR ventricles compared to LR ventricles (1.00±0.14; p<0.0001; Fig.4B).

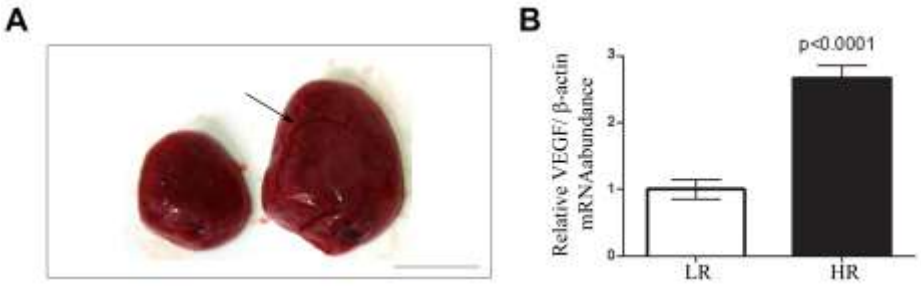

Fig. 4 Vascularization is increased in the HR fish ventricle
Representative image of ventricles of size-matched (47 cm long) LR (left) and HR (right) fish. Extensive vascularization of the HR ventricle is pointed out by an arrow. Scale bar = 1 cm, LR n=12, HR n=10 (**A**). mRNA expression level of the angiogenic marker vascular endothelial growth factor (VEGF) relative to the standard gene β-actin in HR and LR ventricles, presented as mean ± s.e.m. relative to LR expression, n=6 (**B**). Statistical difference was tested using an unpaired t-test and the p-value is indicated.

Increased collagen deposition and expression indicates enhanced fibrosis in the ventricles of HR fish

Excessive fibrosis is a hallmark of pathological myocardial remodeling in mammals, and to examine fibrosis in the trout hearts we stained ventricular sections histochemically with a marker for collagen depositions (AFOG) and measured Col 1a2 and Col 1a1 mRNA expression levels. There was moderate staining for collagen in all examined ventricles of HR fish (n=3), while no staining was observed in the LR ventricles (n=3; Fig. 5A left panel). Magnification of the HR ventricle revealed areas with (Fig. 5A, right panel 1) or without (Fig. 5A, right panel 2) collagen depositions, indicating focal fibrosis and disruption of muscle structure in the HR heart. Further supporting enhanced fibrosis in HR hearts, COL1a2 mRNA levels were 2.16±0.36-fold increased in HR ventricles compared to LR (1.00±0.21; p=0.0207; Fig.5B). We also observed a 1.84±0.39-fold, but not statistically significant, increase in COL1a1 expression in HR ventricles compared to LR (1.00±0.17; p=0.0770; Fig.5C).

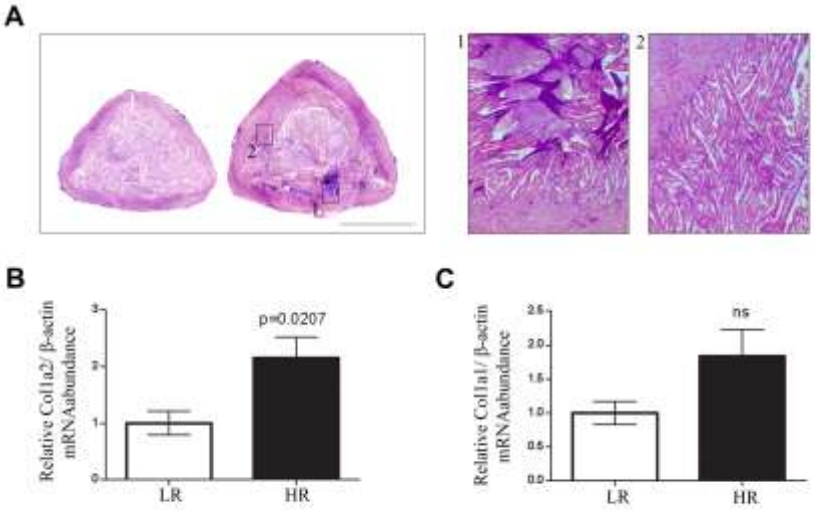

Fig.5 Increased collagen deposition and expression in ventricles of HR fish
Representative image of acid fuchsin-orange G (AFOG)-stained ventricular sections of size-matched (47 cm long) LR (left) and HR (right) fish (n=3) (**A** left panel). AFOG staining is highly sensitive for collagen depositions (fibrin = red/purple; collagen = blue). Scale bar = 1 cm. Magnification of areas in the HR ventricle with (1) or without (2) collagen depositions (**A** right panels). mRNA expression level of the fibrotic markers collagen alpha 2(1) (COL1a2) (**B**) and collagen alpha 1(1) (COL1a1) (**C**) relative to the standard gene β-actin in HR and LR ventricles, presented as mean ± s.e.m. relative to LR expression, n=6. Statistical differences were tested using an unpaired t-test and the p-values are indicated. ns = non significant.

