© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 1077-1083 doi:10.1242/jeb.114314

RESEARCH ARTICLE

Natural selection constrains personality and brain gene expression differences in Atlantic salmon (*Salmo salar*)

Per-Ove Thörnqvist[1,*], Erik Höglund[2,3] and Svante Winberg[1]

ABSTRACT

In stream-spawning salmonid fishes there is a considerable variation in the timing of when fry leave the spawning nests and establish a feeding territory. The timing of emergence from spawning nests appears to be related to behavioural and physiological traits, e.g. early emerging fish are bolder and more aggressive. In the present study, emerging Atlantic salmon (*Salmo salar* L.) alevins were sorted into three fractions: early, intermediate and late emerging. At the parr stage, behaviour, stress responses, hindbrain monoaminergic activity and forebrain gene expression were explored in fish from the early and late emerging fractions (first and last 25%). The results show that when subjected to confinement stress, fish from the late emerging fraction respond with a larger activation of the brain serotonergic system than fish from the early fraction. Similarly, in late emerging fish, stress resulted in elevated expression of mRNA coding for serotonin 1A receptors (5-HT_{1A}), GABA-A receptor-associated protein and ependymin, effects not observed in fish from the early emerging fraction. Moreover, fish from the early emerging fraction displayed bolder behaviour than their late emerging littermates. Taken together, these results suggest that time of emergence, boldness and aggression are linked to each other, forming a behavioural syndrome in juvenile salmon. Differences in brain gene expression between early and late emerging salmon add further support to a relationship between stress coping style and timing of emergence. However, early and late emerging salmon do not appear to differ in hypothalamus–pituitary–interrenal (HPI) axis reactivity, another characteristic of divergent stress coping styles.

KEY WORDS: Animal personality, Behaviour, Boldness, Confinement stress, Hypoxia, Swim-up

INTRODUCTION

Intraspecific variability in behaviour has been described in numerous species. Moreover, behavioural traits are usually correlated, i.e. bold individuals are also aggressive whereas shy individuals are non-aggressive (Wilson et al., 1994). Thus, behavioural traits form divergent clusters, or behavioural profiles. An individual behavioural profile that is consistent over time and context is described as personality, temperament or behavioural syndrome, depending on the scientific discipline in which it is studied (Freeman and Gosling, 2010; Gosling, 2001; Gosling and John, 1999; Sih et al., 2004; Wilson et al., 1994). In addition, behavioural profiles are often correlated with physiological traits, a phenomenon that is referred to as stress coping style, as it is most obvious when an animal is challenged by a stressor (Koolhaas et al., 1999). Generally, individuals with a proactive coping style respond to a challenge with a high adrenaline (epinephrine) and low glucocorticoid release, and trigger a fight–flight behavioural response. They also show a propensity for social dominance and develop and follow routines easily (Basic et al., 2012; Koolhaas et al., 1999; Ruiz-Gomez et al., 2011). When reactive individuals are provoked, they show a freeze–hide response with a low adrenaline and high glucocorticoid release (Koolhaas et al., 1999; Schjolden and Winberg, 2007), avoiding danger by keeping a low profile. In addition, they show a tendency to be socially subordinate and to have a more flexible behaviour (Ruiz-Gomez et al., 2011; Koolhaas et al., 1999; Schjolden and Winberg, 2007).

In salmonid fishes, individual time of emergence of larvae from the spawning nest can vary by up to several weeks (Brännäs, 1995) and has been suggested to be of selective importance (Chandler and Bjornn, 1988; Einum and Fleming, 2000; Vaz-Serrano et al., 2011). There is a well-established relationship between suites of behavioural and physiological traits and the timing of this niche shift. Generally, larvae that emerge early are considered to be more aggressive and to have a propensity for social dominance (Metcalfe and Thorpe, 1992). This relationship has also been verified in studies of selected lines of rainbow trout (*Oncorhynchus mykiss*), where the line having a propensity for social dominance also emerged earlier from artificial spawning nests than the line having a propensity for social subordination (Åberg Andersson et al., 2013). Moreover, these lines, which were selected on post-stress plasma cortisol concentrations, the high responsive (HR) line showing high plasma cortisol and the low responsive (LR) line showing low plasma cortisol in response to confinement stress (Pottinger and Carrick, 1999), also differ in several other behavioural and physiological traits (Øverli et al., 2002; Schjolden et al., 2005a,b, 2006a,b; Schjolden and Winberg, 2007). The behavioural and physiological traits of the HR and LR trout seem to cluster in two distinct profiles, resembling the proactive and reactive stress coping style, respectively. Interestingly, these trout lines also differ in brain neurochemistry when exposed to stress, including differences in brain tissue levels of serotonin (5-hydroxytryptamine, 5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA) (Øverli et al., 2001; Schjolden et al., 2006b). The brain serotonergic system has been suggested to play an important role in stress coping and differences in central 5-HT function have previously been reported in rodents displaying divergent stress coping styles (Koolhaas et al., 1999).

