

Differences in behaviour between rainbow trout selected for high- and low-stress responsiveness

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Summary

Two F₁ lines of rainbow trout *Oncorhynchus mykiss*, divergent for plasma cortisol responsiveness, were generated by individual selection for post-stress cortisol values within the F₀ generation. Adult females of the F₁ generation were transferred to rearing in social isolation in observation tanks. After 6 days, locomotor activity in high-responding (HR) and low-responding (LR) individuals was quantified as time spent moving during a 20 min observation period. Behavioural observations were repeated the next day with a smaller conspecific intruder present in each observation tank. Differential hypothalamus–pituitary–interrenal axis activity in the two lines was subsequently confirmed by a standardised confinement stress test, which resulted in significantly higher plasma cortisol concentrations in HR than LR fish. HR fish displayed higher levels of locomotor activity than LR fish in the presence of an intruder, but not when in

isolation. Aggressive behaviour towards the intruder was not seen, suggesting either a state-dependent lack of territorial aggression, or chronic stress in the experimental fish. A significantly higher incidence of feed intake was seen in LR trout when held in observation tanks (40% versus 0% of the fish took food when in isolation), suggesting that these fish acclimated more successfully to the experimental conditions than HR fish did. These results suggest that selection for stress responsiveness in salmonid fish leads to behavioural alterations, which are of potential importance to the performance of these fish in aquaculture rearing operations.

Key words: stress response, behaviour, rainbow trout, *Oncorhynchus mykiss*, cortisol.

Introduction

Recent studies in mammals suggest that individual differences in stress responsiveness are associated with differences in behaviour. In fact, behavioural and physiological traits are linked in such a way that two opposing stress-coping styles, denoted active (proactive) and passive (reactive) coping, can be identified (Koolhaas et al., 1999). A coping style, or coping strategy, 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). The active stress-coping style is behaviourally characterised by a high level of active avoidance, locomotor activity, aggression and other behavioural patterns indicating an active attempt to counteract the stressful stimulus (e.g. defensive burying in rodents) (Bohus et al., 1987; Benus et al., 1989; Driscoll et al., 1990; Sluyster et al., 1996). Passive coping, on the other hand, is characterised by immobility and low levels of aggression.

Fundamental to the theory of different coping styles is that different behavioural characteristics are associated with consistent neuroendocrine and physiological differences (reviewed by Koolhaas et al., 1999). The active strategy is generally associated with low hypothalamus–pituitary–adrenal (HPA) axis responsiveness, but high sympathetic reactivity, while the opposite is true for the passive strategy (De Boer et al., 1990; Korte et al., 1992; Fokkema et al., 1995).

Limited information is available on whether different individual stress-coping styles have evolved in 'lower' vertebrates. Among teleost fish, consistent behavioural patterns that may reflect alternative coping styles have been observed in cichlids (Francis, 1990; Budaev et al., 1999). It has recently been observed that domestication in anadromous brown trout (*Salmo trutta*) affects both behavioural and physiological stress responses (Lepage et al., 2000), and Van Raaij et al. (1996) found strong individual differentiation in behavioural and

physiological responses of rainbow trout exposed to hypoxia. Furthermore, Pottinger and coworkers have demonstrated that rainbow trout can be segregated into high-responding (HR) and low-responding (LR) individuals on the basis of their plasma cortisol response to a defined stressor, the magnitude of the cortisol response showing both consistency over time and a high degree of heritability (Pottinger et al., 1992; Pottinger and Carrick, 1999). Behavioural stress responses have, however, not been studied in established HR and LR strains of rainbow trout. The current study was designed to quantify several behavioural patterns in these groups of fish following transfer to a previously unfamiliar, potentially stressful environment, thereby testing the hypothesis that differential stress responsiveness in rainbow trout is associated with differences in behaviour.