ANP and BNP mRNA levels are not increased in ventricles of HR fish

To assess further signs of remodeling in HR hearts, we measured expression of ANP and BNP, which are sensitive markers of heart failure in mammals. We found no differences in either ANP (HR: 1.35±0.26 vs. LR: 1.00±0.30; p=0.4019; Fig.6A), nor BNP mRNA expression levels (HR: 0.90±0.10 vs. LR: 1.00±0.17; p=0.6401; Fig.6B).

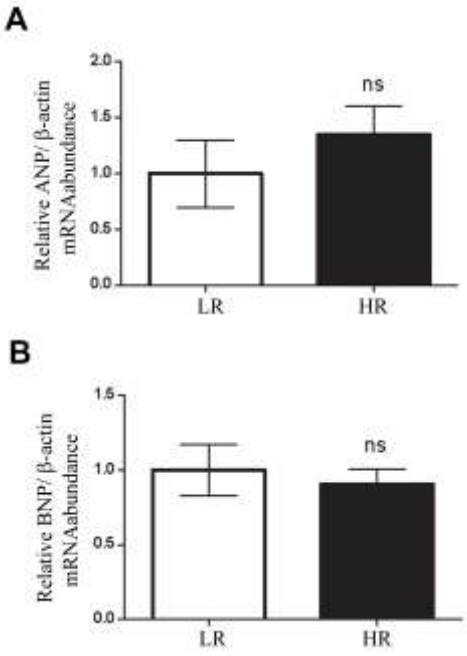

Fig. 6 No increase in the heart failure biomarkers ANP and BNP in ventricles from HR fish
mRNA expression level of the heart failure markers A-type natriuretic peptide (ANP) (**A**) and B-type natriuretic peptide (BNP) (**B**) relative to the standard gene β-actin in HR and LR ventricles, presented as mean ± s.e.m. relative to LR expression, n=6. Statistical differences were tested using an unpaired t-test, ns = non significant.

Myocardial cortisol receptors are increased in the ventricles of HR fish

To assess whether differences in post-stress cortisol levels are accompanied by differences in the expression of myocardial cortisol receptors, we measured MR, GR1, and GR2 mRNA levels in ventricles of HR and LR fish. HR fish, which consistently respond to stress with high plasma cortisol (Pottinger and Carrick, 1999, 2001; Trenzado et al., 2003; Schjolden et al., 2006), displayed increased levels of all three receptors in the ventricle compared to LR fish (Fig.7A-C). In more detail, expression level of MR was 2.08±0.24-fold higher in HR than in LR ventricles (1.00±0.05; p=0.0012; Fig.7A), that of GR1 was 2.36±0.24-fold higher (1.00±0.09; p=0.0003; Fig.7B) and that of GR2 was 2.19±0.10-fold higher (1.00±0.08; p<0.0001; Fig.7C).

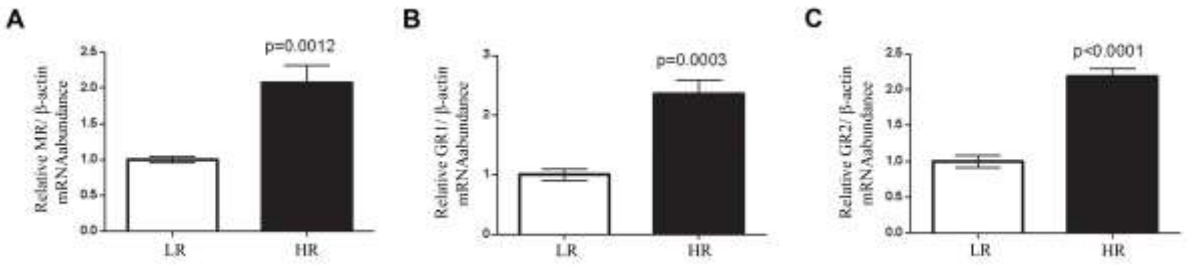

Fig. 7 Cortisol receptors are upregulated in ventricles of HR fish
mRNA expression level of mineralcorticoid receptor (MR) (**A**), glucocorticoid receptor 1(GR1) (**B**) and 2 (GR2) (**C**) relative to the standard gene β-actin in HR and LR ventricles, presented as mean ± s.e.m. relative to LR expression, n=6. Statistical differences were tested using an unpaired t-test and p-values are indicated.