In addition to 5-HT neurotransmission, brain gene expression also differs between LR and HR trout (Thomson et al., 2011; Johansen et al., 2011; Backström et al., 2011). When challenged by confinement, brain expression of calmodulin (CALM), ependymin (EPD1), gamma-aminobutyric acid receptor-associated protein

[1]Department of Neuroscience, Physiology Unit, Biomedical Centre (BMC), Uppsala University, Box 593, Uppsala SE-75124, Sweden. [2]National Institute of Aquatic Resources, Section for Aquaculture, Technical University of Denmark, PO Box 101, Hirtshals DK-9850, Denmark. [3]Research secretariat, University of Agder, 4630 Kristiansand, Norway.

*Author for correspondence (per-ove.thornqvist@neuro.uu.se)

Received 22 September 2014; Accepted 5 February 2015

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(GABARAP), major histocompatability complex class1 (MHC1), retinol binding protein (RETB1), vasotocin receptor (AVTR) (Thomson et al., 2011) and mineralcorticoid receptor (MR) are higher in LR than in HR trout. Differences in the expression of these genes, which are involved in stress responses, aggression and neuronal plasticity, could reflect differences in other brain functions.

Thus, divergent personality traits akin to what has been described as proactive and reactive stress coping styles appear to exist in salmonid fish, and the existence of this intraspecific divergence in coping style has also been indicated in wild populations (Brelin et al., 2008). Moreover, as suggested by differences in neurochemistry and brain gene expression, proactive and reactive coping styles appear to be related to divergent brain functions. However, recent results have questioned the relationship between these traits. Thomson et al. (2011) reported that there was no relationship between boldness and plasma cortisol in rainbow trout of the HR and LR strains. They found that even though HR and LR trout, as expected, differed in post-stress plasma cortisol, they did not differ in boldness. Individuals scoring high on boldness were found among both HR and LR fish, and there was no significant difference in the frequency of bold and shy fish between the HR and LR line. Moreover, brain gene expression differed between HR and LR trout, but there was no difference in either plasma cortisol or brain gene expression between trout classified as bold and shy. However, most of the reported neuroendocrine mechanisms underlying personality differences stem from laboratory studies in inbred lines (Koolhaas et al., 2010). The occurrence of a relationship between differences in personality and expression of genes involved in neural transmission in populations that undergo natural selection may reveal how evolutionary forces constrain individual behavioural variation in nature.

In salmonids, alevins emerging early and late from the redd appear to differ in behaviour and physiology, suggesting that they display divergent stress coping strategies. In the current study, offspring of Baltic salmon, *Salmo salar* L., that had undergone natural selection in a river for at least one generation were sorted according to time of emergence from the redd. As juveniles, these offspring were tested for boldness, neuroendocrine stress responses and brain gene expression. We report behavioural differences, as well as differences in brain neurochemistry and gene expression between early and late emerging Baltic salmon.

RESULTS

Plasma cortisol concentration

Stress had a significant effect on plasma cortisol concentration ($F_{1,22}$=78, P<0.001) but there was no effect of time of emergence ($F_{1,22}$=0.29, P<0.60) and no interaction between stress and time of emergence ($F_{1,22}$=0.20, P<0.65; control early emerging fraction (EE) 2.8±3.2 ng ml^{-1}; control late emerging fraction (LE) 1.9±2.4 ng ml^{-1}; stress EE 52.4±16.9 ng ml^{-1}, stress LE 52.3±8.6 ng ml^{-1}).

Forebrain gene expression

Time of emergence had a significant effect on forebrain serotonin 1Aα receptor (5-HT$_{1Aα}$) mRNA levels ($F_{1,23}$=9.8, P<0.01), with LE fish showing higher mRNA concentrations than EE fish (Table 1). Moreover, there was a significant interaction effect of time of emergence and stress on 5-HT$_{1Aα}$ mRNA levels ($F_{1,23}$=8.9, P<0.01). In LE fish, 5-HT$_{1Aα}$ increased with stress, an effect not seen in EE fish (P<0.05, Table 1). However, there was no effect of stress by itself on 5-HT$_{1Aα}$ mRNA concentration ($F_{1,23}$=0.39, P<0.50). By contrast, there was no effect of either stress ($F_{1,25}$=0.98, P<0.25) or time of emergence ($F_{1,25}$=1.2, P<0.40), and no interaction effect ($F_{1,25}$=3.0, P<0.10), on 5-HT$_{1Aβ}$ mRNA levels (Table 1).

