Covariation between behaviour and physiology indicators of coping style in zebrafish (Danio rerio)

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Abstract

All vertebrates exhibit physiological responses to a wide variety of stressors. The amplitude and profile of the response depend on the intensity, duration, controllability and predictability of the stressor, but there is also individual variation in the response, termed coping style. A better understanding of the expression of coping styles is of great value for medical applications, animal welfare issues and conservation. Here, we investigated the effect of repeated netting stress on proactive and reactive zebrafish (Danio rerio) as an upcoming model system for stress research. Fish were separated by coping styles according to the order of entering a novel environment. Subsequently, repeated netting stress was applied as stressor, over a period of 21 days. Full-body cortisol levels were determined at 0, 15, 30, 60 and 120 min after the last repeated stress event. Our results show that reactive fish display i) increased basal cortisol concentrations after being repeatedly stressed, ii) higher cortisol secretion over time and iii) slow recovery of cortisol concentration towards basal levels after the last repeated stress event. This study shows for the first time in zebrafish that different coping styles are associated with different cortisol responses during the recovery from stress over time and that coping styles can explain otherwise unaccounted variation in physiological stress responses.

Introduction

All vertebrates exhibit physiological responses to stress, which are at the basis of appropriate behavioural adaptation. The amplitude and profile of these responses depend on the intensity, duration, controllability and predictability of the stressor (Kloet et al. 1998, Korte et al. 2005). The stressor-induced secretion of cortisol, arising from activation of the hypothalamus–pituitary–adrenal (HPA) axis (or hypothalamus–pituitary–interrenal (HPI) axis in fish, is a major index of stress level (Wendelaar Bonga 1997). Especially, long-term elevated levels of cortisol can cause a number of deleterious effects (Pankhurst & van der Kraak 2000, Schreck et al. 2001, Consten et al. 2002, Bernier et al. 2004), but there is a large degree of individual variation in this response.

Different individuals cope with exposure to stressors in different ways, which is an inherent feature of natural variation. The so-called stress coping styles are defined as a coherent set of individual behavioural and physiological differences that remain consistent across time and context (Koolhaas et al. 1999, Øverli et al. 2007). Other commonly used definitions, such as personalities (Gosling 2001), boldness (Dingemanse et al. 2007) or behavioural

MacKenzie et al. (2009) showed that proactive and reactive common carp (Cyprinus carpio) responded oppositely to inflammatory challenge regarding 80% of investigated immune-related gene transcripts. Similarly, it has been repeatedly shown that there is a strong link between genetics, physiology and behaviour of stress coping in fishes (e.g. Sih et al. 2004, Øverli et al. 2007, MacKenzie et al. 2009). For example, individual rainbow trouts (Oncorhynchus mykiss) with low blood cortisol levels exhibit consistently different behavioural patterns, acclimating more rapidly to a novel environment, and being more likely to become socially dominant in pairing tests, than fish with high blood cortisol levels (Øverli et al. 2005). Interestingly, during such pairing tests in rats, the main difference between winning and losing a social interaction appears to be the speed of recovery of the stress hormone concentrations to baseline levels (Koolhaas et al. 2011). Consequently, we believe that individual variation in coping style, incorporated as an explanatory variable, could also account for unexplained variation in the stress response of zebrafish (Moretz et al. 2007, Norton & Bally-Cuif 2012, Maximino et al. 2013).

Zebrafish (Danio rerio) have become an important model organism for neuropharmacological and behavioural research, and they are increasingly exploited for anxiety and stress research, as their genome, brain patterning, structure and function of neurochemical and behavioural systems show large similarities to those of terrestrial vertebrates, such as humans (for a review, see Steenbergen et al. (2011)). In zebrafish, the plateau of the stress response measured as full-body cortisol concentration is reached after about 15 min and recovery to baseline is accomplished after 60 min, although with a considerable amount of individual variation in the data (Ramsay et al. 2006).

A better understanding of the endocrine stress response in terms of individual behaviour and stress coping styles can be useful for medical applications (e.g. Smith & MacKenzie 2006, Boersma et al. 2011), animal welfare issues (e.g. Huntingford & Adams 2005, Brown et al. 2009, Weiss et al. 2011) and conservation (e.g. Conrad et al. 2011, Vegvari et al. 2011, Gherardi et al. 2012). The objective of the current study, therefore, was to investigate the association between behavioural traits related to coping style, corticosteroid receptor expression and stressor-induced cortisol concentrations at different recovery time points after repeated stress, using the zebrafish as a model system.