Post-stress cortisol response is positively correlated to CSI in wild-type brown trout

Finally, to validate our results from the HR and LR selection model in wild-type trout, plasma cortisol levels were measured and CSI calculated in a European brown trout population in an established stress model (acute confinement stress). There was a positive correlation between CSI and post-confinement plasma cortisol in these wild-type fish (n=27; R^2=0.347; p=0.0012; Fig.8), supporting our results from the HR and LR animals of an association between stress-responsiveness and myocardial remodeling.

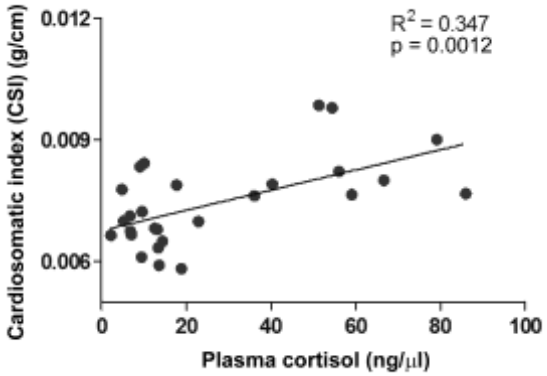

Fig. 8 Post-stress cortisol response is positively correlated to cardiosomatic index in wild-type brown trout

Post-stress plasma cortisol (ng/µl) was measured after confinement of wild-type brown trout (n=27) and correlated to cardiosomatic index (CSI) (g heart/cm body length) of each individual fish (one point represents one fish). Regression line slope statistically different from zero is indicated by p-value. The statistical analysis was performed using linear regression analysis (least squares method) with Pearson's product moment correlation coefficient as a measure of the resulting linear relationship (R^2 and p values).

Discussion

Our results show that fish responding to stress with high post-stress plasma cortisol levels have significantly bigger hearts than fish responding with low cortisol, and this phenomenon was observed in both a genetic selection model of rainbow trout and in wild-type brown trout. Immunohistochemical studies suggested that this was a result of growth of the compact myocardium, as this area, relative to the spongious myocardium, was bigger in HR ventricles compared LR ventricles. Such an increase of compact myocardium is also seen during ontogenetic growth in salmonid fishes, during which the compact layer increases in both amount and thickness (Poupa et al., 1974; Farrell et al., 1988).

In mammals, cardiomyocytes may under certain physiological (i.e. training or pregnancy) or pathological (i.e. hypertension or myocardial infarction) conditions undergo hypertrophy leading to increased muscular mass. Although recent findings suggest that cardiomyocytes within the diseased adult human heart can proliferate (Beltrami et al., 2001), most evidence to date indicates that myocyte proliferation is not a significant component of the mammalian cardiac growth response (Pasumarthi and Field, 2002). In adult teleostean hearts, including in rainbow trout, previous studies have shown that ventricular growth occurs through both hyperplasia and hypertrophy (Farrell et al., 1988; Clark and Rodnick, 1998; Poss

et al., 2002). In the current study, there was a consistent upregulation of genes involved in the hypertrophic gene program. For instance, mRNA levels of commonly used hypertrophy markers in mammals (RCAN1, SMLC2 and the fish homologue of β-MHC, VMHC) (Swynghedauw, 1999; Lim et al., 2001) were significantly higher in HR compared to LR ventricles. Furthermore, although the hypertrophy marker MLP did not differ significantly between LR and HR ventricles, it displayed a trend towards being increased in the HR ventricles. We also found a trend towards increased expression of the proliferation marker PCNA (Yu et al., 1992; Köhler et al., 2005), indicating increased cell proliferation in the HR ventricles compared to LR ventricles. Nevertheless, based on the combined gene expression profile of the current study, we believe that the bigger relative heart size in fish responding to stress with high plasma cortisol is primarily due to hypertrophy of existing compact cardiomyocytes. In cold-acclimated rainbow trout, Vornanen et al. (2005) observed an increase in heart size of 33%. This heart growth was, similar to the results of our study, associated with increased expression of VMHC and SMLC2. Furthermore, they reported a 5-fold increase in the expression of MLP mRNA. The authors interpreted these results as an activation of genes associated with adaptive cardiac hypertrophy without the pathological features that impair the function of the hypertrophic mammalian heart. Fish hearts can undergo hypertrophy (and hyperplasia) as a routine remodeling mechanism (Farrell et al., 1988). Therefore, as opposed to mammals, where cardiac hypertrophy is often associated with a failing heart (Lloyd-Jones et al., 2002), a bigger fish heart is not necessarily detrimental. For example, an increased ventricular mass in cold-acclimated rainbow trout serves to offset the reduction in cardiac power output observed with acute decreases in temperature and also enhances stroke volume (Graham and Farrell, 1989). Also, in the growing eel (*Anguilla anguilla L*) structural remodeling of the heart, such as growth of the compact myocardium, is associated with enhanced mechanical performance (Cerra et al., 2004).

