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Personality, plasticity, and predation: linking endocrine and behavioural reaction norms in stickleback fish

Ines Fürtbauer*[a], Alice Pond[a], Michael Heistermann[b] & Andrew King[a]

[a]College of Science, Department of Biosciences, Swansea University, Swansea, UK
[b]Endocrinology Laboratory, German Primate Center Göttingen, Germany

* Corresponding author: I.Fuertbauer@swansea.ac.uk

Summary

1. Predation plays a fundamental role in evolutionary processes, driving changes in prey morphology, physiology, and behaviour. With organisms being increasingly exposed to rapid environmental changes, there is growing interest in understanding individual phenotypic plasticity in responses to changes in predation pressure.

2. Behavioural and physiological responses to predator exposure are of particular interest as differences in predation pressure are often reflected in correlated suites of behavioural and hormonal profiles across populations. Within populations, the association between endocrine profiles and behaviour is less understood, and often lacking.

3. Adopting a reaction norm approach and a repeated measures design, we assessed within-population effects of changes in perceived predation risk on endocrinology and behaviour in three-spined sticklebacks (*Gasterosteus aculeatus*). We repeatedly exposed subjects to a robotic model predator, and assessed their behavioural response. The fish showed consistent behavioural profiles and were less active and shyer when predation risk was higher.

4. Using non-invasive waterborne hormone analysis, we assessed basal cortisol as well as the cortisol response to changes in predation risk. Individuals showed significantly higher cortisol levels following exposure to the model predator. Individual post-predator exposure cortisol was repeatable but unrelated to behavioural responses. Accounting for between versus within subject effects, we found that basal cortisol and shyness were positively related within individuals, i.e. individuals overall were shyer on days they had higher cortisol levels. We also tested if basal testosterone predicted risky behaviour and found no evidence for this hypothesis.

5. No individual differences in hormonal or behavioural responses to changes in predation risk were found, suggesting that individuals are not constrained by their personalities in their ability to cope with a potentially harmful threat.

6. Overall, we show that individuals of different personalities are equally “flexible” in their response to changes in predation pressure. Our study offers novel insight into consistent individual differences and plasticity in hormones and behaviour as well as their interplay within populations. Future studies should assess the applicability of these findings to other changes in the environment, as well as the effects of social context on endocrine and behavioural reaction norms.

Keywords: Anti-predator response, Coping styles, Cortisol, Individual differences, Shyness, Stress, Testosterone, Waterborne hormone analysis

Introduction

Predation plays a fundamental role in animal evolutionary processes (Abrams 2000), driving changes in phenotypic traits of prey (and predators). For example, predation risk can induce changes in nest-site selection and clutch size in the Siberian jay (*Perisoreus infaustus*, Eggers et al. 2006), and can cause lizards (*Anolis sagrei*) to alter their habitat use (Losos, Schoener & Spiller 2004). Such adaptive changes can occur through natural selection across generations (Cousyn et al. 2001), or within the lifetime of an individual, with predation risk influencing the expression of heritable variation during development (Dingemanse et al. 2009), or in direct phenotypic responses to recent experience (Magnhagen et al. 2012). With organisms being increasingly exposed to rapid environmental changes such as habitat disruption or ecological invasions that can increase exposure to novel threats (Sih 2013), there is a growing interest in understanding individual phenotypic plasticity in response to change (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010; Sih et al. 2012; Dingemanse & Wolf 2013; Wingfield 2013).

There is vast evidence documenting individual differences in behaviour, which are consistent across time and contexts, i.e. 'animal personalities' (or 'coping styles' , 'behavioural types', 'temperament', 'behavioural syndromes' ; e.g. Koolhaas et al. 1999; Sih, Bell & Johnson 2004; Reale et al. 2007; Réale et al. 2010). However, only recently have these consistent individual differences in behaviour been linked to differences in the responsiveness to environmental change, by adopting the concept of "reaction norms" (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010; Dingemanse & Wolf 2013). Behavioural reaction norms include information on how an individual behaves on average (i.e. its personality or statistically its intercept) and how its behaviour changes over a specific environmental gradient (i.e. its plasticity or statistically its slope) (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010; Dingemanse & Wolf 2013). Importantly, reaction norms allow one to assess the effects or constraints of personality upon individual responses to changing environments. In tree swallows (*Tachycineta bicolour*), for instance, more aggressive individuals are better able to adjust to variation in temperature (Betini & Norris 2012), and in great tits (*Parus major*), personality predicts responsiveness to predation risk (Quinn et al. 2012).