Materials and methods

Animals

Zebrafish (D. rerio, ABTL, stock from Europet Bernina International BV, Gemert-Bakel, The Netherlands) were reared in densities of 12 individuals (male:female 1:1) per 7.5 l tanks in standardised recirculation systems (Fleuren & Nooijen, Nederweert, The Netherlands; 14 h light:10 h darkness cycle; 24 °C water temperature). Fish were fed daily with dry food (DuplalinM, Gelsdorf, Germany) and frozen Artemias (Dutch Select Food, Aquadistri BV, Klundert, The Netherlands). Fish used in the experiment were ~3.5 months old. All experimental procedures were approved by the animal welfare committee of Leiden University (DEC# 11023).

Behavioural assay

Behavioural indicators of coping styles were determined according to MacKenzie et al. (2009) and Huntingford et al. (2010). Briefly, we used a glass tank, consisting of a darkened holding compartment and an uncovered compartment, separated by a wall with a closable hatch. A total number of 100 adult zebrafish were transferred from their housing tank to the holding compartment. After acclimation for 5 min, the trap door was opened so that fish could come out. Fish were divided into three approximately equally sized subgroups according to the order of emergence: first (defined as proactive coping style), intermediate (not used any further in this study) and last (defined as reactive coping style), based on the order they passed the hatch. This procedure was repeated until a stock of ~140 proactive and 140 reactive fish was created. Subsequent housing conditions were similar to rearing conditions, but with six fish per tank of both
sexes and separated coping styles in duplicate, resulting in 12 fish per condition.

**Stress procedure**

We applied a netting stressor according to Ramsay *et al.* (2010). Briefly, fish were gently removed from the holding tank using a hand net (11×24 cm). All six fish per tank were netted simultaneously, suspended in air for 3 min, submerged in water for 3 min and suspended again for 3 min (Fig. 1). This stressor was applied once daily at different times of the day during a period of 21 days (repeatedly stressed) or only once (singularly stressed) before sampling (Fig. 1). We sampled all six fish per tank, from two different tanks (resulting in n=12) simultaneously before the experimental period (control), at t=0 (before netting), 15, 30, 60 and 120 min after the onset of the stressor. Fish were placed on blotting paper for a few seconds, subsequently placed in liquid nitrogen for 10 s and stored in plastic bags at −80 °C until full-body cortisol measurement.

**Full-body cortisol measurement**

Cortisol levels were measured according to a protocol previously described by Canavello *et al.* (2010). Briefly, frozen fish were weighed and pulverised in liquid nitrogen. Approximately half of the material was transferred to a bullet tube and weighed. Subsequently, 500 μl ice-cold 1× PBS buffer was added and the samples were vortexed for 1 min. Samples were split into three parts (allowing triplicate measurements) and each third of the sample volume (~300 μl) was transferred to separate bullet tubes. Subsequently, 1 ml diethyl ether (BDH, Lutterworth, UK) was added to each new sample. Each sample was vortexed for 1 min and then centrifuged at 1370 g for 5 min. Following centrifugation, the organic layer of each sample containing cortisol was transferred to a separate test tube (3× per sample for maximal cortisol extraction). Samples were kept overnight in the fume hood for evaporation of ether. The next day, determination of cortisol concentration was performed using an ELISA kit (Demetic Diagnostics GmbH, Kiel, Germany).

