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Plasticity of boldness in rainbow trout, *Oncorhynchus mykiss*: do hunger and predation influence risk-taking behaviour?

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Abstract

Boldness, a measure of an individual's propensity for taking risks, is an important determinant of fitness but is not necessarily a fixed trait. Dependent upon an individual's state, and given certain contexts or challenges, individuals may be able to alter their inclination to be bold or shy in response. Furthermore, the degree to which individuals can modulate their behaviour has been linked with physiological responses to stress. Here we attempted to determine whether bold and shy rainbow trout, *Oncorhynchus mykiss*, can exhibit behavioural plasticity in response to changes in state (nutritional availability) and context (predation threat). Individual trout were initially assessed for boldness using a standard novel object paradigm; subsequently, each day for one week fish experienced either predictable, unpredictable, or no simulated predator threat in combination with a high (2% body weight) or low (0.15%) food ration, before being reassessed for boldness. Bold trout were generally more plastic, altering levels of neophobia and activity relevant to the challenge, whereas shy trout were more fixed and remained shy. Increased predation risk generally resulted in an increase in the expression of three candidate genes linked to boldness, appetite regulation and physiological stress responses – ependymin, corticotrophin releasing factor and $GABA_A$ – but did not produce a significant increase in plasma cortisol. The results suggest a divergence in the ability of bold and shy trout to alter their behavioural profiles in response to internal and exogenous factors, and have important implications for our understanding of the maintenance of different behavioural phenotypes in natural populations.

Introduction

Boldness defines how individuals respond to risk and novelty: bold animals are generally more active, more likely to explore novel objects or environments and spend more time in the open compared with shy conspecifics (Sih et al., 2004; Sneddon, 2003), and this variation exists along a continuum from bold to shy. Whilst many behaviours are, at least partly, heritable (Giles and Huntingford, 1984; van Oers et al., 2004), they can also be shaped by experience and animals may vary their degree of boldness according to extrinsic (environmental; e.g. Chapman et al., 2010; Frost et al., 2007) or intrinsic (age, size, etc.; Bell and Stamps, 2004; Brown and Braithwaite, 2004) factors. Since boldness influences decision making, the fitness consequences of bold or shy behaviour may be determined by the immediate environment (for example, where territory or food is limited, bolder animals may be more successful due to higher aggression and exploration tendency; Dingemanse et al., 2004); the ability to alter behaviour therefore has important implications for fitness, particularly in a fluctuating environment, since an individual may be able to adapt to the environmental conditions. The present study tested this behavioural plasticity by determining the degree to which bold or shy behaviour changes in the context of extrinsic (risk, measured as predation threat) and intrinsic (nutritional status) factors.

The wrong behavioural choices when exposed to predation threat can lead to mortality, but the optimal behavioural strategy to deal with this threat remains unclear: whilst high threat may encourage risk-taking behaviour to forage (Brown et al., 2005b), a shy strategy limits exposure through reduced activity and exploration (Archard and Braithwaite, 2011; Brydges et al., 2008). The most appropriate behavioural response may depend on additional factors such as habitat stability (Brydges et al., 2008), age (Magnhagen and Borcherding, 2008), size (Werner et al., 1983) and food availability (Borcherding and Magnhagen, 2008). Exposure to predation threat can drive the expression of boldness (Bell and Sih, 2007), but little is known as to how this process may be modulated by individual hunger levels in prey. Nutritional status is an important determinant of activity levels since animals with low energy reserves need to increase activity to forage (Borcherding and Magnhagen, 2008; Vehanen, 2003). Like predator threat, foraging profitability varies spatiotemporally; animals therefore need to adjust foraging rates and activity dependent on both profitability (Croy and Hughes, 1991) and prevailing predation risk (Lima and Bednekoff, 1999; Metcalfe et al., 1987; Vehanen, 2003; Werner et al., 1983), and it is likely these decisions may be modulated by an individual's propensity for taking risks.

The stress response in fish is controlled through activation of the hypothalamo-pituitary-interrenal (HPI) axis, resulting in the release of cortisol (Wendelaar Bonga, 1997). Likewise, much of the control of food intake takes place in the hypothalamus (Kalra et al., 1999), and utilises some of the same biochemistry. Predation threat necessarily invokes a stress response and thus may evoke a reduction in feed intake both through physiological (Scheuerlein et al., 2001) and behavioural (Metcalfe, 1987) changes to reduce feeding rates in the presence of a predator. Coping style theory predicts that bold (proactive) and shy (reactive) animals respond to stress with low or high HPI activity, respectively (Koolhaas et al., 1999; Øverli et al., 2002; Pottinger and Carrick, 2001), and previous studies using lines of rainbow trout, *Oncorhynchus mykiss*, bred for divergent stress responses revealed significantly different patterns of gene expression between low and high stress responsive fish (Backström et al., 2011; Johansen et al., 2011; Thomson et al., 2011). However, how individual differences in HPI activity and coping style reflect antipredator and foraging strategies remains relatively under-studied.