Stress had a significant effect on the expression of CALM ($F_{1,25}$=6.5, P<0.02) and GABARAP ($F_{1,25}$=11, P<0.02), CALM being down-regulated while GABARAP was up-regulated by stress (Table 1). Moreover, there was a significant interaction effect of time of emergence and stress on forebrain GABARAP mRNA concentration ($F_{1,25}$=12, P<0.01), with only LE fish showing a significant up-regulation of GABARAP expression in response to stress (P<0.05, Table 1).

There was a significant interaction effect of stress and time of emergence on forebrain EPD1 mRNA expression ($F_{1,25}$=6.3, P<0.001), the *post hoc* analysis showing that stressed LE fish display significantly higher EPD1 mRNA levels than EE fish (P<0.05, Table 1). However, neither stress ($F_{1,25}$=0.04, P<0.90) nor time of emergence ($F_{1,25}$=2.7, P<0.20) had any effect on EPD1 mRNA expression (Table 1).

Table 1. Concentrations and ratios of monoamines in the hindbrain together with relative gene expression values in the forebrain of salmon sorted by emergence

				EE		LE	
	Emergence	Stress	Emergence×stress	Control	Stress	Control	Stress
5-HIAA	**0.049**	0.09	0.08	400±27	400±40	410±28	540±37
5-HT	0.14	**0.043**	1	560±39	740±85	680±74	890±140
5-HIAA/5-HT	0.82	0.48	0.14	0.72±0.05	0.58±0.07	0.61±0.07	0.66±0.08
DA	0.06	0.12	0.9	300±83	490±150	490±150	820±250
DOPAC	0.41	0.93	0.33	30±3.5	30±2.10	32±3.1	27±3.3
DOPAC/DA	0.053	0.082	0.56	0.13±0.02	0.08±0.02	0.08±0.01	0.06±0.02
NA	0.12	0.41	0.69	780±130	990±140	860±74	1000±150
CALM	0.7	**0.016**	0.09	1.0±0.27	0.79±0.06	1.3±0.27	0.56±0.04
EPD1	0.15	0.9	**0.001**	1.0±0.10^{a}	0.72±0.07^{a,b}	0.9±0.15^{a}	1.1±0.07^{a,c}
GABARAP	0.65	**0.012**	**0.003**	1.0±0.14^{a}	0.97±0.19^{a}	0.51±0.11^{a,b}	1.5±0.09^{a,c}
RBP1	0.47	0.3	**0.01**	1.0±0.14^{a}	1.50±0.15^{a}	1.5±0.15^{a}	1.1±0.07^{a}
5-HT$_{1Aα}$	**0.01**	0.5	**0.01**	1.0±0.09^{a}	0.94±0.04^{a,b}	1.35±0.46^{a}	3.1±0.5^{a,c}
5-HT$_{1Aβ}$	0.41	0.25	0.09	1.0±0.09^{a}	0.94±0.04^{a}	0.94±0.13^{a}	1.2±0.08^{a}

The fish were also subjected to 0 (non-disturbed controls) or 60 min of confinement stress. Values are presented as means+s.e.m. together with the calculated *P*-values. Bold indicates P<0.05. Different superscript letters (a,b,c) indicate significant differences at the level of P<0.05.

EE, early emergence; LE, late emergence; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, 5-hydroxytryptamine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; NA, noradrenaline; CALM, calmodulin; EPD1, ependymin; GABARAP, gamma-aminobutyric acid receptor-associated protein; RBP1, retinol binding protein 1; 5-HT$_{1Aα}$, 5-HT 1Aα receptor; 5-HT$_{1Aβ}$, 5-HT 1Aβ receptor.

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Similarly there was a significant interaction effect of stress and time of emergence on forebrain RBP1 mRNA expression ($F_{1,25}$=10, P<0.01) but the *post hoc* analysis did not detect any difference between experimental groups. Also, neither stress ($F_{1,25}$=0.69, P<0.30) nor time of emergence ($F_{1,25}$=0.14, P<0.50) had any individual significant effects on RBP1 mRNA expression.

Hindbrain concentrations of monoamines and monoamine metabolites

There was no effect of stress, time of emergence or interaction effects of these variables on hindbrain levels of noradrenaline (norepinephrine, NA), dopamine (DA) and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) (Table 1). There was, however, a significant effect of emergence on hindbrain 5-HIAA concentration ($F_{1,26}$=4.33, P<0.05) and of stress on hindbrain 5-HT levels ($F_{1,26}$=4.59, P<0.05) (Table 1). There was also a tendency towards an interaction between stress and time of emergence, the trend being that LE fish responded to stress with a larger elevation of hindbrain 5-HIAA levels than EE fish.

Behavioural response to hypoxia and novel objects

In two trials with five fish from each fraction, in total, eight out of 10 EE and two out of 10 LE moved over to the illuminated tank from the home tank during hypoxia (Fig. 1). Thus, when reducing the oxygen levels in the familiar home tank, significantly more EE than LE fish moved to the unfamiliar but oxygenated environment of the illuminated tank (P<0.05, Fisher's exact test).

