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Brain cortisol receptor expression differs in Arctic charr displaying opposite coping styles

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ABSTRACT

Individually consistent behavioral and physiological responses to stressful situations (often referred to as coping styles) has been reported in many animal species. Differences in hypothalamic-pituitary axis reactivity characterize individuals, and it has been proposed that the glucocorticoid (*gr*) and mineralocorticoid (*mr*) receptors are fundamental in regulating coping styles. We sorted individuals into reactive and proactive coping styles by collapsing behavioral outputs from net restraint and confinement stress tests in a principal component analysis. We then analyzed plasma cortisol levels, serotonin neurochemistry and the relative mRNA expression of *gr1 and mr* in stressed individuals per coping style. Proactive fish were characterized as having a lower serotonergic activity and being more active under stress. In addition, proactive fish had higher hypothalamic *gr1* and *mr* abundance and a higher *mr/gr1* ratio, compared to reactive fish. We found no significant differences in cortisol or telencephalic mRNA, *gr1* and *mr* expression, or their ratio. Brain MR and GR have been proven to have an important role in the appraisal, coping and adaptation to stressful stimuli, so that a higher expression of these receptors in proactive fish suggests increased tolerance and performance under stress, compared to reactive individuals. We present evidence of a conserved neuroendocrine mechanism associated with coping styles in a fish species which is ecologically very diverse and considered to be the most cold-adapted fish in freshwater. We propose that this may be a first step into exploiting this model in order to better understand climate-change related effects in sub populations and ecophenotypes.

1. Background

The response to a stressful situation is found to vary consistently among individuals of the same species, and this topic has recently received much attention [1–5]. Stress coping styles are typically identified as varying along a gradient from proactive to reactive and have been studied in many taxa, including birds, mammals and teleost fish [1,5]. Proactive animals respond to stress with a lower reactivity of the hypothalamic-pituitary-adrenocortical (HPA, in mammals) or hypothalamic-pituitary-interrenal (HPI, in fish) axis, and high sympathetic nervous system activity, while reactive animals exhibit an opposite pattern of correlated traits [1,2,5,6]. Differences in brain monoamine reactivity, cortisol levels and expression of cortisol receptor genes have been previously reported for fish displaying contrasting coping styles, with proactive fish exhibiting low post-stress cortisol (*e.g.* [7]) serotonergic activity (*e.g.* [5]), as well as higher glucocorticoid receptors (*gr*; *e.g.* [8]) and mineralocorticoid (*mr*) receptor expression (*e.g.* [9]).

Glucocorticoid and mineralocorticoid receptors (GR and MR) have a wide tissue distribution and are fundamental in the negative feedback control of the cortisol stress response in fish [10,11]. While in many fish species there has been a duplication of the GR gene (i.e. *gr1* and *gr2*), in mammals there is only one [12]. However, *gr1* has been found to be functionally homologous to the mammalian GR [13] and it is considered the "stress receptor" in fish since its activated at higher cortisol concentrations than both *gr2* and *mr* [12,14,15]. Importantly, in mammalian studies it has been found that the low-concentration activated MR amplifies the onset of the stress response and that this plays an important role in the appraisal and coping of stressful stimuli, whereas GR is essential for management and adaptation to stressful

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situations [16]. In this context, it has been proposed in mammals that the MR/GR ratio may be more relevant when studying the regulation of the stress response [17,18], where a low MR/GR ratio is associated with increased HPA axis reactivity and increased anxiety [19], which has been proposed to be also important in fish [20].

Arctic charr (*Salvelinus alpinus*) is ecologically very diverse and consists of many subtypes [21,22], which makes the species an ideal model organism to study the evolution of proximate and ultimate mechanisms underlying the evolution of correlated phenotypic traits. Arctic charr is considered to be the most cold-adapted freshwater fish [23]. It is therefore imperative to understand how neural mechanisms are regulated in this species, particularly in response to stress, in order to understand better climate-change related effects in subpopulations and ecophenotypes. Previous studies on Arctic charr have established that behavioral outputs, such as activity and struggling movements during restraint, are often correlated with post-stress cortisol levels, in which lower cortisol levels were associated with increased activity (i.e. a proactive coping style) [24,25]. In addition, during pair-wise contests, dominant proactive fish were characterized by lower cortisol levels, and lower brain serotonergic activity [26], but the glucocorticoid neuroendocrine regulation of the stress response is yet to be elucidated in this species.

In order to characterize the neuroendocrine regulation of Arctic charr subjected to stress, we here investigate the relationship between plasma cortisol, monoamine neurochemistry and the *gr* and *mr* transcript abundance, since all these play an important role in the regulation of the HPI axis [9,27]. In addition, we investigated the relationship between cortisol levels and brain stem serotonergic activity, since serotonin and cortisol tend to increase proportionally in response to stress and their interaction is fundamental in the regulation of the HPI axis [28–31]. We expect, therefore, that these 2 variables will be positively correlated in all fish. As mentioned above, many fish species have 2 types of *gr*, including salmonid fish, such as rainbow trout (*Oncorhynchus mykiss*) [12]. However, in the zebrafish (*Danio rerio*) genome only one *gr* gene is found and in some fish species like Japanese flounder (*Paralichthys olivaceus*) and brown trout (*Salmo trutta*) only one *gr* gene is currently known [14]. As far as we know, there is only one *gr* currently found in Arctic charr. We therefore present results in the *gr* transcript abundance (hereafter referred to as *gr1*, since it is recognized as 98% similar to the rainbow trout *gr1* sequence when blasted on NCBI) of Arctic charr. We predict that, in agreement with previous studies, proactive charr will be characterized by increased activity to restraint/confinement, lower cortisol levels and lower serotonergic activity. Furthermore, we hypothesize that proactive fish will have higher *gr1* and *mr* abundance as well as a higher *mr/gr1* ratio, compared to reactive individuals.