Materials and methods

Experimental fish

Two F_1 lines of rainbow trout *Oncorhynchus mykiss* (Walbaum) divergent for the magnitude of their plasma cortisol response to a standardised stressor (confinement) were generated by individual selection for consistently high or low post-stress cortisol values within the F_0 generation. Full details of the procedures followed and results obtained are presented elsewhere (Pottinger and Carrick, 1999). Evaluation of the cortisol response of the progeny groups to confinement established a heritability (h^2) for the trait of 0.4–0.6 (Pottinger and Carrick, 1999; Fevolden et al., 1999). HR and LR F_1 families were maintained in 1500 l outdoor tanks, supplied with lake water (25 l min^{-1} ; stocking density approximately 20 g l^{-1}) at ambient temperature, and fed three times weekly with commercial feed at the manufacturer's recommended rate (Trouw Standard Expanded 40).

Experimental protocol

The experiment was carried out during April 2000. Female HR ($N=18$) and LR ($N=18$) rainbow trout weighing $987.5 \pm 39.5 \text{ g}$ (mean \pm S.E.M.) were transferred individually from communal holding tanks to rearing in isolation in outdoor observation tanks. The polypropylene observation tanks (250 l , flow rate 15 l min^{-1}) were covered with plastic mesh (mesh diameter 1.5 cm) in order to prevent escape of the fish, while allowing for behavioural observations from above. The water temperature increased gradually from 6.2°C at the start of the experiment to 8.5°C at the end of the experiment.

From day 1 after transfer to rearing in isolation, fish were fed daily by hand (1.5% of body mass). After distribution of the food the observer stepped back and stayed motionless for 3 min while registering any food intake by the fish. All behavioural observations were carried out visually from a position 1 m away from and at an angle of approximately 45° above the tanks, thus the observer was visible to the fish during behavioural testing. Only two experimenters (Ø.Ø. and E.Ø.) carried out behavioural observations, taking care to behave similarly on all occasions and to cause minimal disturbance to

the fish. Although an influence of the presence of the observer on the behaviour of the fish cannot be excluded, each fish was approached identically and the identity of the fish (HR, LR) was unknown to the observer. All behavioural observations and blood sampling (see below) were carried out between 10.00 h and 14.00 h.

After being held for 6 days in isolation, locomotor activity was quantified as the time spent moving during a 20 min period for each fish, starting 1 h after feeding. Time spent moving was measured by triggering a stop-watch each time the fish was in motion, and stopping it when the fish was motionless. The definition of 'moving' was restricted to active locomotion transporting the fish more than 10% of its body length (as judged against the mesh covering each tank). Locomotor activity was quantified again the next day, this time with an intruder fish present in the observation tank. Intruder fish were smaller (<50% body mass of the resident fish) group-reared rainbow trout from a hatchery population, and previously unfamiliar to the test fish. The purpose of the intruder test was to stimulate aggressive behaviour in the resident fish, which inevitably leads to the intruder's defeat (Höglund et al., 2001; Winberg et al., 2001). Thus, aggressive behaviour of the resident fish can be quantified in a simulated territorial conflict with a highly predictable outcome. Behavioural observations, which this time also included observations for aggressive behaviour, started when an intruder was introduced to an observation tank, and lasted for 20 min before the intruder was removed and killed. The intruder test was only performed once with each test fish, and all intruder fish were naive to the test situation.

Blood sampling and analysis

On the day after the intruder test, 50% of the fish from each line (HR or LR) were transferred to 50 l confinement tanks that had previously been utilised to quantify the cortisol response during selection of HR and LR rainbow trout (Pottinger and Carrick, 1999). After 1 h in the confinement tanks, fish were netted and anaesthetised in 0.5 ml l^{-1} 2-phenoxyethanol, then a blood sample was obtained from the caudal sinus into a heparinised syringe. Blood samples were kept on ice for less than 1 h before centrifugation (3000 g at 4°C for 10 min). Separated plasma was transferred to polypropylene tubes and stored frozen (-20°C) until analysis for plasma cortisol levels, determined using a previously validated cortisol radioimmunoassay (Pickering et al., 1987). Following blood sampling, fish were killed by decapitation and dissected, the sex was confirmed, and the presence or absence of food in the stomach and/or intestines was recorded.