The calcium/calcineurin-regulated NFATc family is thought to have arisen following recombination about 500 million years ago, producing a new group of signaling and transcription factors (the NFATc genes) found only in genomes of vertebrates. It has been proposed that the recombination enabled Ca^{2+} signals to be redirected to a new transcriptional program, which provided part of the groundwork for vertebrate morphogenesis and organogenesis, including the cardiovascular system (Wu et al., 2007). Calcineurin-NFAT signaling was first shown by Molkentin et al. (1998) to be important in development of cardiac hypertrophy and today extensive evidence exist showing that this intracellular

signaling pathway is essential, and activated in pathological hypertrophy only (Wilkins et al., 2004). As the NFATc proteins are transcription factors, their activity can be measured by expression of target genes. RCAN1 is known to be a direct NFATc target in the heart (Rothermel et al., 2003; Oh et al., 2010). In this study we show that HR hearts, which are bigger than LR hearts mainly due to increased hypertrophy of the compact myocardium, have almost 3-fold higher RCAN1 expression. Such an increase in RCAN1 expression indicates increased NFAT signaling and hence that the growth of HR hearts has a pathological character. Alternatively, NFAT activation in fish may serve a more physiological role given their adaptive hypertrophic potential. To our knowledge, this is the first time NFAT activation, well-known in the hypertrophic mammalian heart, is shown to occur in fish. Our results thus imply that NFAT activation in cardiac hypertrophy is evolutionary conserved between mammals and fish, indicating that fish can serve as models with biomedical relevance.

In mammals, catecholamines (CAs) are important mediators of stress-related cardiac remodeling as they induce myocardial hypertrophy and cardiomyocyte death. In fish, though, the effects of CAs on the myocardium seem less pronounced (Tota et al., 2010). As it has been shown that LR fish respond to stress with higher plasma levels of the CA epinephrine than HR fish (Schjolden and Winberg, 2007), and the HR hearts were bigger than LR despite lower post-stress plasma CA, we propose that cortisol constitutes the main stress-induced influence on the trout heart.

We found a higher expression of the angiogenic gene VEGF in the HR ventricles compared to LR, which likely reflects increased vascularization necessary to support the thicker layer of compact myocardium (Cerra et al., 2004), as this is the muscular layer vascularized by coronary vessels. Increased vascularization was indeed extensive when visually examining the HR ventricles.

The fibrillar collagen network serves as an elastic element in the heart. Structural remodeling of this network is, however, associated with pathological conditions such as fibrosis (Abrahams et al., 1987; Caulfield and Bittner, 1988). Fibrosis is the dominant histological reaction to injury in the mammalian heart and adversely increases tissue stiffness and impairs normal cardiac function (Schnitt et al., 1993; Weber et al., 1994). In fish, though, collagen remodeling has been postulated to play an important role in the maintenance of the mechanical cardiac performance (Cerra et al., 2004; Icardo et al., 2005). We also find enhanced collagen deposition in the HR ventricles compared to LR as evaluated by AFOG-

staining. Still, as the collagen depositions were non-homogenously distributed, revealing fibrosis and disruption of muscle structure in certain areas (see Fig. 5A), we believe this may still represent pathological collagen production, similar to what is found during pathological focal fibrosis in mammals. In adaptive cardiac hypertrophy in trout, however, collagen gene expression was reduced approximately 5-fold (Vornanen et al., 2005). Thus, a higher expression of COL1a2 and a trend towards higher COL1a1 in the HR hearts compared to LR, as observed in our study, indicates a different response than in adaptive cold-acclimation, and could be interpreted as related to pathological fibrosis.

Because expression of genes regulating hypertrophy and fibrosis could indicate pathological myocardial remodeling in the HR trout, we wanted to investigate the expression of ANP and BNP, which are commonly used markers of heart failure in mammals (Lerman et al., 1993; Maisel et al., 2002). Generally, little data is available concerning secretion of natriuretic peptides associated with cardiac growth and remodeling in non-mammalian species (Tota et al., 2010) and the regulation and/or function of ANP and BNP can be different in fish and mammals (Loretz and Pollina, 2000). For instance, the interplay between CA and natriuretic peptides seems to differ. As LR fish respond to stress with higher plasma CA (Schjolden and Winberg, 2007), this could have predicted differences in ANP and BNP production. Yet, the expression of ANP and BNP did not differ between LR and HR ventricles. Alternatively, assuming a similar upregulation of natriuretic peptides with heart failure in fish such as in mammals, our results indicate that the HR fish had not developed heart failure despite the indications of pathological remodeling.