On a proximate level, shifts in behavioural traits are often mirrored or mediated by changes in hormone profiles and there is increasing interest in understanding the relationship between physiology and behaviour (McGlothlin & Ketterson 2008; Ketterson, Atwell & McGlothlin 2009; Killen et al. 2013). In general, the endocrine system responds to environmental changes to help the body cope with challenges, i.e. “stressors”, with the release of glucocorticoids (cortisol in most mammals and fish; corticosterone in birds, amphibians, reptiles, and rodents) from the hypothalamic-pituitary-adrenal (HPA) axis (e.g. Wingfield 2013).

Stress coping styles, i.e. “a coherent set of behavioral and physiological stress responses which is consistent over time and which is characteristic to a certain group of individuals” (Koolhaas et al. 1999) are often found on a population-level. For instance, populations of the Panamanian bishop (*Brachirhaphis episcope*) exposed to high predation pressure are more exploratory and active and exhibit lower cortisol release rates in response to a stressor compared to populations exposed to low predation pressure (Archard & Braithwaite 2011; Archard et al. 2012; see Figure 1A). Likewise, guppies (*Poecilia reticulata*) from high-predation sites have lower basal cortisol levels than conspecifics from low-predation sites (Fischer et al. 2014), and rainbow trout (*Oncorhynchus mykiss*) selected for high cortisol responses are less bold and aggressive than those selected for low cortisol responses (Pottinger et al., 1992). Within populations, however, the link between hormones and behaviour is often lacking (Bell et al. 2007; Archard et al. 2012). A potential reason for this is that researchers tend to adopt a single, rather than repeated measures, sampling design (see Figure 1AB). Single measure designs, by definition, do not allow estimation of within (versus between) subject effects. Given the extensive individual variation in the glucocorticoid stress response across vertebrates (Cockrem 2013), repeated measures designs have potentially important implications for understanding the evolution and maintenance of coping styles (Figure 1C).

Another endocrine axis, closely linked to the HPA axis, which has been associated with coping styles but has received far less attention is the hypothalamic-pituitary-gonadal (HPG) axis which controls the secretion of sex steroids (androgens, oestrogens, and progestogens). Most research on sex steroids and personality has been on humans, focussing on the positive effect of testosterone on risk-taking and sensation-seeking behaviours in men (reviewed by Caramaschi et al. 2013). In non-human animals, e.g. birds and rodents, testosterone levels have been linked to aggression, coping, and exploration (reviewed by Caramaschi et al., 2013). Although, to date, relatively little is known about HPG functioning and personality traits, the (potential) effect of testosterone on risk-taking may, in addition to glucocorticoids, be an important factor in determining antipredator behaviour.

Here, we adopt a reaction norm approach using wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*; Linnaeus, 1758; Figure 2) from the same population, allowing us to investigate potential effects of coping styles on responses to changes in predation risk. We manipulate ‘perceived predation risk’ (sensu Dingemanse et al. 2010), and repeatedly collect phenotypic data on waterborne hormone levels and behavioural measures from the same individuals. Crucially, the use of a non-invasive sampling method (see methods) allows the same subjects to be sampled repeatedly, which is necessary for assessing individual variation in the glucocorticoid stress response (see Cockrem 2013), and within versus between subjects effects of hormones on behaviour (see Figure 1B,C). Specifically, we aimed: (1) to assess individual consistency and repeatability in behavioural and hormonal responses (i.e. personality), (2) to assess the plasticity, and potential constraints of personality, on the behavioural and glucocorticoid stress response to changes in perceived predation risk, and (3) to test whether hormonal and behavioural responses are linked (across and/or within subjects), i.e. whether cortisol and/or testosterone levels predict phenotypic variation in risky behaviour.