Divergent bold/shy phenotypes reflect differences in how animals respond to threat, but whilst the strategies of bold and shy fish are established under risk or food-deprived regimes it remains unclear how animals exhibiting bold or shy strategies, and consequently differential physiological activity, respond to an interaction between risk and hunger. Individual genes are important in shaping behaviour (e.g. Greenwood et al., 2008; Sneddon et al., 2011), and to understand this relationship it is thus imperative to identify correlations between gene expression and behavioural or physiological responses to such challenges. The aim of this study was therefore to determine how behavioural decisions in bold and shy rainbow trout may be influenced by exposure to varying levels of predator threat and metabolic demand. Circulating plasma cortisol levels were assessed to determine variation in stress levels in these fish, and behavioural and physiological differences were related to the expression of three specific genes known to be involved in processes of behaviour, the physiological stress response and appetite regulation: ependymin, CRF and the GABA$_A$ receptor (Table 1). Ependymin is involved in behaviours strongly linked with boldness, such as aggression (Sneddon et al., 2011) and behavioural plasticity and learning (Shashoua, 1985), but is also involved in responses to environmental stress (e.g. Tang et al., 1999). CRF plays an integral role in the corticosteroid response to stress, initiating the HPI axis through binding to CRF Type I receptors in the pituitary to stimulate the secretion of ACTH (Chrousos and Gold, 1992). CRF is also a critical hormone for the integration of sensory cues and dietary (or energetic) information with stress status, and translating this into orexigenic or anorexigenic signals (see Bernier, 2006, and references therein). Finally, the GABA$_A$ receptor, and the GABA system in general, has broad functionality and has been linked with fearfulness (Caldji et al., 2000) and aggression (Miczek et al., 2003), both indicators of boldness and stress responsiveness or coping style (Koolhaas et al., 1999). Furthermore, evidence suggests GABA$_A$ receptors may be linked with the control of appetite and feed intake (Wu et al., 2009). Divergent mRNA expression for each of these genes has previously been demonstrated in lines of rainbow trout bred for divergent stress responses (Backström et al., 2011; Thomson et al., 2011), and they are therefore excellent candidate genes to investigate links between boldness and stress responsiveness in unselected trout in the contexts presented.

Specifically, we hypothesised that (1) individuals would vary their behaviour according to prevailing risk, with the prediction that satiated fish would reduce risk-taking activity whilst food-deprived animals would take more chances; (2) fish under higher predation and/or restricted dietary regimes would experience elevated activation of the stress response, and alterations in the expression of three candidate genes implicated in feeding- and boldness-related behavioural processes.

Methodology

Test Animals

The following experiment was conducted under Home Office, UK, guidelines according to the Animal (Scientific Procedures) Act 1986, and following local ethics approval. Rainbow trout, *Oncorhynchus mykiss*, were maintained in stock tanks (2 x 2 x 0.5 m) with a semi-recirculating system on a 14:10 h light:dark cycle at 13 ± 1°C, and fed 1% body weight per day on commercial trout feed (Skretting, UK). Experimental fish (n = 75, 93.48 ± 3.94 g) were caught at random and transferred to individual glass aquaria (90 x 50 x 45 cm) which were screened from visual disturbance, and maintained at 10 ± 1°C with constant aeration. Feed were provided 1% body weight feed per day at the same time each day. The next day trout were netted, anaesthetised in 0.033 g l^{-1}, benzocaine (Sigma-Aldrich Co., UK) and weighed, and then returned to their individual aquaria and allowed to acclimate for at least one week or until the resumption of feeding. Fish that did not resume feeding after 14 days were not used in the study.

Novel Object Tests

Boldness was assessed using a standard novel object paradigm (Frost et al., 2007; Thomson et al., 2011) whereby a novel object was placed centrally into the tank and the behaviour was recorded for ten minutes (after which the object was removed). Novel objects comprised an orange rubber stopper (7.1 cm mean diameter, 4.9 cm height) and a blue transparent box weighted with gravel (7.5 x 5.3 x 3.8 cm). We focussed on two key behavioural responses (see Thomson et al., 2011 for details): 1) latency to approach to within 5cm of the novel object (s); and, 2) The duration of passive behaviour (s), which included the subject resting at the base of the tank, pivoting on its own axis, and drifting across the tank, but excluded swimming greater than one body length. Bold fish were defined as those approaching the novel object within 180 s (n = 35) and shy fish as those which did not approach within 300 s (n = 36); the remainder were classed as intermediate (n = 4) and discarded from further analysis. These were therefore clearly distinct behavioural groups, and trout exhibiting discrete suites of behavioural differences in response to a novel object have previously been successfully selected on this basis (Thomson et al., 2011).

Predation Risk and Diet Manipulations

Once each day, subsequent to the first behavioural test, fish were subjected to one of three treatments varying the level of perceived predation risk, and imposing one of two levels of feed provision (Table 2). Predation risk was simulated by using a plastic heron head (*Ardea cinerea*) mounted on a pole to simulate a predator attack (see Johnsson et al., 2001b; Jönsson et al., 1996). Attacks were made from behind a screen to prevent association with the presence of a human, and consisted of three swift strikes into the water followed by immediate removal of the model. Simulated attacks coincided with the injection of 20 ml trout alarm substance into the water to provide a chemical stimulus of risk. Alarm substance extracted from conspecific skin has been demonstrated to increase antipredator behaviour in fish (Smith, 1992) with rainbow trout increasing cover use and decreasing activity and feeding (Ashley et al., 2009; Brown and Smith, 1998). Alarm substance was prepared from dissected skin from non-experimental trout that was then washed with sterile distilled water (SDW) and homogenised in 50 ml Falcon tubes containing 6.25 ml SDW per 1 g skin. After centrifugation at 4°C, the supernatant was aliquoted and frozen at -20°C. For experiments, aliquots were further diluted (1 ml pheromone per 7 ml SDW) and a final volume of 20 ml solution used for each tank.

Fish experiencing no risk did not experience a simulated predator attack. Low risk fish were subjected to a simulated attack each day at the same time, one hour after feeding, to create a predictable threat. High risk trout were exposed to an unpredictable environment where the timing of the attack varied each day at random.