In the novel object test, the latency to approach within a 5 cm radius of the novel object was determined. There was a significant difference between EE and LE fish in approaching the novel object, with EE fish being more willing to approach (P<0.05 Fisher's exact test). Within 15 min of the novel object being introduced, eight out of 11 EE fish had approached the novel object compared with three out of 11 LE fish (Fig. 2).

DISCUSSION

The results of the current study show that salmon emerging early from the nest clearly differ from those emerging late. The EE fish appear to be bolder, more readily inspecting a novel object as well as being more willing to enter an unfamiliar environment than their LE littermates. The different behavioural profiles of EE and LE fish appear to be reflected in brain function as well. Confinement stress resulted in a larger activation of the brain 5-HT system in LE than in EE fish, as indicated by hindbrain 5-HIAA concentrations. Moreover, LE fish showed higher 5-HT$_{1A\alpha}$ expression than EE fish, and stress resulted in an up-regulation of 5-HT$_{1A\alpha}$ expression in LE but not EE salmon. Thus, brain 5-HT function appears to differ between EE and LE fish. Similar results have been obtained in studies on HR and LR trout. When subjected to confinement stress, only HR trout respond with elevated brain 5-HIAA concentrations (Øverli et al., 2001; Schjolden et al., 2006b).

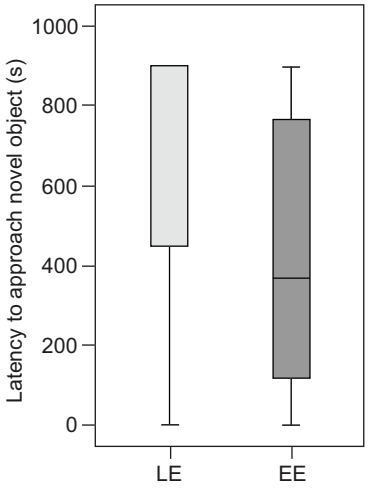

Fig. 2. The latency to approach a novel object in EE or LE salmon. N=11 for both LE and EE. P<0.05, Fisher's exact test.

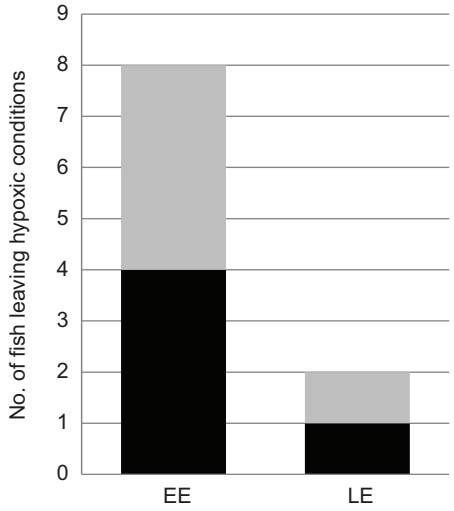

Fig. 1. The number of fish leaving hypoxic conditions in early (EE) or late (LE) emerging salmon. N=5 for both EE and LE. The results are from an experiment run in two consecutive sessions, represented by black (1) and grey (2). P<0.05, Fisher's exact test.

The organization of the brain 5-HT system is evolutionarily conserved, and 5-HT appears to have similar behavioural effects across the vertebrate subphylum (Lillesaar, 2011). The importance of the central 5-HT system in the inhibitory control of aggressive and impulsive behaviour is well established and the brain 5-HT system is also strongly implicated in mechanisms mediating divergent stress coping styles (Koolhaas et al., 1999; Mahar et al., 2014).

The different phenotypes of EE and LE salmon could represent different stress coping styles (Schjolden and Winberg, 2007). Differences in behaviour and brain 5-HT function between EE and LE salmon are very similar to those observed between LR and HR trout (Øverli et al., 2001; Schjolden et al., 2006b). The LR and HR trout have been suggested to show divergent stress coping styles, LR trout being proactive whereas HR trout respond to challenges by a reactive coping style (Schjolden and Winberg, 2007). The time of emergence from the redd also seems to differ between LR and HR trout, with LR fish being significantly more frequent in the early emerging fraction than HR fish (Åberg Andersson et al., 2013). Divergent hypothalamus–pituitary–interrenal (HPI) axis reactivity has been suggested to be one of the characteristics of different coping styles, with reactive animals responding to stress with higher plasma cortisol than proactive conspecifics (Koolhaas et al., 1999; Schjolden and Winberg, 2007). However, in the current study, we could not detect any difference in HPI axis reactivity between EE and LE salmon. The HR and LR trout were created by selective breeding for divergent HPI axis reactivity, which could explain the divergence in post-stress plasma cortisol observed in these strains. Recently, it has been questioned whether HPI/hypothalamus–pituitary–adrenal (HPA) axis reactivity is part of the coping style or rather reflects differences in emotionality (Koolhaas et al., 2010).