The remaining 50% of the fish from each line were sampled directly from rearing in isolation in the observation tanks, to serve as undisturbed controls. The fish were anaesthetised and blood sampled as described above. Thus, for plasma cortisol analysis four experimental groups were established: HR controls, LR controls, HR stressed and LR stressed ($N=9$ in all groups). Both controls and stressed fish had previously been tested for behavioural activity when isolated, and in the

presence of an intruder. The work was carried out within the requirements of the Animals (Scientific Procedures) Act 1986, UK.

Data analysis

Behavioural activity was quantified as the percentage of time spent moving during the 20 min observation period, and the results subjected to Kruskal–Wallis analysis of variance (ANOVA; median test) followed by groupwise comparisons by the Mann–Whitney *U*-test. The following four comparisons were made using this test: LR control *versus* LR with intruder, LR control *versus* HR control, LR with intruder *versus* HR with intruder, and HR control *versus* HR with intruder. The *P* level was adjusted for multiple tests using the Bonferroni correction method (adjusted $P=0.013$). A non-parametric method was chosen for comparing the behavioural data since variance was very different between groups (Levene's test, $P<0.001$). Cortisol concentrations in control and stressed HR and LR fish fulfilled the criteria for parametric statistics, and were analysed by ANOVA, followed by the Tukey's HSD *post-hoc* test. The frequencies of fish within the HR and LR groups feeding during the acclimation period were compared by the Fisher's exact test.

Results

Plasma cortisol

Plasma cortisol values of HR and LR rainbow trout sampled directly from observation tanks and following 1 h confinement stress are shown in Fig. 1. Both HR and LR rainbow trout had higher cortisol levels after a 1 h confinement than controls sampled directly from observation tanks ($P=0.009$ and $P=0.022$, respectively). Furthermore, HR fish had higher cortisol levels than LR fish after a 1 h confinement ($P=0.04$),

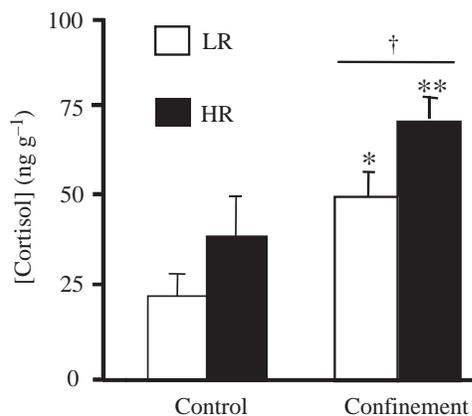


Fig. 1. Plasma cortisol levels in low-responsive (LR) and high-responsive (HR) rainbow trout sampled after rearing in isolation (Control) and following a 1 h confinement stress period (Confinement). Values are means \pm S.E.M., $N=9$ for each group. *Post-hoc* significance levels are indicated; * for a difference between controls and stressed fish, and † for a difference between LR and HR fish (*, † $P<0.05$, ** $P<0.01$).

but this difference was not significant in isolated controls ($P=0.15$).

Behaviour

Locomotor activity in isolated HR and LR female rainbow trout, when in isolation and when challenged with a conspecific intruder, is depicted in Fig. 2. The Kruskal–Wallis ANOVA indicated highly significant differences in locomotor activity between groups ($P<0.001$). Groupwise comparisons revealed that both HR and LR rainbow trout increased their activity level when the intruder was present ($P<0.001$ and $P=0.01$, for HR and LR, respectively). Furthermore, HR rainbow trout displayed higher locomotor activity than LR trout in the presence of an intruder ($P=0.006$), while there was no significant difference between the two groups in the isolated condition.