To generate different levels of hunger in the subjects, fish were fed one of two quantities of feed. Those on a high diet were fed 2% ± 0.01g body weight per day, whilst those fed on a low feed routine were only provided with 0.15% ± 0.01g body weight per day to induce fasting. Short-term food deprivation occurs in the wild due to limited food availability or low temperatures, and therefore fasting is a non-stressful natural process in rainbow trout causing a reduction in weight and condition factor (Pottinger et al., 2003); upon resumption of feeding, individuals quickly return to their former health status with no further ill-effects. Indeed, the subjects in this study did not lose weight during the trial period (Fig. 3). Fish were fed at the same time each day regardless of threat or diet regime. These conditions were applied each day for seven days, at which point a second novel-object test was used to reassess behavioural phenotype.

Hormone Analysis and Quantification of Gene Expression

After reassessing behaviour, fish were killed by concussion at the same time each day (15:00 ± 1 hr) to account for diel fluctuations in levels of plasma cortisol (Pickering and Pottinger, 1983). The fish were reweighed and a 2 ml blood sample taken from the caudal vessels into a heparinised syringe. After centrifugation, the plasma was divided into aliquots and frozen at -20°C. Plasma cortisol levels were determined by radioimmunoassay (see Pottinger and Carrick 2001 for details of protocol). The antibody employed was rabbit anti-cortisol antibody IgGF2 (IgG Corporation, Nashville). Sensitivity (minimal detection limit) of the assay was 0.3 ng ml^{-1}. The inter-assay coefficients of variation for a low (5 ng ml^{-1}) and high (53 ng ml^{-1}) plasma sample were 8.4 and 8.1% (n = 8) and the corresponding intra-assay coefficients of variation were 5.5 and 5.1% (n = 8). Cross-reactivity of the antibody with cortisone, the most significant potential competitor in rainbow trout plasma, was 2.6%.

Whole brains were removed and stored at -80°C until RNA extraction. Total RNA was extracted using TRIzol® (Invitrogen Life Science, UK). For each sample, ~1 µg of mRNA was reverse-transcribed into first strand cDNA using random hexamers and SuperScript^{TM} III reverse transcriptase (Invitrogen Life Science, UK). For RT-PCR, ~0.15 µg cDNA was amplified in a 10 µl PCR (using 5 µl Fast SYBR Green, Invitrogen Life Science, UK) primed with 2 pmol of each primer. Four primer pairs were developed using Primer Express® 3.0 (Applied Biosystems, USA) software against *O. mykiss* sequences, which were: ependymin, 5-CTCATGCTCACGCTCTGGAA-3 and 5-CCAAAAACAGCTCAACCTGATG-3; CRF: 5-GTGGTTCTGCTCATTGCTTTCTT-3 and 5-CGCCAGGGCTCTCGATAG-3; GABA$_A$ Receptor: 5-CTCATCCGAAAGCGAATCCA-3 and 5-CACACTCTCGTCACTGTAGG-3; GAPDH: 5-TGTTGTGTCTTCTGACTTCATTGG-3 and 5-CCAGCGCCAGCATCAAA-3.

Thermal cycling conditions, using a 7500 Fast Real-Time PCR System (Applied Biosystems), were: 10 min at 95°C, followed by 40X [95°C 3 s, 60°C 30 s] and then [95°C for 15 s, 60°C for 6 s, 95°C for 15 s and 60°C for 15 s], and the relative quantitation of PCR product was determined using comparisons of ΔCt (Ct of target – Ct of reference [GAPDH]).

Data Analysis

All analyses were performed in R (ver. 2.7.0; R Development Core Team, 2009). Response variables (change in behaviour between the trials; change in weight (%); plasma cortisol (ng μl^{-1}); ΔCt of target genes) were analysed with a factorial analysis using a generalized linear model; non-significant terms were systematically removed, and degrees of freedom and AIC values compared using the stepAIC function (MASS package; Venables and Ripley, 2002) to obtain the minimum adequate model (Table 3). With the exception of cortisol, all response variables fit the assumptions of GLM; cortisol data were $\log_{10}$-transformed for analysis. Since bold and shy fish exhibited a difference in the degree to which they changed their latency to approach to within 5 cm of the object, a Wilcoxon signed rank test was used to determine if these changes between trials was significant. Mann-Whitney U Tests were subsequently utilised to determine whether either 5 cm latency or the duration of passive behaviour were equivalent for bold and shy fish after the second trial.

Plasma cortisol concentration data included three points with large residuals: one shy individual (high risk) had a cortisol concentration of 110.4 ng ml^{-1}, typical of the response to a moderate stressor in this species (Øverli et al., 2002; Pottinger and Carrick, 1999) and far in excess of those exhibited by the remainder of the group. Two bold fish, one each in the no threat and low threat group exhibited cortisol concentrations (20.5 and 19.9 ng ml^{-1} respectively) which, whilst high, are of a magnitude previously observed in unstressed trout

(Øverli et al., 2002). However, it is unlikely these fish exhibited the same physiological profile as the remainder of their groups, which exhibited cortisol concentrations <4ng ml^{-1} which are more typical of an unstressed state (Balm and Pottinger, 1995; Thomson et al., 2011). On these bases, and verified through Grubbs' Tests, these data were assumed to be outliers and all analyses are thus presented with these data excluded.