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The salmon used in the current study were offspring of wild-caught parents subjected to natural selection during at least one generation. In offspring of wild parents from six different Swedish populations of anadromous brown trout (*Salmo trutta*), Brelin et al. (2008) reported the presence of two distinct behavioural profiles, one being bold and showing high post-stress plasma catecholamine levels whereas the other was more shy and responded to stress with a more modest elevation of plasma catecholamine concentrations. However, these divergent phenotypes did not differ in post-stress plasma cortisol levels (Brelin et al., 2008). Thus, the relationship between HPI axis reactivity and behavioural profile appears to be ambiguous in wild salmonid populations.

Moreover, recent results also question the relationship between HPI axis reactivity and behaviour in the HR/LR trout lines. Thomson et al. (2011) found that HR and LR trout differ in post-stress plasma cortisol concentrations as well as in brain gene expression, with LR trout showing higher expression of EPD1, MHC1, CALM, GABARAP, AVTR and RBP1 mRNA than HR trout. However, when they screened trout of the HR and LR line for boldness, bold and shy individuals were observed in both these selected lines, and frequencies of bold and shy fish did not differ significantly between the HR and LR line. Moreover, fish classified as bold or shy did not differ in post-stress plasma cortisol levels or in brain gene expression (Thomson et al., 2011).

Similarly, in the current study, EE and LE salmon did not differ in plasma cortisol but they did differ in brain gene expression and behaviour. For instance, stress resulted in an increase in forebrain 5-$HT_{1A\alpha}$ and GABARAP mRNA concentrations in LE fish, an effect not seen in EE salmon. There was also an interaction between stress and time of emergence on EPD1 mRNA expression, with LE fish showing higher expression than EE fish. An interaction effect of stress and time of emergence on forebrain RBP1 mRNA levels was also observed. Thus, divergent gene expression in EE and LE salmon appears to be related to differences in how these phenotypes respond to stress. In the study by Thomson et al. (2011), stress had no effect on the expression of the target genes in HR and LR trout.

Differences in forebrain gene expression between EE and LE salmon could reflect their divergent behavioural and neuroendocrine profiles. The expression of CALM and RBP1, gene products known to affect neuronal plasticity, was affected by stress or stress and time of emergence, respectively. Specifically, the protein RBP1 is implicated in the distribution of retinol (vitamin A), which in its active form (retinoic acid) is involved in the control of neural plasticity (for a review, see Shearer et al., 2012). CALM is a key protein in the transduction of signals in response to increases in intracellular Ca^{2+}. Upon Ca^{2+} binding, it will activate Ca^{2+}/calmodulin-dependent kinases, leading to an enzymatic cascade that regulates the activity of several transcription factors (for a review, see Racioppi and Means, 2008). Stress has been reported to have effects on neuronal plasticity processes in mammals as well as in teleosts (von Krogh et al., 2010). Moreover, mechanisms like neuronal plasticity are likely to be of importance for mediating differences in behavioural plasticity and neuroendocrine mechanisms between animals displaying divergent stress coping styles (Sørensen et al., 2013).

The forebrain expression of 5-$HT_{1A\beta}$, GABARAP and EPD1 was also affected by stress and time of emergence. There is evidence that stress and aggression are linked, so exposure to confinement stress may not only activate specific brain regions and neural circuitries regulating stress but also activate neural circuitries involved in aggressive behaviour (reviewed in Summers and Winberg, 2006). The 5-HT system has a well-documented role in the regulation of aggression (Edwards and Kravitzt, 1997; Popova, 2006; Miczek et al., 1994, 2007; Summers and Winberg, 2006) and stress responses (Winberg and Nilsson, 1993; Summers and Winberg, 2006) throughout the animal kingdom. The involvement of 5-HT_{1A} receptors in aggression has been demonstrated in several animal studies with inhibition of aggression via agonists of 5-HT_{1A} (Miczek et al., 1994). Low aggressiveness has also been shown to be associated with higher 5-HT_{1A} densities in several brain regions and higher 5-HT_{1A} mRNA levels in the midbrain (Popova, 2006). Other genes associated with serotonin synthesis, regulation, uptake and degradation also affect aggressive behaviour. A long-term elevation of brain serotonergic activity leads to suppression of aggressive behaviour. This effect has been demonstrated in rainbow trout, where elevated dietary intake of L-tryptophan, the precursor of 5-HT, resulted in the suppression of aggressive behaviour and suppressed post-stress plasma cortisol as a consequence of the increase of serotonin synthesis and levels of serotonin in the brain (Winberg et al., 2001; Lepage et al., 2002).