Sticklebacks are a major model in behavioural ecology (Huntingford & Ruiz-Gomez 2009) and are ideally suited for studying endocrine and behavioural responses to predators in the context described above. They exhibit consistent inter-individual variation in behavioural responses to predation (reviewed by Huntingford & Coyle 2007), and predation pressure generates different behavioural (Dingemanse et al. 2007), morphological (Reimchen 1994), and physiological (Bell, Henderson & Huntingford 2010) profiles. Also, repeated exposure to predator cues leads to significant transcriptomic changes in the brain (Sanogo et al. 2011). Furthermore, sticklebacks show increased glucocorticoid concentrations in response to both acute and chronic stressors (Pottinger, Carrick & Yeomans 2002; Bell et al. 2007; Fürtbauer, King & Heistermann, revision under review).

Materials and Methods

STUDY ANIMALS

Three-spined sticklebacks are known to display both sex-differences in personality (King et al. 2013) and stress-induced cortisol responses (Pottinger et al. 2013). Therefore, this study focussed on non-gravid females (n=20; mean±standard deviation (sd), body mass and length 1.5±0.3 g and 5.3±0.3 cm), wild-caught on Swansea University Campus (Figure 2). Subjects were initially housed in a large holding tank (30x39x122 cm), containing gravel substrate, plants, and drift wood. Fish were kept at a constant temperature/photoperiod regime (17°C/8L:16D). Two weeks prior to behavioural tests, the subjects were transferred to individual 2.8l gravel-lined aerated tanks in which they were housed throughout the entire test period. Fish were fed once daily between 0830 and 0900 h with defrosted bloodworms. All procedures described were approved by Swansea University's Ethics Committee (IP-1213-3).

HORMONE SAMPLE COLLECTION, EXTRACTION, AND ANALYSIS

In fish, exposure to a stressor leads to the activation of the hypothalamic-pituitary-interrenal (HPI) axis, resulting in increased cortisol secretion within minutes (reviewed by Pankhurst 2011; Cockrem 2013). Free cortisol (and other steroid hormones) in fish diffuses from the bloodstream into the water through the gills (Vermeirssen & Scott 1996; Ellis, James & Scott 2005). Confining a fish to a known volume of water for a known period of time allows to extract these steroids and quantify their concentrations (Hirschenhauser et al. 2004; Ellis, James & Scott 2005; Scott & Ellis 2007; Sebire, Katsiadaki & Scott 2007; Wong et al. 2008; Sebire, Katsiadaki & Scott 2009; Kidd, Kidd & Hofmann 2010; Archard et al. 2012; Fischer et al. 2014; Fürtbauer, King & Heistermann, revision under review) which correlate with concentrations of the free fraction of hormones in the blood (Scott & Ellis 2007; Sebire, Katsiadaki & Scott 2007). This method of measuring waterborne hormones in teleost fish is extremely practical with smaller fish since it negates the need for sacrificing the fish to obtain blood samples for hormone measurements (reviewed by Scott & Ellis 2007).

A total of n=286 (mean±sd: 14.3±2.2 per female, n=20 females; two individuals died during the study) waterborne hormone samples were collected in order to assess (i) habituation effects across five consecutive days (Wong et al. 2008; Fischer et al. 2014) prior to behavioural and hormone data collection, (ii) basal cortisol and testosterone levels (i.e. before the predation risk phase) and (iii) post-predator exposure cortisol concentrations (post-predator exposure testosterone was not measured as individuals were tested in a non-social context, see e.g. Oliveira et al. 2002). Subjects were weighed prior to each hormone sample collection. Following previously described procedures for sticklebacks (Sebire, Katsiadaki & Scott 2007; Sebire, Katsiadaki & Scott 2009, Fürtbauer, King & Heistermann, revision under review), fish were confined individually for 1 h in a 150 ml glass beaker (rinsed with 99.9% methanol and distilled water prior to use), filled with 50 ml water (same source as used for tanks). To remove particulate matter, water samples were filtered through a net rinsed with distilled water, and transferred into 60 ml polypropylene tubes, and stored at -18°C until further processing.