**GRα, GRβ and MR expression**

The relative expression levels of GRα and GRβ were evaluated using qPCR according to Stockhammer *et al.* (2009). Briefly, five proactive and five reactive fish were killed immediately after separation according to coping style, in an overdose of clove oil (10% in ethanol, >30% in water). The brains were dissected out, and brains and body were subsequently snap-frozen in liquid nitrogen for storage at −80 °C. After thawing the samples in RNA later, the tissue was homogenised in 1 ml TRIzol reagent (Invitrogen), and subsequently, total RNA was extracted according to the manufacturer’s instructions. cDNA synthesis reactions were performed in a 20 μl mixture of 500 ng RNA, 4 μl 5× iScript reaction mix (Bio-Rad Laboratories) and 1 μl iScript reverse transcriptase (Bio-Rad Laboratories). The reaction mixtures were incubated at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min.
Real-time PCR was performed using the Chromo4 Real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer’s instructions. Each reaction was performed in a 25 μl volume comprising 1 μl cDNA, 12.5 μl 2× iQ SYBR Green Supermix (Bio-Rad Laboratories) and 10 pmol of each primer. Cycling parameters were 95 °C for 3 min to activate the polymerase followed by 40 cycles of 95 °C for 15 s and 59 °C for 45 s. Fluorescence measurements were taken at the end of each cycle. Melting curve analysis was performed to verify that no primer dimers were amplified. All reactions were performed as technical triplicates. The primer sequences for GRα, GRβ and MR are as follows: forward and reverse, AACTGGCAACGGTCTTATCACGCTCA and TTCTGGTAAAGAGCACAGGG; GATGAACTACGAATGTCTTAGCAACAGACAGCCAGA-CAGCTCAG (Schaaf et al. 2008) and CCCATTGAGGACCAAATCAC and AGTAGACATTGCGGTG (Alsop & Vijayan 2008) respectively. To test whether genomic sequences were amplified, a control with no reverse transcriptase was used (non-RT control). Results were analysed using the control-independent qPCR method. In short, the C_T value of the proactive samples was set at 100% and the difference between proactive and reactive C_T was calculated as C_Tactive−C_Treactive=ΔC_T. Reactive relative expression was calculated as 100/2^ΔC_T and expressed in %. Statistical analysis was performed on the raw C_T values.

Statistical analyses

Two-way ANOVA and Bonferroni post-hoc test were used to test statistically significant differences at P<0.05 with coping style as the independent variable and cortisol concentrations or relative expression levels as the dependent variable. All values are given as mean ± S.E.M. All tests were conducted using SigmaStat 3.0 (Systat Software, Inc., San Jose, CA, USA).

Results

Basal cortisol levels of proactive and reactive fish

First, basal cortisol levels of proactive and reactive fish were assessed before the onset of the stress paradigm repetition of 21 days. These levels were not different between proactive and reactive fish (Table 1). Secondly, basal cortisol levels were measured again at t=0, i.e. before the start of the stressor (singularly stressed group) or at day 21 before the last stressor (repeatedly stressed group; Table 1 and Fig. 2). At this stage, reactive fish in the repeatedly stressed group showed ~100% higher values than fish in the other three groups: proactive fish in the repeatedly stressed group and fish from both coping styles in the singularly stressed group (Table 1, P<0.05).

Stress-induced cortisol levels of proactive and reactive fish

In addition, full-body cortisol concentrations were determined after the netting treatment for the different behaviourally determined coping styles, revealing considerable difference between the groups. All groups reached similar cortisol levels at 15 min after the netting treatment, but differences between coping styles and stress regimes were visible when comparing the subsequent downward slopes of the plots of full-body cortisol concentrations in ng/g against time in minutes (Fig. 2). In singularly and repeatedly stressed proactive fish, the recovery to baseline levels was already complete after 60 min, while this took 120 min in reactive fish, both repeatedly and singularly stressed. The resulting regression patterns with the highest correlation coefficient (r^2) varied with treatment and coping style. Proactive fish, both singularly and repeatedly stressed, showed an exponential decay, while singularly stressed reactive fish showed a linear decay over time. Decay in repeatedly stressed

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<th>Coping style</th>
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<th>Reactive</th>
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<tr>
<td>Stress regime</td>
<td>Singular</td>
<td>Repeated</td>
<td>Singular</td>
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<tr>
<td>Control concentration (ng/g)*</td>
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<tr>
<td>Baseline concentration (t=0; ng/g)*</td>
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<td>6.66 ± 2.21a</td>
<td>6.66 ± 1.63a</td>
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<tr>
<td>Area under the curve (AU)</td>
<td>5.66 ± 2.80a</td>
<td>6.50 ± 1.84a</td>
<td>775</td>
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*Superscript letters indicate significant differences between values (ANOVA, P<0.05, n=6, mean ± S.E.M.).
reactive fish followed a three-parameter power curve. The area under the curve is given in Table 1. Values in arbitrary units (AU) show that singularly stressed reactive fish display a 2.1- to 2.3-fold increase and repeatedly stressed reactive fish an increase of 2.7–2.9 times when compared with proactive fish.

MR, GRα and GRβ expression of proactive and reactive fish

In order to investigate whether different expression levels of GRα, GRβ and MR may underlie the observed differences between proactive and reactive fish, absolute C_T values of reactive and proactive fish brain and the remaining body tissue were compared statistically with each other. There was no significant difference between coping styles or tissue (P > 0.05, n = 6). The relative differences are shown in Fig. 3.