Contrary to our expectations, none of the fish tested showed any aggressive behaviour during intruder testing. Food intake was not observed during the 1-week acclimation period, but examination of gut contents following termination of the experiment revealed that food was present in the stomach of 7 out of 18 LR fish, but 0 out of 18 in the HR fish. Thus, approximately 40% of the LR fish had eaten during the experiment (although never when an observer was watching), while HR fish apparently did not ingest any food during the same period. This difference was highly significant (two-tailed Fisher exact $P=0.0076$).

Discussion

These data confirm that rainbow trout selected for a high (HR) and low (LR) plasma cortisol response to a standardised stressor show differences in behaviour. When challenged with a conspecific intruder, HR rainbow trout showed significantly

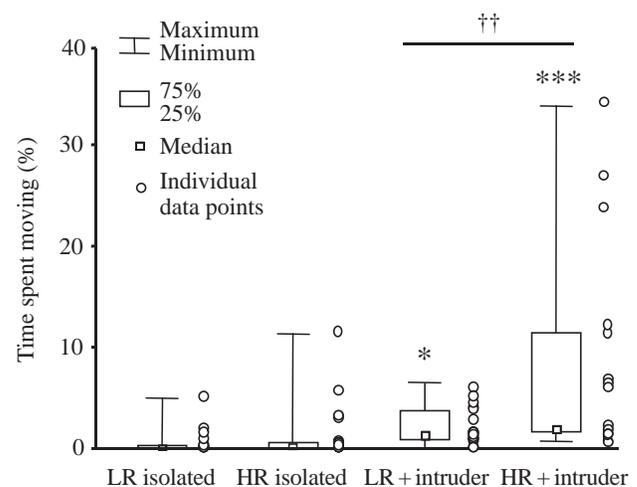


Fig. 2. Box plot showing locomotor activity in low-responsive (LR) and high-responsive (HR) rainbow trout, when alone (isolated) and when challenged with a conspecific intruder (+ intruder). *Post-hoc* significance levels are indicated; * for a difference between fish in isolation and when intruder-tested, and † for a difference between LR and HR fish (* $P<0.05$, † $P<0.01$, *** $P<0.001$) ($N=18$ for each group).

higher levels of locomotor activity than LR fish, a difference not seen in socially isolated fish. Differential cortisol responses were subsequently confirmed in the same individuals by a confinement test. Contrary to our expectations, neither HR nor LR fish exhibited aggressive behaviour when confronted with a conspecific intruder. The resident-intruder regime has previously been used as a model to quantify aggressive behaviour in juvenile rainbow trout (Höglund et al., 2001; Winberg et al., 2001), but adult rainbow trout have not been tested with this regime. Genetic factors, gender, age, season and territory quality are all factors that might affect intraspecific aggression and motivation to fight in animals. Any of these factors, or a combination of them, could explain the lack of aggressiveness towards the intruder in the present study. However, the same lines of fish did show aggressive behaviour in a pilot experiment where HR and LR rainbow trout of the F₁ generation were tested for social dominance in staged fights (T. G. Pottinger and T. R. Carrick, unpublished results). The test fish may have experienced a mild chronic stress in the observation tanks, which could have caused an inhibition of aggressive behaviour. Prolonged stress has been shown to inhibit aggression and other behaviours in fish as well as mammals (McNaughton, 1993; Winberg and Nilsson, 1993). A significant elevation of plasma cortisol levels was seen, however, in response to confinement stress in both HR and LR fish, when compared to fish sampled directly from observation tanks. Fish sampled directly from rearing in isolation are therefore referred to as controls throughout this manuscript.