For hormone extraction, samples were thawed and loaded onto Waters Sep-Pak Plus C18 solid phase extraction cartridges placed onto a 12-port vacuum manifold connected to a vacuum pump. Prior to sample loading, cartridges were primed with 5 ml methanol followed by 5 ml distilled water. After the samples had been passed through, the cartridges were washed with 5 ml distilled water, followed by 20 ml air to remove water. Steroids were eluted with 5 ml absolute methanol, collected in a glass tube and evaporated under nitrogen at 45°C. The dried extracts were sent to the Endocrinology Laboratory of the German Primate Center, Göttingen, Germany.

Steroids were redissolved in 350 µl assay buffer, and analysed for immunoreactive cortisol and testosterone using enzyme immunoassays (Palme & Möstl 1994, 1997). All samples were run in duplicate, and samples with a CV above 7% between duplicates were re-measured. Sensitivity of the both assays at 90% binding was 0.5 pg. Intra- and inter-assay coefficients of variation, calculated from replicate determinations of high- and low- value quality controls were 7.4% (n=16) and 11.0% (n=20) (high) and 8.9% (n=16) and 15.2% (n=20) (low) for cortisol, and 6.6% (n=16) and 7.4% (n=16) (high) and 8.9% (n=16) and 14.3% (n=16) (low) for testosterone. Hormone data are expressed as ng/g/h.

BEHAVIOURAL TESTS AND TRACKING

Throughout five consecutive weeks, behavioural tests were conducted Mon-Thu. Five fish were tested per day, and each individual was tested each week on the same weekday but at a different time. Behavioural tests were conducted in transparent plastic “test tanks” (WxLxH: 15x54x24), filled up with water to 12.5 cm. A green plastic plant was positioned on one end to provide cover (Figure 3). In order to prevent potential cues from affecting the response of subsequently tested fish, five separate test tanks were used. The test tank was positioned in a rectangular test arena, covered with white sheets on all sides. Behaviours were filmed using a Panasonic HDC-SD60 high definition video camera, mounted above the test tank (Figure 3). Following hormone sample collection to assess cortisol and testosterone baseline concentrations, the subject was placed in the test tank for 20 minutes (“low predation risk phase”). Subsequently, we simulated an aerial predator attack using a polymer clay model heron head which was attached to a Robot Arm with USB PC Interface (Figure 3). The ‘robotic heron’ was hidden behind a curtain before the simulated attack. After exposure to the model predator, the behaviour of the fish was recorded for 20 minutes (“high predation risk phase”). After completion of the tests, water hormone samples were collected as described above, and subjects were returned to their individual tanks.

Automated video tracking was performed using EthoVision XT 9 software (Noldus Information Technology Inc.; Noldus, Spink & Tegelenbosch 2001). The detection settings for each video were set to differencing and a sample rate of 5 frames per second. Six behavioural variables were extracted for both low and high predation risk phases: total distance moved [cm], mean speed [cm/s], time spent in cover [s], and transitions out of cover (i.e. the frequency of movements out of cover into the open area of the tank), and two measures of mobility: “immobility” and “high mobility”. These were calculated based on comparisons of the locations of the tracked fish between consecutive images: where there is a perfect overlap of two consecutive images (i.e. the fish is still), there is a 0% change; where there is no overlap (i.e. fish has moved to a new location) there is a 100% change. Immobility (or “freezing”) was the duration of time [s] for which the tracked fish image change was <10%, and high mobility was the duration of time [s] when the velocity of fish movement was >80%.