GABARAP has a role in the increase in GABA activity as it will increase cell surface expression of $GABA_A$ receptors, possibly by augmenting the trafficking of receptors towards the cell membrane along microtubules, and promote the insertion of or stabilize the surface receptors (for a review, see Chen and Olsen, 2007). It has been proposed that GABA, which is the major inhibitory neurotransmitter, has an inhibitory effect on aggression as GABA and its synthetizing enzyme, glutamic acid decarboxylase, are low in brain areas such as the striatum and the olfactory bulbs in mice and rats that display aggressive behaviour (for a review, see de Almeida et al., 2005). Pharmacological studies also support this view as aggressive behaviour is inhibited in mice and rats when GABA-transaminase is blocked with sodium *n*-dipropylacetate or valproate, or when reuptake is inhibited by diaminobutyric acid or nipecotic acid amide. In addition, a correlation between low plasma GABA levels and aggressiveness was found in psychiatrically healthy adults with a family history of psychiatric disorders (Bjork et al., 2001).

Ependymin, which is the most abundant glycoprotein in the cerebrospinal fluid of fish (Shashoua, 1976; Sneddon et al., 2011), has been shown to be involved in memory consolidation (Pradel et al., 1999) and environmental stress (Smith et al., 2009; Tang et al., 1999). In a recent study, EPD1 was demonstrated to also have a role in the control of aggressive behaviour (Sneddon et al., 2011). Antibodies were injected into zebrafish brains in order to inactivate EPD1, which resulted in increased aggression in subdominant individuals and decreased aggression in dominant zebrafish. By measuring EPD1 protein levels in zebrafish according to their social rank, the authors could conclude that the most dominant fish had intermediate levels of EPD1 while the subdominant fish had high and the subordinate fish low levels of EPD1. So, a reduction of EPD1 by injection of antibodies reduced the level of EPD1 in subdominant fish to levels similar to those of dominant fish and in dominant fish to levels similar to those of subordinate fish.

The current study does not include agonistic behaviour. However, LE salmon show behavioural and neuroendocrine traits similar to those characterizing a reactive stress coping style. Reactive animals are recognized as non-aggressive individuals, that when kept in groups tend to become subordinate (Schjolden et al., 2006a). This is a behavioural profile that seems to fit with elevated expression of 5-$HT_{1A\beta}$, GABARAP and EPD1 in EE salmon.

In conclusion, the results from the current study suggest that individual variation in time of emergence from spawning nests is related to behavioural traits and brain function in Atlantic salmon. EE fish display a bolder behavioural profile, being more explorative and

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Table 2. Genes, accession numbers and primer sequences for qPCR analysis

Gene	Accession no.	Forward primer	Reverse primer
AVTR	AGKD01053513*	ACGGGTTCATCTGCCACAGCA	TGACAGTTCTCAATTTCGCTCTGGA
CALM	BT057678	TGCTGCAGAGCTGCGTCACG	AGCCTCCCGGATCATCTCATCCA
CRF1	NM_001141590	CACACCCACATCCTAGGCTACTCAA	TAGCGGGGTTGGAAGGCACCA
CRHBP	NM_001173799	TTGAGAAGCGTGCGGTGCGT	AGCTGCTCTCGGAAAGTCCCCT
CRFR1	AKD01020666*	ATCATCCATTGGAACCTGAT	ATCCAGAAGAAGTTTGTCAC
EPD1	NM_001140909	TCTGTGAGGGTGTGGAGCTGGAG	TTGGTTGGTTGGTTGGGGCTG
GABARAP	NM_001142717	ACTCCCCCTCCTTCCCTCATCCA	ATCCCCATCTCGGCGACCCG
GR	GQ179974	TGGCCTGTATCCCCCACTGCC	CCGCTGGGCTTGGCTGACG
MHC1	AF504021	AATGGATCGCCCCAACGCCA	CTGTCGCGTGGCAGGTCACT
MR	AGKD01011423*	AGCTGGCTGGGAAACAGATGA	TCAGGGTGATTTGGTCCTCTATGG
RETB1	NM_001140773.2	GTGGCGGGGCCCTACGCTAT	TCCTGTGCCCAGCATGTCGC
5-HT$_{1A\alpha}$	AGKD01067361*	ATGCTGGTCCTCTACGGGCG	CGTGGTTCACCGCGCCGTTT
5-HT$_{1A\beta}$	DY694524	TTGATCATGCGTTCCCAGCCGA	AAAGGAATGTAGAACGCGCCGA

*Salmon DB *Salmo salar* Genome AGKD01053513.1:3663..4137, AGKD01011423.1:11794..11942; 13768..13997, AGKD01067361.1:7182..7844, AGKD01020666.1:14908..15064; 10183..10305; 15178..15240.