Results

Behaviour

Median (min - max) initial latency to approach to within 5 cm of the object were 19.49 s (0.00 – 135.12 s) for bold fish and 600.00 (403.04 – 600.00) for shy fish. The change in latency to approach to within 5 cm of the novel object differed between bold and shy trout ($F_{1,66} = 26.04$, $p < 0.001$; Fig. 1). This was reflected by a significant increase in latency in bold fish between trials ($W_{33} = 462.0$, $p < 0.001$), but shy fish exhibited a non-significant decrease in latency ($W_9 = 8.0$, $p = 0.097$). Despite these changes, after the second trial bold trout did not become as neophobic as shy trout (median [min - max] latency after treatments = 137.86 s [0.00 – 600.00 s] for bold fish and 600.00 s [4.01 – 600.00 s] for shy fish; $W = 293.0$, $p < 0.001$, $n_1n_2 = 33, 35$).

Bold fish were also less passive than shy trout during the first trial (median [min - max] passive duration = 476.84 s [163.79 – 582.75 s] for bold fish and 572.95 s [442.42 – 600.00 s] for shy fish; $W = 151.0$, $p < 0.001$, $n_1n_2 = 33, 35$). The change in the duration of passive behaviour between the trials varied according to a three-way interaction of all parameters ($F_{2,56} = 4.95$, $p = 0.010$; Fig. 2), and varied more in bold fish than shy. Median passive duration generally increased for bold fish except for some individuals showing a decrease in activity when on a low diet and confronted with low or high predation risk. In contrast, shy fish generally did not alter their level of passive behaviour, with the exception of an increase in activity for animals under high threat and high diet.

Unsurprisingly, those fish on a higher diet gained proportionally more weight than those on a low diet ($F_{1,62} = 72.82$, $p < 0.001$; Fig. 3), but no other treatment had a significant effect.

Cortisol Measurements

After removal of outliers, no significant trend was observed between $\log_{10}$-transformed plasma cortisol and any treatment (risk, diet or boldness, or any interaction thereof: $p > 0.05$), although a possible interaction between risk and initial boldness cannot be discounted ($F_{2,54} = 2.82$, $p = 0.068$). The greatest plasma cortisol was recorded from shy fish under a low-threat regime, whilst all fish in the high risk group generally showed higher concentrations than those under low risk (Fig. 4).

Gene Expression

Diet did not influence gene expression and all effects of diet were removed from the model. Relative expression of all three genes varied according to the levels of threat experienced by individual trout. Expression of CRF was significantly greater in fish under high threat than those under no threat ($F_{2,51} = 9.20$, $p < 0.001$; Fig. 5). In contrast, expression of both ependymin ($F_{2,51} = 3.65$, $p = 0.033$) and GABA$_A$ receptor ($F_{2,50} = 4.46$, $p = 0.016$) varied according to an interaction between threat and boldness: in each case, expression generally increased with increasing threat but was highest in shy fish under unpredictable predation threat (Fig. 5).

Discussion

Being able to alter behaviour may provide an adaptive advantage, particularly in fluctuating environments, but not all individuals exhibit the same degree of behavioural plasticity. Here we demonstrate that, at least in some contexts, personality (in this case boldness) may be one determinant of the extent to which individuals can alter their behavioural profile. Bold fish were generally labile, and altered their strategy according to context, but in contrast the behaviour of shy fish was relatively inflexible in these contexts. Risk itself appeared to induce physiological and gene expression changes in these fish regardless of food availability or, to some extent, the level of boldness; thus, bold and shy behavioural profiles may arise from individual differences in the ability to respond to both external and internal cues.

Behaviour

In response to high predation threat either bold (e.g. Brown et al., 2005b) or shy (e.g. Brydges et al., 2008) strategies can be preferred, suggesting both can be adaptive antipredator strategies but likely dependent upon additional environmental contexts (Coleman and Wilson, 1998). Bold fish in this study may have behaved according to the risk allocation hypothesis and allocated food acquisition behaviour to periods when risk was low (Lima and Bednekoff, 1999); these fish altered levels of activity dependent upon predation risk and dietary regime. In general, bold trout decreased activity levels which may be an attempt to lower the encounter rate with any potential predator given the unpredictability of attacks (Anholt and Werner, 1995; Ferrari et al., 2008). Inactivity would, furthermore, conserve energy if fish were habituated to a particular feeding time (Chen and Tabata, 2002). When threat was unpredictable and food availability low, however, some bold fish increased activity, possibly to maximise food acquisition (Vehanen, 2003); greater risk-taking behaviour in bold animals may therefore be adaptive in these conditions (Brown et al., 2005b).

In comparison, shy fish exhibited an ostensibly reduced behavioural plasticity in response to a novel object compared to bold trout. Whilst potentially adaptive, phenotypic plasticity is expensive (DeWitt et al., 1998), and shy fish may be able to place less investment in plasticity as some costs (e.g. the acquisition of environmental information) may favour risk-taking behaviour. One argument may be that shy behaviour was simply the most suitable strategy in response to the presented treatments. However, changes to internal state can lead to a need to change behaviour: for instance, the potential cost of exposure to threat may be mitigated by a need to forage driven by low nutritional state, and thus below a certain nutritional threshold shy animals may begin to forage even in risky environments (Dall et al., 2004; Höjesjö et al., 1999). However, even animals in the low dietary groups in this study gained weight, and thus this threshold may not have been reached and the need to forage regardless of danger not achieved. Since shy animals are less prone to taking risks than bold conspecifics, the threshold nutritional level before which shy animals are prepared to forage under threat may be lower. Reduction in feeding (such as that which could be caused by the threat of predation; Metcalfe et al., 1987) can result in a reduced body condition (e.g. Höjesjö et al., 1999) which was not observed in the present study despite similar quantities of feed being provided, possibly suggesting a difference in the quality of feed. Nutrient profiles of feed may thus be a more useful measure in future studies than supply by weight (e.g. in Borcherding and Magnhagen, 2008). However, whilst growth rates were positive in the present study, the high threat group presented a possible lower rate of growth compared to low and no threat, and a sustained period of high threat may result in a more conspicuous deterioration in weight gain relative to exposure to lower threat levels.