DATA ANALYSIS

In order to summarise the six behavioural variables (see above), we used Principal component analysis (PCA) in SPSS Statistics 17.0. We used Linear Mixed Models (LMMs) in R (R Development Core Team; package lme4, Bates & Maechler, 2010) to analyse behavioural and hormonal patterns across low- and high predation risk phases. Hormone data were log-transformed to achieve normality. To assess whether environmental change (E; i.e. differences in perceived predation risk; fixed effect), the individual (I; random intercept) or the interaction between individual and changes in predation risk (I x E; ID as random intercept; predation risk as random slope) affected cortisol levels and behaviours, we used a reaction norm approach (Nussey, Wilson & Brommer 2007; Dingemanse et al. 2010) and performed three sets of simple model comparisons (for cortisol, activity, and shyness) using log-likelihood ratio tests following Carter, Goldizen & Heinsohn (2012). In each model, we included "day" (i.e. the date) as a random effect to control for temporal effects. To investigate whether cortisol and testosterone concentrations predicted behaviour, and to account for between versus within subject effects, we included daily hormone levels per subject (centred to a mean of zero per subject) as well as the mean hormone levels per subject as fixed effects (van de Pol & Wright 2009). Repeatability of hormones and behaviour was assessed by calculating intraclass correlation coefficients (ICCs) and 95% confidence intervals (CIs) (Lessels & Boag 1987) in SPSS Statistics 17.0.

Results

INDIVIDUAL CONSISTENCY AND REPEATABILITY IN BEHAVIOURAL AND HORMONAL MEASURES

Principal Component analysis of the six behavioural variables revealed two Principal Components (PCs), explaining 75% of the total variance (Table 1). Distance, high mobility, speed, and transitions out of cover positively loaded on PC1 (hereafter referred to as "activity"), and immobility and time spent in cover positively loaded on PC2 (hereafter referred to as "shyness"). Activity was repeatable during both low and high perceived predation risk across weeks (low risk: ICC=0.26, $p=0.001$; high risk: ICC=0.36, $p<0.001$, Table 2). Average activity during low and high predation risk phases were significantly positively correlated (Pearson's rho=0.641, $p=0.002$, $n=20$; Figure 4A). Shyness was not repeatable during low predation risk phase, and a non-significant trend was found for the high predation risk phase (low: ICC=0.08, $p=0.157$; high: ICC=0.11, $p=0.076$, Table 2). Average shyness during low and high predation risk phases were significantly positively correlated (Pearson's rho=0.451, $p=0.046$, $n=20$; Figure 4B).

Cortisol concentrations varied significantly across the five habituation days (Friedman test: $\chi^2=29.32$, df=4, $p<0.001$, $n=20$) and decreased significantly from day 1 to day 3 after which they remained stable (Wilcoxon signed ranks test: day 1 to day 3: $Z= -2.389$, $p=0.017$, $n=20$; Figure 5). Post-predator exposure cortisol concentrations were repeatable (ICC=0.34, $p<0.001$; Table 3) and a non-significant trend was found for basal cortisol (ICC=0.11, $p=0.087$; Table 3). Cortisol levels before and after exposure to the model predator were not correlated (Spearman's rho=0.179 $p=0.450$, $n=20$). No significant difference was found in testosterone concentrations across the five habituation days (Friedman test: $\chi^2=2.08$, df=4, $p=0.721$, $n=20$; Figure 4), and testosterone was not repeatable between weeks (ICC=-0.01, $p=0.515$; Table 2).

BEHAVIOURAL AND GLUCOCORTISCOID STRESS RESPONSES TO PERCEIVED PREDATION RISK

Both activity and shyness altered in response to the change in perceived predation risk (E; Table 3). Individuals showed significantly lower activity during the high- compared to low predation risk phase (estimate±standard error (se)=-0.43±0.12, t=-3.62, p=0.003; Figure 6A). Shyness was higher during the high- compared to the low predation risk phase (estimate±se=0.40±0.12, t=2.91, p=0.004; Figure 6B). Individual had a significant effect on activity but not shyness (I, Table 3). Neither activity nor shyness showed a significant E x I interaction (Table 3), indicating no individual differences in the behavioural response to changes in predation risk.

Comparing basal and post-predator exposure cortisol revealed that cortisol was significantly higher after exposure to the simulated aerial predator (estimate±se=0.62±0.10, t=6.26, p<0.001; Figure 6C, Table 3). Individual had a significant effect on cortisol levels but no E x I interaction was found (Table 3), indicating no individual differences in HPI axis sensitivity to perceived predation.