Discussion

This study shows that differences in the pattern of cortisol secretion during recovery from a physical stressor are associated with differences in behaviourally determined coping style. In reactive fish, the baseline cortisol levels were significantly elevated after a period of daily repeated stressors, which was not the case for proactive fish. Peak cortisol levels measured at 15 min after the stressor did not vary among coping styles and stress regimes. However, the shape of the recovery phase of the cortisol response curves varied considerably. Proactive fish returned to baseline levels after 60 min, while reactive fish had reached full recovery only after 120 min. The area under the curve (reflecting the total amount of cortisol) showed similar low values in proactive fish but a twofold increase in singularly stressed and a threefold increase in repeatedly stressed reactive fish. The full-body baseline and control cortisol levels presented here are consistent with previous studies on stress response in zebrafish, as were peak levels after 15 min (Ramsay et al. 2006, 2010). This consistency in peak cortisol levels suggests a general physiological response to strong physical stressors independent of coping style, while only after repeated exposure to stressors significant variation in cortisol levels emerges that is strongly dependent on coping style.

Our results show that recovery phase of the cortisol response curves after stress differs greatly between coping styles (as assessed by separating fish based on a behavioural assay of emergence into the open from a smaller enclosure). The exponential decline curves in proactive

Figure 2
Full-body cortisol concentrations in ng/g body weight over time in minutes after netting stress; (a) in proactive singularly, (b) in proactive repeatedly, (c) in reactive singularly and (d) in reactive repeatedly stressed fish. The t = 0 data points indicate baseline values before the stress event. Values are mean ± S.E.M., n = 6. Letters indicate significant differences between, and * significant difference from baseline value.
Relative difference in control-independent qPCR mRNA expression of mineralocorticoid receptor (MR), glucocorticoid receptor α (GRα) and glucocorticoid receptor β (GRβ) in brain (black) and body (grey) tissue. The bars indicate the relative expression in reactive fish in % proactive expression, with proactive expression set to 100% (line). Values are mean ± S.E.M., n = 5. Statistical analysis was done on raw Ct values. There is no significant difference between coping style and tissues (P > 0.05).

Figure 3

Our use of descriptive models, a method adopted from ecological studies (Mangel & Clark 1989), has proven to be a suitable tool to reveal patterns that suggest divergent physiological processes among individuals of different coping style. This method is supported by the additional analysis of the areas under the curves, which also differed with coping style and stress regime. The variation in curves among coping style and exposure regime groups allows some speculation about underlying processes, but more studies are needed to get a better understanding of the consistency and physiological basis of these patterns.

Chronically elevated baseline cortisol levels due to repeated stressors can lead to a variety of negative physiological and behavioural effects on the individual, such as a reduction in foraging behaviour, a decrease in body weight or a disruption of the immune response (Koolhaas et al. 2011). Consequently, stress may reduce reproductive success (Schreck et al. 2001) and disrupt trophic interactions (Archard et al. 2012), from which it follows that stress may not only negatively affect individuals but also contribute to population decline and the possible extinction of species (Schaaf et al. 2008).

Nevertheless, natural populations typically consist of individuals of a range of different copying styles and such a mixture means that individuals will vary in how well they fare under stressful situations but also provides a population with some flexibility to persist (Koolhaas et al. 1999, Korte et al. 2005). Similarly, studies on sticklebacks (Gasterosteus aculeatus) have revealed a personality-dependent predation risk (Bell & Sih 2007) and personality distribution in natural lakes determined by the presence of a large natural predator (Dingemans et al. 2007). Also for this particularly strong selection pressure, the presence of a mixture of coping styles allows a variable subset of individuals to survive and therefore populations to persist under various predation regimes. Consequently, we argue that understanding the impact of stressors on a particular species requires the analysis of individuals of divergent phenotype. Mean values for a population may correctly reflect the stress physiology of only few individuals and may lead to misunderstandings about individuals of extreme coping style (which may be critical for medical cases in humans) and about population developments (which may be critical for applications in conservation).

In conclusion, this study stresses the value of taking coping styles into account when using the zebrafish as a model system in stress research. Our data show that behavioural aspects of different coping styles are associated with different cortisol responses after stress. Given the popularity and versatility of the zebrafish as a model in...
biomedical research, this indicates that there will be plenty of opportunity to study molecular mechanisms underlying this association. We expect that such studies will contribute significantly to our insights into the potential impact of behavioural and physiological aspects of coping styles on individual fitness.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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