The antipredator response often derives from experience (Kelley and Magurran, 2003), without which animals may have impaired behavioural and physiological reactions to a threat

(Brown et al., 2005a; Johnsson et al., 2001a) which may be evident in cultured fish such as those used here (Álvarez and Nicieza, 2003). Whilst this should be considered when interpreting behaviour, alarm pheromone does elicit antipredator responses even in farmed trout (Ashley et al., 2009), though comparisons between wild and farmed individuals could be explored in future studies.

Cortisol Measurements

In contrast to the behavioural responses of these fish, the endocrine and gene expression data suggest that both bold and shy fish were experiencing similar physiological and genetic responses to the stimuli. Cortisol levels were marginally higher, although not significantly so, in both bold and shy fish under high predation threat compared to none: in general, fish in or from a riskier environment tend to show heightened stress indicators (Brown et al., 2005a), though in this case the cortisol increase was not as profound as what would be expected after an acute and substantial stress (cf. confinement: Øverli et al., 2002; Pottinger and Carrick, 1999; emersion: Sloman et al., 2001; Thomson et al., 2011). Rather than being an acute response to the behavioural test, these values may therefore reflect baseline HPI activity which has been modified by exposure to the treatment regimes. The functional significance of variation in blood cortisol levels of unstressed fish remains relatively unknown. Here, no significant differentiation in plasma cortisol concentrations was observed, yet the data suggest a possible divergence in HPI activity between bold and shy fish exposed to a predictable threat. Rainbow trout are able to precisely anticipate daily feeding times (Chen and Tabata, 2002), and it seems likely that other types of routine event can likewise be predicted. Animals of different personality or coping style may have divergent abilities to predict such events, and certainly there are marked differences in cognitive performance between trout exhibiting divergent coping styles (Moreira et al., 2004). Shy (reactive) animals may be better at dealing with unpredictable events than bold (proactive) animals, which are better at learning and retaining conditioned stimuli and dealing with routines (Koolhaas et al., 1999; Sneddon, 2003, Ruiz-Gomez et al., 2010). Therefore, elevated cortisol levels in shy fish may represent an anticipatory response to the stimulus which the bold fish have already learned is not a threat. On the other hand, unpredictability may drive the development of bold behaviour as risk-taking, not risk-aversive, strategies will be favoured when environmental variables cannot be accurately predicted (Chapman et al., 2010). Current literature, therefore, provides inconsistent evidence in behavioural and physiological comparisons of animal responses to predictable and unpredictable events, aversive or positive (Bassett and Buchanan-Smith, 2007). Additionally, the influence of personality on the physiological responses to predictability of an aversive stimulus remains unclear, and future work should address this.

Gene Expression

Two of the genes utilised in this study, CRF and $GABA_A$ receptor, have been implicated as having roles in neural pathways controlling appetite and feed intake (Bernier and Craig, 2005; Pu et al., 1999), yet expression of these genes in this study was not correlated with dietary regime. Instead, since these genes are also linked with stress reactivity (CRF: Chrousos and Gold, 1992; GABA, Makara and Stark, 1974), and $GABA_A$ receptor in the expression of fear (Caldji et al., 2000), it is likely that increased expression of these genes with higher risk represents a molecular response to predation risk. Risk can, however, reduce foraging without affecting appetite (Metcalfe, 1987), and since exposure to a predator induces a stress response it seems likely that upregulation of CRF and $GABA_A$ receptor in high threat conditions may represent simultaneous activation of both physiological stress response and appetitive pathways.

Ependymin plays an important role in behavioural responses to the environment (Shashoua, 1991). One such role is that of memory formation (Shashoua, 1991), and variation in its expression may therefore be linked with the anticipation of events, such as predator attacks as observed in this study. This effect was strongest in shy fish, which are known for reduced competitive ability compared to bold animals, an effect also linked with upregulation of ependymin (Aubin-Horth et al., 2005; Sneddon et al., 2011). These data may therefore suggest an important link, at the level of gene expression, between individual boldness and how individuals react to the regularity of predator threat.

Conclusions and Implications

In the present study we demonstrated clear differences in the behavioural responses of bold and shy rainbow trout to variations in nutritional state and predation threat, where bold fish exhibited greater behavioural plasticity in their response to novelty than shy fish. In contrast, only slight differences between bold and shy fish in plasma cortisol concentrations were observed and trends in gene expression were dominated by responses to predation threat rather than initial boldness. These data therefore highlight the divergence of phenotypic plasticity in response to a particular stimulus within a species whilst providing information on the roles of physiology and gene expression in response to these contexts. State and context-dependent effects, such as prevailing predation pressure, may generate evolutionary constraints particular to one species or population. Future work therefore needs to focus on how personality affects responses to multiple challenges, and apply this to complex environments to elucidate the functional significance of variation in both behaviour and its plasticity in nature. Furthermore, studies should continue to attempt to determine how behaviour and plasticity are related to key physiological and genetic mechanisms which may drive these differences in personality.

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Table 1: Genes used in this study, their abbreviations and relevant roles.