THE LINK BETWEEN HORMONAL AND BEHAVIOURAL RESPONSES TO PERCEIVED PREDATION RISK

Neither basal nor post-predator exposure cortisol predicted activity (p>0.05; Table 4). Post-predator exposure cortisol did not predict shyness (p>0.05; Table 4). No relationship of basal cortisol on shyness was found across subjects (p=0.816; Table 4) but there was a significant within-subjects effect (p=0.038; Table 4). A post-hoc investigation into immobility and time spent in cover (see Table 1), revealed a significant within-subject effect of basal cortisol on the time spent in cover (estimate±se=50.35±23.01, t=2.19, p=0.035) but not immobility (estimate±se=38.98±26.96, t=1.45, p=0.160), i.e. individuals, across low- and high-predation risk phases, spent more time in cover on days where they had higher basal cortisol levels. Testosterone neither predicted activity or shyness ($p>0.05$; Table 4).

Discussion

How animals respond to changes in their environment and whether certain individuals are better able to cope with challenges are crucial questions given the rapid environmental changes caused by human activity. Our repeated measures sampling design allowed us to (1) investigate behavioural and endocrine personalities and (2) their potential impact on phenotypic plasticity in response to changes in perceived predation risk, and (3) to account for within- versus between-subject effects of hormones on behaviour (see Figure 1B,C).

First, we found individual behaviours and hormone levels to be consistent and repeatable, indicative of personality. In the case of individual differences in behaviour, we found that both “activity” and “shyness” were significantly correlated across low and high predation risk phases, demonstrating behavioural consistency within our test treatments. Interestingly, we only found significant individual differences and repeatability in activity and not shyness. This suggests that our measure of shyness does not represent an individual trait (see e.g. Carter, Goldizen & Heinsohn 2012). However, the two variables that loaded onto PCA axis of shyness ‘immobility’ and ‘time spent in cover’ were affected by ‘individual’, and thus, can be considered personality traits (data not shown). Measuring multiple behaviours can be important, since some may be representative of individual traits whilst others may not (Carter, Goldizen & Heinsohn 2012; Carter et al. 2013; Weiss & Adams 2013). Our study, further suggests that caution is required when applying Principal Component Analysis to combine correlated behavioural traits.

Regarding individual differences in HPI axis activity, we found that post-predator exposure cortisol concentrations were repeatable, indicating a strong individual consistency in the physiological stress response, i.e. the sensitivity of the HPI axis, to perceived predation risk (‘endocrine personalities’). In contrast, we only found a trend for repeatability in basal cortisol. Across vertebrates, most of our knowledge on repeatabilities of glucocorticoid responses comes from studies on birds which generally show high repeatabilities (reviewed by Cockrem 2013). Comparable data on individual differences in the glucocorticoid stress response for fish are scarce (reviewed by Cockrem 2013); nevertheless, similar repeatabilities have been reported for bluegill sunfish (*Lepomis macrochirus*: Cook et al. 2012) and, on a population level, for rainbow trout and the Panamanian bishop (Pottinger, Pickering & Hurley 1992; Archard et al. 2012).

Second, we were interested if and how subjects respond to changes in perceived predation risk, both behaviourally and hormonally, and whether different personalities (see above) respond differently. Both activity and shyness were affected by changes in predation risk, i.e. individuals were significantly less active and shyer, and also had significantly higher cortisol levels after, compared to before, exposure to the model predator. Since individuals habituated to the hormone sampling method within three days (for similar findings see Wong et al. 2008; Fischer et al. 2014), we are confident that confinement, which itself is a stressor (e.g. Cockrem 2013, this study), did not - or only marginally - affected the ‘true’ physiological state/response of our subjects. Despite the individual differences in activity and cortisol levels (see above), we found no evidence for individuals varying in the level of endocrine and behavioural plasticity with respect to predation risk (E x I interaction), suggesting that individuals are not constrained by their personality in their response to changes in their environment. Having said this, sticklebacks, as numerous other animals, are not solitary – they are social and respond to the movements of others (e.g. Harcourt et al., 2009). Sociality, in fact, can have moderating effects upon personality (e.g. King et al. in press; for review see Webster & Ward 2011) as well as the glucocorticoid stress response (e.g. Fürtbauer et al. 2014). Also, phenotypic plasticity could be context-dependant, making it entirely possible that individuals differ in their response to other (non-predator) stimuli, or when trade-offs are involved (see e.g. Quinn et al., 2012).