AVTR, vasotocin receptor; *CRF1*, corticotropin-releasing factor 1; *CRHBP*, corticotropin-releasing factor binding protein; *CRFR1*, corticotropin-releasing factor receptor 1; *GR*, glucocorticoid receptor; *MHC1*, major histocompatability complex class1; *RETB1*, retinol binding protein.

risk taking than LE fish. Moreover, when subjected to stress, EE fish show a less pronounced activation of the brain 5-HT system than LE fish. The fact that LE fish responded to stress with an up-regulation of forebrain gene expression that has previously been associated with suppressed aggression (GABARAP, EPD1 and 5-HT$_{1A}$) adds further support to a relationship between stress coping style and timing of emergence. However, EE and LE salmon do not appear to differ in HPI axis reactivity, another characteristic of divergent stress coping styles.

MATERIALS AND METHODS

Study material and experimental design

In November 2009, eggs from six female salmon (*S. salar*) were fertilized on the same day by a single male per female, and incubated at the Älvkarleby salmon hatchery by the river Dalälven in Sweden. In February 2010, eyed eggs were transferred to six incubators containing golf balls to mimic natural gravel as described in Vaz-Serrano et al. (2011); 600 eggs from a single female were placed in each incubator. Larvae were sorted by time to emerge by flushing downstream to a collecting tank when they emerged from the bottom and reached the surface. The collecting of emerging larvae started on 5 May and ended on 26 May. During these weeks of emergence there was a continuous rise in water temperature from 8°C to 13°C. The first 25% (150 from each incubator) of fish were collected as the EE fraction, the middle 50% as intermediate fraction and the last 25% as the LE fraction. From the intermediate fraction, 900 fish were kept, so, as each fraction was divided into three tanks (1 m^2), about 300 fish were put in each tank.

Confinement stress and collection of brains

Confinement and collection of blood and brain samples were performed in November 2010. Confinement boxes (15×10 cm with a water level of 2.5 cm) with lids were continuously supplied with running river water. Each fish was held in the confinement box for 1 h before it was anaesthetized in benzocaine solution (100 mg l^{-1}). Control fish were netted from the holding tank and rapidly anaesthetized. A blood sample was immediately collected from the caudal vasculature using a heparinized syringe and the blood cells were separated from the plasma by centrifugation at 1500 ***g*** for 3 min; plasma samples were then frozen on dry ice. Cortisol levels in the plasma were determined by radioimmunoassay (RIA) as described by Pottinger and Carrick (2001). The fish was decapitated immediately after the collection of blood and the brain was dissected out and divided into two parts: the forebrain (excluding the olfactory bulb) and hindbrain. Each brain sample was placed in a 1.5 ml tube, frozen on dry ice and kept at −80°C.

Analysis of gene expression using quantitative PCR (qPCR)

Extraction of RNA from individual brains was performed using GenElute mammalian total RNA mini prep kit (Sigma, RTN70-1KT) together with an on-column DNAse 1 digestion set (Sigma, DNASE70-1SET) according to the manufacturer's instructions. For quality and quantity measures, the total RNA was analysed by spectrophotometry (Nanodrop, Thermo Scientific). cDNA was prepared from 0.6 µg total RNA (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, K1641, Fermentas) according to the manufacturer's instructions. After cDNA synthesis, the reaction volume of 20 µl was diluted to 800 µl, divided into aliquots, and 4 µl of diluted cDNA was used in each qPCR reaction. Primers were 19–25 nucleotides in length with a melting point around 60°C and formed products in the range 82–219 bp (Table 2). From an original set of seven reference genes, three genes that displayed the smallest variation across treatment were selected – peptidylprolyl isomerase A (*PPIA*), elongation factor 1 α (*EF1α*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) – and used for subsequent normalization of qPCR data using geNorm (Vandesompele et al., 2002). Nine genes were selected for expression studies, six that have previously been shown to be up-regulated in the brain of LR fish compared with HR fish when exposed to stress: *CALM*, *EPD1*, *GABARAP*, *MHC1*, *RETB1* and *AVTR* (Thomson et al., 2011). The remaining genes analysed were *MR*, shown to have a higher expression in LR fish (Johansen et al., 2011) and two serotonin receptor genes, 5-HT$_{1A\alpha}$ and 5-HT$_{1A\beta}$, which are two forms of the 5-HT$_{1A}$ receptor found in some fish species as a result of the third whole genome duplication in teleosts.

Novel object and hypoxia

Three consecutive sets of novel object tests were performed. In each set, four EE and four LE fish were transferred and individually placed in plastic aquaria (50×25 cm) with a water level of 25 cm. After transfer to the new environment, the fish were left to acclimate for 1 week. The outer walls of the aquaria were covered in black plastic and a black plastic curtain was used to prevent the fish from being exposed to other disturbances. Transparent plastic lids were placed on top of the aquaria, and a camera was placed to record from above without disturbing the fish. The aquaria were continuously supplied with river water at ambient temperature. Before a fish experienced any novel object, it was recorded for 15 min without disturbance. The novel object was placed at the centre of the back wall and the behaviour of the fish was recorded for a further 15 min. The novel object consisted of two pieces of white and blue Lego bricks (15.6×31.2×9.6 mm), which, together with an M8 stainless steel screw-nut as a weight, was attached to a fishing line. Response time to approach within 5 cm from the novel object was used as measurement for boldness.