Gene	**Abbreviation**	**Role**
Ependymin	Epd	A glycoprotein implicated in environmental adaptation, particularly linked to boldness[1].
Corticotrophin Releasing Factor	CRF	A hypothalamic neurotransmitter hormone which activates the HPI axis by binding with CRF Type I receptors in the anterior lobe of the pituitary to stimulate the release of adrenocorticotrophic hormone[2]. Acts as an anorexigenic factor[3].
γ-Aminobutyric Acid A Receptor	$GABA_A$	A receptor protein with diverse functionality, including roles in the control of ACTH release in the stress response[4], the control of appetite[5], and also lined with the expression of boldness (e.g. aggression[6]).
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Reference gene

[1]Sneddon et al. (2011); [2]Chrousos and Gold (1992); [3]Bernier and Craig (2005); [4]Makara and Stark (1974); [5]Pu et al. (1999); [6]Miczek et al. (2003).

Table 2: Treatment combinations and sample size for individual rainbow trout, *Oncorhynchus mykiss*, used in this study; each trout was exposed to one level of threat alongside one diet regime throughout the seven-day test period. Asterisks indicate groups where a subject was removed from analysis due to high cortisol levels (see Methodology).

Threat regime	**Diet regime**	***n***	
		Bold	***Shy***
None	Low (0.15% bw)	7	8
	High (2% bw)	7*	8
Low (Predictable)	Low (0.15% bw)	5	5
	High (2% bw)	4*	5
High (Unpredictable)	Low (0.15% bw)	5	4*
	High (2% bw)	5	5

Table 3: Akaike Information Criteria (AIC) and residual degrees of freedom (d.f.) for full models and, where appropriate, minimum adequate models for each response variable. Full model was defined as *response* ~ *risk* × *diet* × *boldness*, and terms retained in the selected model are also presented.

	Full Model		**Minimum Adequate Model**		
Response Variable	**AIC**	**d.f.**	**Retained terms**	**AIC**	**d.f.**
Δ5cm Latency (s)	955.5	56	*boldness*	940.9	66
ΔPassive Duration (s)	875.5	56	-	-	-
ΔWeight (%)	474.5	50	*risk*, *diet*	465.5	58
log$_{10}$-Cortisol (ng ml^{-1})	120.1	48	[*risk* × *boldness*]	108.6	54
ΔCt(Ependymin)	193.5	45	[*risk* × *boldness*]	187.8	51
ΔCt(CRF)	206.7	45	[*risk* × *boldness*]	200.5	51
ΔCt(GABA$_A$)	192.6	44	[*risk* × *boldness*]	185.0	50

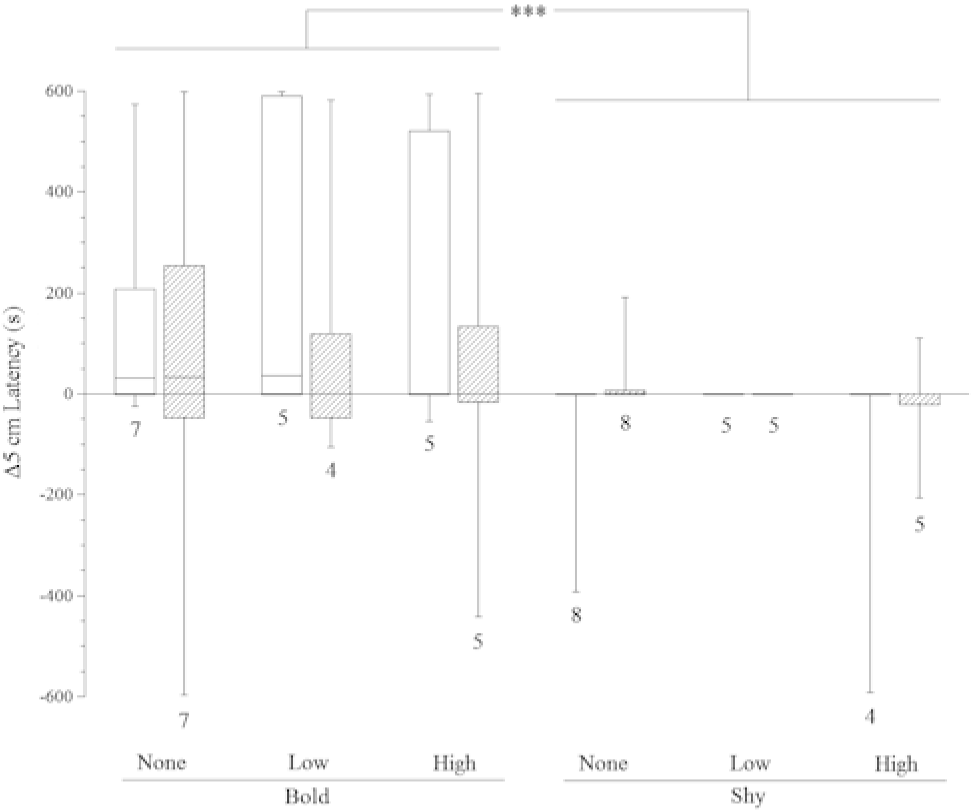

Figure 1: Median (upper and lower boundaries are 25th and 75th percentiles) change in latency between trials (s) for bold and shy rainbow trout, *Oncorhynchus mykiss*, to approach within 5 cm of a novel object. Individual trout experienced no, low or high predator threat and had either low (white, left box) or high (hatched, right box) feed availability. *n* for each treatment indicated below boxes. Asterisks represent a significant difference between groups (***, $p < 0.001$).