A limiting factor determining the sensitivity of a cell to glucocorticoids is the intracellular concentration of glucocorticoid receptors (Vanderbilt et al., 1987; Dong et al., 1989). Hence, altering the expression of these receptors renders a tissue capable of adjusting the biological response according to the requirements of the environment. In rainbow trout, cortisol actions are mediated through three different glucocorticoid receptors, GR1, GR2 and MR (Colombe et al., 2000; Bury et al., 2003). In the HR ventricles, expression of all three receptors was significantly higher than in the smaller LR ventricles. Thus, it is probable that increased cortisol receptor expression in HR fish makes them more sensitive to the actions of cortisol and that cortisol-dependent intracellular signaling is increased. In addition, these fish experience higher post-stress cortisol levels regularly throughout life, at least in stressful environments such as intensive aquaculture. This assumption implies a role for cortisol not

only in cardiac hypertrophy of fish, but also as one of the causative factors of pathological heart conditions in the fish farming industry.

In mammals, the mineralcorticoid aldosterone has been shown to induce fibrosis (Funder, 2001). Also, mineralcorticoid-induced cardiac hypertrophy is associated with increased NFAT-activity through increased calcineurin activity (Takeda et al., 2002; Tong et al., 2004). Salmonid fish, however, lack aldosterone (Sangalang and Uthe, 1994) and the main corticosteroid is thought to be cortisol, acting both as a mineralcorticoid and a glucocorticoid hormone (Bern and Madsen, 1992; Wendelaar Bonga, 1997). Thus, cortisol in fish might mimic the effect of aldosterone on the mammalian heart and induce fibrosis and increased NFAT-activity as reflected by increased RCAN1 expression in the current study.

In our study, the HR-LR selection model provided a tool to study the effects of transiently elevated cortisol levels. However, the HR and LR rainbow trout have been bred for several generations during the selection regime. Thus, founder effects, unique mutations or random genetic drift could in fact account for the observed divergence in CSI. Nevertheless, a positive correlation between cortisol-responsiveness following stress and relative heart size was revealed also in wild-type European brown trout. This finding suggests an evolutionary conserved correlation between heart size and cortisol-responsiveness and that this trait correlation is not an incidental artifact of the HR-LR selection regime. Indeed, a link between high cortisol-responsiveness to stress and increased cardiovascular morbidity and mortality has been reported also in humans (Pedersen and Denollet, 2003).

In conclusion, high post-stress cortisol levels seem to be associated with cardiac remodeling and altered gene expression in salmonid fish. While the significance of cortisol-related cardiac remodeling for the fish remains speculative, both hypertrophy (Lloyd-Jones et al., 2002) and high serum cortisol (Yamaji et al., 2009) have been identified as independent risk factors for adverse cardiac events in humans. Thus, a follow-up study should involve experiments to determine if HR fish have impaired cardiac function and are more prone to stress-induced mortality. In aquaculture there is increasing worry about stress-induced mortality of fish carrying cardiac anomalies (Brocklebank and Raverty, 2002; Poppe et al., 2007), and we here suggest that cortisol-induced cardiac myocardial remodeling may be one of the explanatory factors.

List of symbols and abbreviations

AFOG = acid-fuchsin-orange G

ANP = A-type natriuretic peptide

BNP = B-type natriuretic peptide

CA = catecholamine

COL1a1 = collagen alpha 1(1)

COL1a2 = collagen alpha 2(1)

CRP = cysteine rich protein

CSI = cardiosomatic index

Cp = crossing point

GR = glucocorticoid receptor

HR = high responder

LR = low responder

NFAT = nuclear factor of activated T-cell

MLP = muscle LIM protein

MCIP = modulatory calcineurin-interacting protein 1

MR = mineralcorticoid receptor

PCNA = proliferating cell nuclear antigen

PFA = paraform aldehyde

Qt-RT-PCR= quantitative real time- polymerase chain reaction

RCAN1 = regulator of calcineurin 1

RIA = Radioimmunoassay

SMLC2 = slow myosin light chain 2

VEGF = vascular endothelial growth factor

VMHC = ventricular myosin heavy chain

β-MHC = β-myosin heavy chain

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