2. Methods

2.1. Experimental animals, facilities and experimental procedure

This study was carried out in May 2013 at the Aquaculture Centre North in Kälarne, Sweden, using 1-year old juvenile Arctic charr from the 7th generation of the Swedish Arctic charr breeding program, for further details see Nilsson et al. [32]. The fish were bred and kept at the stocking facilities in holding tanks (area 7 m^2, depth 1 m) supplied with running water from the nearby lake Ansjön, kept at natural temperatures (5–6 °C), and artificial light using a photoperiod of 12D:12L. The fish were fed by automatic feeders according to standardized feeding models for Arctic charr.

Fish were randomly selected from the rearing tank, and 28 individuals were subjected to acute stress in order to determine coping style. The acute stress consisted of one minute net restraint followed by 30 min confinement as described by Magnhagen et al. [25]. In brief, the net restraint test consisted of capturing a fish from the storage tank, putting it into a 10 l container with water, and then gently pouring it into a fish net (24 × 20.5 × 13 cm, mesh size 1.5 × 0.75 cm), which was then left in air for 1 min. We video recorded the fish in the net from above in order to analyze struggling (i.e. escape) movements. Thereafter the fish were placed for 30 min in a small container (ca 30 × 17 × 18 cm) with water (ca 3 l) and an air stone (i.e. confinement test, following Kittilsen et al. [3]). Individually confined fish were video recorded in order to quantify swimming activity during confinement. Immediately after the confinement period, fish were euthanized with an overdose of buffered MS222 (0.3 g/l) until there was no opercular movement (within 10 s of immersion). Samples of blood were taken from the caudal vessels, using a 23-gauge syringe with heparin as an anti-coagulant. Blood samples were centrifuged at 10000*g* for 5 min, and plasma was collected and stored at − 20 °C until further analyses. The brains were dissected out and telencephalon, hypothalamus and brainstem were immediately placed in liquid nitrogen and stored at − 80 °C until further analysis. Size did not differ between coping styles (Reactive: mean ± SD: body length 19.2 ± 1.6 cm; mass 94 ± 21.6 g and Proactive: body length 19.6 ± 1.6 cm; mass 95.0 ± 20 g; Length: $t_{(26)} = -0.65$, $p = 0.52$; Mass: $F_{(26)} = -0.12$, $p = 0.9$).

2.2. Analysis of behavior

Video recordings of the net restraint and confinement stress were used in order to quantify behavior. In the net restraint test 2 behavioral outputs were measured: 1. *Struggling*, consisting of the amount of time (s) that fish spent vigorously wriggling and jumping calculated for the entire test time (60 s) and 2. *Time* to immobility, that is, the amount of time (s) that it took for individuals to stop moving (not struggling) from the moment fish were placed in the net, within the total test time of 60 s. Due to restriction of movement within the confinement box, fish were only able to move by bending their bodies from side to side (wriggling) or stay immobile. Therefore, time spent wriggling in min (within the total test time of 30 min) was quantified for this test. This data was later statistically analyzed (for further details see Section 2.6) in order to categorize fish into proactive and reactive individuals.

2.3. Plasma cortisol analysis

Plasma cortisol was analyzed using a commercial enzyme linked immunosorbent assay (ELISA) kit, following the manufacturer's instruction (product # 402710, Neogen corporation, Lexington, USA). Each sample was run in duplicate during a single assay with an intra-assay coefficient variation of 3.08%.

2.4. Brain stem serotonergic neurochemistry

Frozen brain stems were homogenized in 4% ice cold perchloric acid (PCA) containing 0.2% EDTA and 3,4-dihydroxybenzyl amine hydrobromide (DHBA, 40 ng/ml) as an internal standard using either a Potter–Elvehjem homogenizer or an MSE 100 W ultrasonic disintegrator, respectively. After spinning samples for 10 min at 15,493 rcf and 4 °C, the supernatant was analyzed by means of high-performance liquid chromatography (HPLC). The mobile phase was made up of 12 μM EDTA, 86 mM sodium phosphate and 1.4 mM sodium octyl sulphate in deionized water (resistance 18.2 MW), containing 7% acetonitrile set to pH 3.1 using phosphoric acid. The system contains a solvent delivery system (Shimadzu, LC-10AD), an auto-injector (Famos, Spark), a reverse phase column (4.6 mm 100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at − 40 mV and + 320 mV. A conditioning electrode with a potential of + 40 mV was used to oxidize possible contaminants before analysis. Brain stem concentrations of serotonin (5-hydroxy-tryptamine; 5-HT) and its main catabolite 5-hydroxyindoleacetic acid (5-HIAA), were quantified by comparison with standards and corrected for recovery of the internal standard using HPLC software