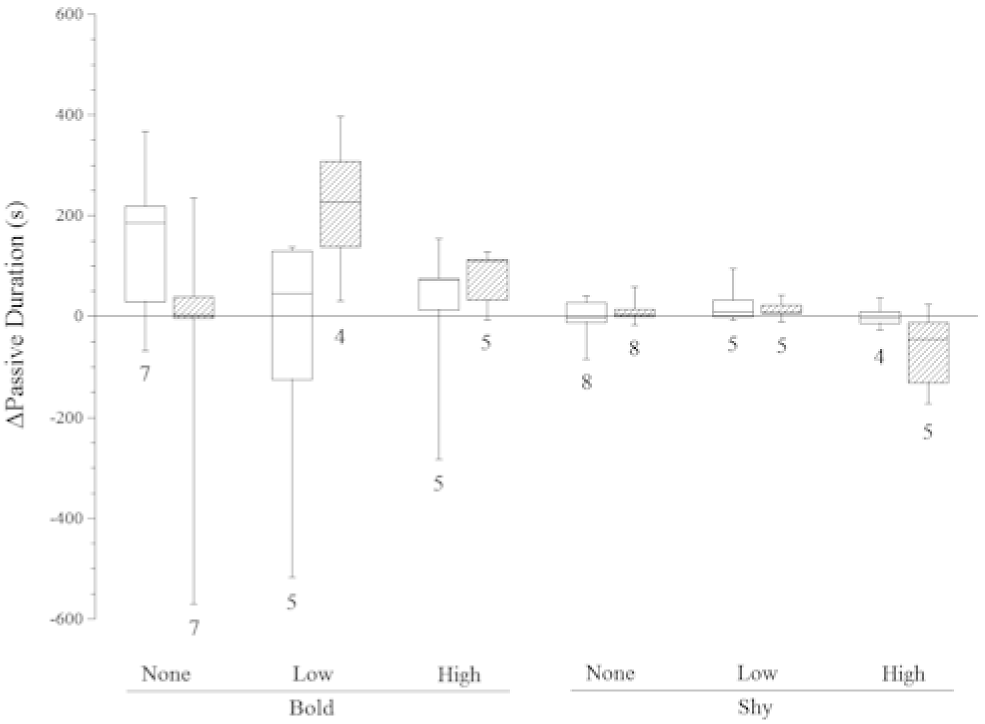

Figure 2: Median (upper and lower boundaries are 25th and 75th percentiles) change in duration of passive behaviour between trials (s) for bold and shy rainbow trout, *Oncorhynchus mykiss*, under a no, low or high threat regime and low (white) or high (hatched) food availability. *n* for each treatment indicated below the boxes. Overall change in passive duration differed between treatments according to a three-way interaction between level of risk, diet and initial boldness ($p = 0.010$).

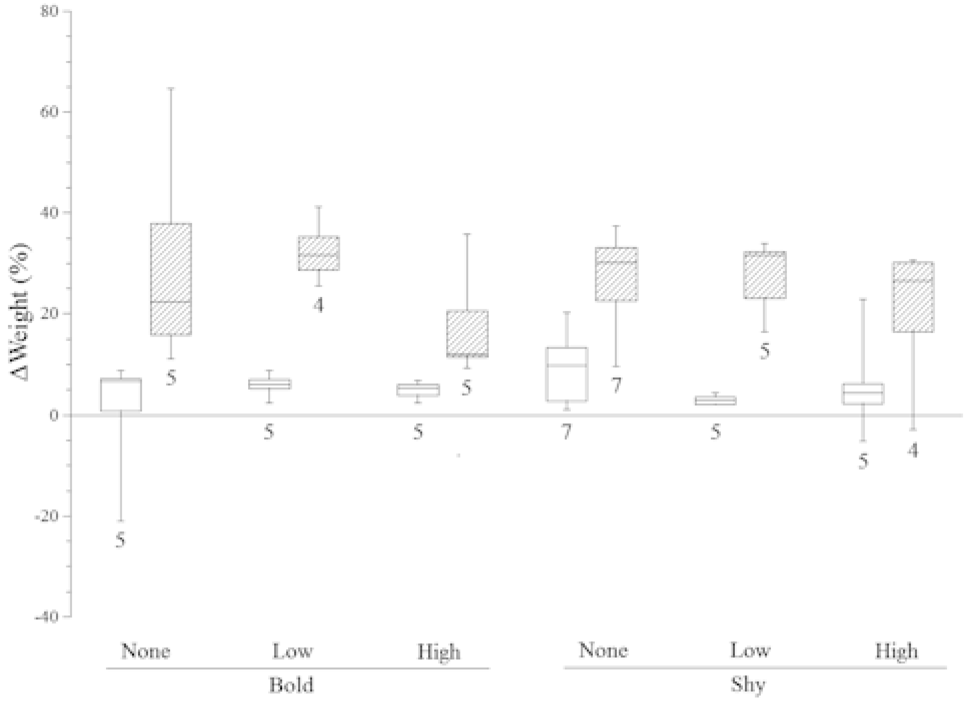

Figure 3: Mean (± SE) change in weight (%) in rainbow trout, *Oncorhynchus mykiss*, under no, low or high predation threat and low (white) or high (hatched) feed availability. *n* for each treatment indicated below the boxes. Means that do not share a common lowercase letter were significantly different ($p < 0.05$).