A notable behavioural difference between HR and LR rainbow trout was the higher incidence of feeding in LR fish. Stressful situations, like social subordination (Øverli et al., 1998), or transfer to a new environment (Ø. Øverli, E. Larsson, and S. Winberg, unpublished results) have previously been shown to lead to a reduction or cessation of food intake in salmonid fish. In fish, as in mammals, stress-induced appetite reduction is partly mediated by signal substances that are simultaneously involved in central control of the neuroendocrine stress response (De Pedro et al., 1998a,b). Thus, the resumption of feeding after a stressful experience probably reflects a downregulation of the neuroendocrine stress response (Øverli et al., 1998). The higher incidence of feeding in LR fish thus indicates that LR fish habituated more quickly to the new environment than fish from the HR strain.

The neuroendocrine mechanisms involved in the coselection of concurrent differences in behaviour stress responsiveness remain unidentified in teleost fish. Both glucocorticoid- and mineralocorticoid-like receptors have been cloned in rainbow trout (Ducouret et al., 1995; Takeo et al., 1996; Colombe et al., 2000), and the expression and distribution of glucocorticoid receptor mRNA in behaviourally important areas of the rainbow trout forebrain have been described by Teitsma et al. (1997). The precise role of these steroid receptors in behaviour has not, however, been studied in fish. Glucocorticoid hormones may affect behaviour through genomic and non-genomic effects in the brain (Oitzl et al., 1997; Haller et al., 1998; Moore and Evans, 1999), or through their effects on energy homeostasis

(Haller, 1995). Furthermore, glucocorticoids are involved in extensive cross-talk with other behaviourally important signal substances in the brain (e.g. Marinelli et al., 1998; Schulkin et al., 1998; Chaouloff, 2000), and glucocorticoids also regulate the expression of steroid receptors in the brain and elsewhere (Lowy, 1991; Spencer et al., 1991). Accordingly, effects of glucocorticoids on behaviour are often time, dose and context dependent (e.g. Kovacs et al., 1977; Hayden-Hixon and Ferris, 1991; Sandi et al., 1996; Haller et al., 1998; Cash and Holberton, 1999; Breuner and Wingfield, 2000). Thus, although the behavioural profile of HR rainbow trout in this study is consistent with some reported effects of cortisol in poikilotherms, such as decreased appetite (Gregory and Wood, 1999) and increased locomotor activity (Cash and Holberton, 1999), the behavioural and physiological characteristics of HR and LR rainbow trout may be functionally linked through a number of factors which influence both endocrine and behavioural responses, as well as through direct behavioural effects of cortisol.

Concurrent differences in behaviour and hypothalamus–pituitary–interneural (HPI)-axis activity suggest that the HR and LR rainbow trout may represent selection for different stress-coping styles, as defined by Koolhaas et al. (1999). However, the behavioural and endocrine traits of HR and LR rainbow trout do not necessarily correspond to the active (proactive) and passive (reactive) coping styles identified in mammals. For instance, the active stress-coping style in mammals generally involves low HPA-axis responsiveness (De Boer et al., 1990; Korte et al., 1992; Fokkema et al., 1995). In our study, fish with high plasma cortisol levels following confinement stress (HR trout) also displayed the highest levels of swimming activity during the intruder test. Great care should be taken, however, when interpreting animal behaviour in terms of intentional or motivational factors. We do not know whether the behavioural activity shown by rainbow trout in this experimental setting represents an active attempt to counteract a stressor, comparable to active avoidance or defensive burying in rodents, or if it reflects anxiety or a panic reaction.

In conclusion, this study shows that differential stress responsiveness in rainbow trout is associated with differences in behaviour. HR rainbow trout increased their level of activity more than LR fish when challenged by an intruder, did not eat when reared in social isolation, and had higher cortisol levels after confinement stress. The reported behavioural and physiological differences between HR and LR fish are all potentially important to the performance of fish under conditions of artificial rearing. Furthermore, these observations raise the interesting possibility that animals with differing stress responsiveness could be identified (to be included in breeding programmes or for other purposes) by observations of simple behavioural patterns, as well as by analysis of physiological stress responses.

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