The hypoxia tests were modified from Laursen et al. (2011) and were carried out using two circular black plastic storage bins (70 l, height 100 cm) connected to one another by a tunnel made of a plastic tube (70 mm in diameter) placed 50 cm above the base. A slit was also made in the connecting tube where a piece of plastic could be placed in order to prevent fish from moving between the bins. When filled, the water level was 5 cm above the connecting tunnel. Throughout the duration of the experiment, one of the bins had a black lid in order to provide a dark, more safe, place. This was the 'home' tank in which fish were placed and allowed to settle overnight before the start of

the hypoxia experiment. Both tanks had an air supply and the home tank had also a nitrogen supply for the induction of hypoxia. The connecting tank was continuously illuminated and is referred to as the 'illuminated' tank. Running river water was continuously supplied to the illuminated tank while the water outlet was placed in the home tank. As the illuminated tank will appear more unsafe, the fish will not voluntarily move from the safe home tank, but with lower oxygen levels the hypoxic conditions in the home tank may trigger them to move over to the illuminated unfamiliar tank. One week before the experiment, three to four fish from each holding tank containing the EE and LE were anaesthetized, weighed and individually tagged with a visible plastic tag and left to recover together in a new holding tank. The hypoxia experiment was performed twice with 10 fish in each trial, five from early and five from late emergence. The fish were transferred to the home tank and left overnight. The following day, a supplement of nitrogen gas started just before the air and river water supplement was turned off and the plastic barrier was removed. The oxygen levels decreased gradually throughout the duration of the experiment to approximately 1.8 mg l^{-1} and lasted until five of the 10 individuals had moved over to the illuminated tank, which took about 30 min. The tunnel opening in the illuminated tank was monitored by a camera in order to determine at what time and which fish moved through the tunnel. Hypoxia and novel object experiments were performed in mid-March to the beginning of May 2011.

Analysis of brain monoamines

Hindbrain levels of 5-HT and the 5-HT metabolite 5-HIAA, DA and the DA metabolite DOPAC, and NA were analysed using high performance liquid chromatography with electrochemical detection (HPLC-EC) as described by Øverli et al. (1999) with some modifications. Briefly, the frozen hindbrains were homogenized in 4% (w/v) ice-cold perchloric acid containing 10 ng ml^{-1} 3,4-dihydroxybenzylamine (DHBA, the internal standard) using a Sonifier cell disruptor B-30 (Branson Ultrasonics, Danbury, CT, USA) and centrifuged at 21,000 ***g*** for 10 min at 4°C. The supernatant was used for HPLC-EC, analysing the amount of monoamines in the samples. In short, the HPLC-EC system consisted of a solvent delivery system model 582 (ESA, Bedford, MA, USA), an autoinjector Midas type 830 (Spark Holland, Emmen, The Netherlands), a reverse phase column (Reprosil-Pur C18-AQ 3 µm, 100×4 mm column, Dr Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) kept at 40°C and an ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) with two electrodes at reducing and oxidizing potentials of −40 and +320 mV. A guarding electrode with a potential of +450 mV was employed before the analytical electrodes to oxidize any contaminants. The mobile phase consisted of 75 mmol l^{-1} sodium phosphate, 1.4 mmol l^{-1} sodium octyl sulphate and 10 µmol l^{-1} EDTA in deionized water containing 7% acetonitrile brought to pH 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations. DHBA was used as the internal standard to correct for recovery using HPLC software Clarity™ (DataApex Ltd, Prague, Czech Republic). For normalization of brain monoamine levels, brain mass was used.

Statistical analysis

Statistical analyses of novel object and hypoxia experiments were performed using Fisher's exact test. The effects of stress and time of emergence on plasma cortisol concentrations, brain monoamines and monoamine metabolites, and gene expression were analysed using two-way ANOVA. Normal distribution of data was investigated by Lilliefors test. DA, DOPAC, 5-HT, 5-HIAA and 5-HT concentrations, gene expression and [DOPAC]/[DA] ratios were log transformed to obtain normal distribution. Unequal *N post hoc* tests were used to determine where the significances occurred. Values are given as means±s.e.m.

Acknowledgements

We are grateful to the staff at SLU Research Station for fisheries in Älvkarleby.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.-O.T., E.H. and S.W. conceived and designed the experiments; P.-O.T. performed the experiments; P.-O.T., E.H. and S.W. interpreted the data, and drafted and revised the paper.

Funding

This work was supported by the Swedish Research Council Formas through the project SMOLTPRO.

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