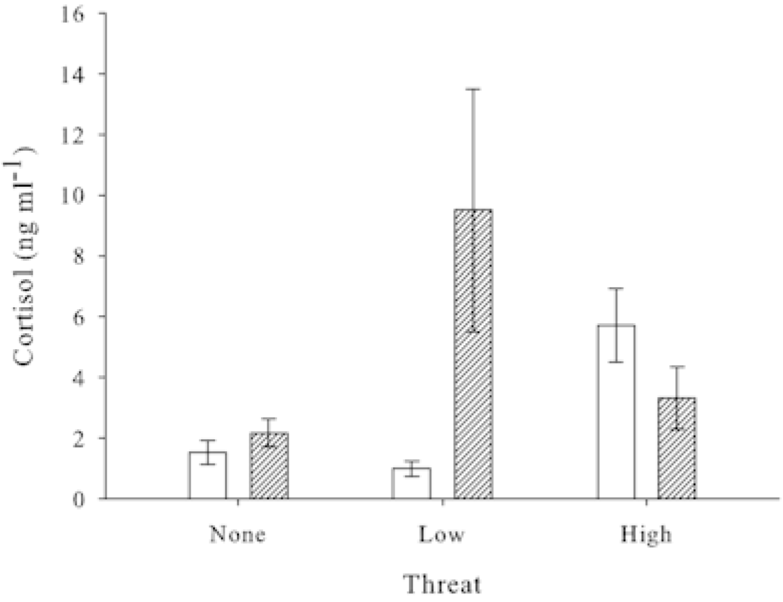

Figure 4: Mean (±SE) plasma cortisol (ng ml^{-1}) in bold (white) and shy (hatched) rainbow trout, *Oncorhynchus mykiss*, exposed to no, low or high levels of predation threat. *n* (no, low, high threat) for bold trout = 10, 9, 10 and for shy trout = 14, 10, 10, with three outlier points removed.

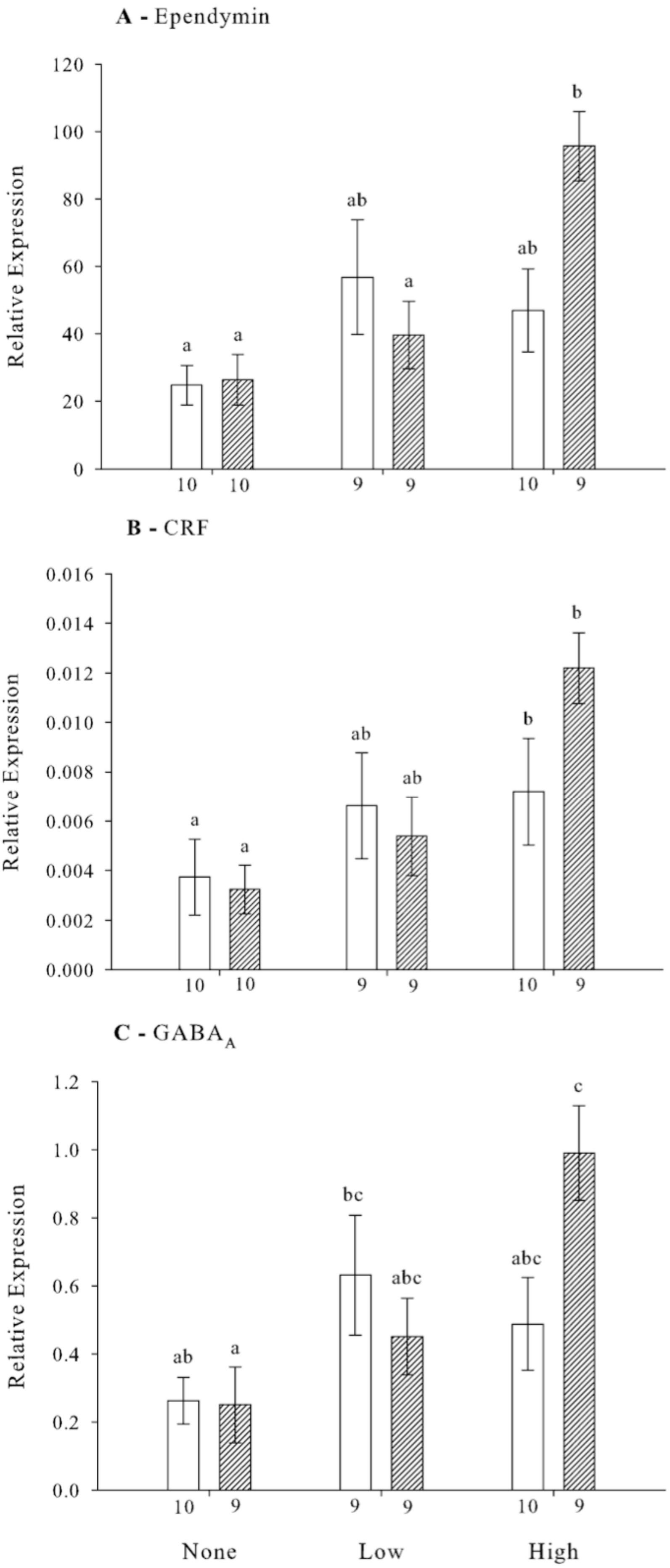
A - Ependymin
Relative Expression
120
100
80
60
40
20
0
a
a
ab
a
ab
b
10
10
9
9
10
9
B - CRF
Relative Expression
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
a
a
ab
ab
b
b
10
10
9
9
10
9
C - GABA$_A$
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ab
a
bc
abc
abc
c
10
9
9
9
10
9
None
Low
High

Figure 5: Mean (± SE) relative expression, $[(E_{\mathrm{Ref}}) \wedge (\mathrm{Ct}_{\mathrm{Ref}})] / [(E_{\mathrm{Target}}) \wedge (\mathrm{Ct}_{\mathrm{Target}})]$ where E = efficiency of the reaction, of (A) ependymin, (B) CRF (corticotrophin releasing factor) and (C) $\mathrm{GABA_A}$ (γ-aminobutyric acid A) receptor in the brains of bold (white) and shy (hatched) rainbow trout, *Oncorhynchus mykiss* under no, low and high predation threat. Sample sizes are presented beneath each bar. Means that do not share a common lower case letter were significantly different (Tukey HSD, $p < 0.05$).