Our third and final aim was to investigate whether cortisol predicted behaviour across low and high predation phases. Neither basal cortisol nor post-predator exposure cortisol predicted activity and we found no link between post-predator exposure cortisol and shyness. Conversely, basal cortisol levels predicted shyness; however only within, but not across, individuals (see Figure 1B,C). Together, these findings have several potentially important implications for past and future studies: (1) it is possible that a relationship between hormones and behaviour (here, cortisol and shyness) is present within, but not across, individuals (Figure 1). Such a relationship is only quantifiable by measuring cortisol and behaviour repeatedly in the same individuals (Figure 1C), rather than just once, or taking the average of multiple measures (Figure 1B). Notably, the existence of a within-subject effect of cortisol on shyness within a population may shed new light on coping styles found across populations (see e.g. Archard & Braithwaite 2011; Archard et al. 2012; Figure 1A). (2) Some behavioural variables may be linked to cortisol levels whereas others are not, e.g. in our study, basal cortisol was linked to the time spent in cover which essentially is the amount of time the subjects were 'hiding' and, in our opinion, probably the most meaningful measure of 'boldness' (c.f. Carter et al. 2013). (3) There may be an important distinction in the relationship between basal versus post- predator exposure cortisol and behaviour, i.e. basal versus reactive cortisol levels. Single measures studies usually measure post-predator exposure cortisol (e.g. Pottinger, Carrick & Yeomans 2002; Bell et al. 2007) which, in our study, was unrelated to behaviour. Clearly, more careful consideration of study and sampling designs is needed when linking endocrine and behavioural profiles in an animal personality framework.

In addition, we tested whether testosterone, a sex steroid hormone which has been linked to risk-taking and sensation-seeking behaviours (reviewed by Caramaschi et al. 2013), affects antipredator behaviour in our female sticklebacks. We found no evidence in support of this hypothesis; however, it remains to be investigated whether testosterone is a marker of male personalities. Generally, sex differences in testosterone (a “male hormone”) concentrations may, at least partly, be the reason why testosterone has been devoted more attention with respect to phenotypic variation in males, compared to females (Ketterson & Nolan 2005; Ketterson, Atwell & McGlothlin 2009). Testosterone is an interesting hormone regarding its presence/function in fish. Usually, in teleost fish, 11-keto testosterone is the most important androgen in males in terms of secondary sexual characters, spermatogenesis, and reproductive behaviour (Borg 1994), and can only be found in very low concentrations or is absent in females. Testosterone, in contrast, is ubiquitous in males and females (Borg 1994; Sebire, Katsiadaki & Scott 2007), and when breeding, females show increased plasma testosterone levels compared to males (Borg & Mayer 1995), and are bolder towards a predator than non-breeding females (Frommen, Mehlis, & Bakker, 2009). These findings, despite the non-significant result in our study, provide an interesting basis for future research into the link between testosterone and risky behaviour in different, e.g. reproductive, contexts.

In summary, our study has revealed consistent individual differences in behaviour and cortisol levels (i.e. behavioural and physiological personalities), plasticity in hormonal and behavioural responses to changes in predation risk, and a relationship between behaviour and basal cortisol within, but not across, individuals. However, we found no individual differences in behavioural and endocrine plasticity, indicating that individuals are not constrained by their personalities, and thus, are equally capable to respond to a potentially harmful threat. Future research should focus on assessing the effects and constraints that conspecifics may impose on personality (see Webster & Ward 2011) but also phenotypic plasticity in the response to environmental change.

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Data accessibility

All data analysed in this study are available online in the supporting information.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Data analysed in this study

Figures

Figure 1:

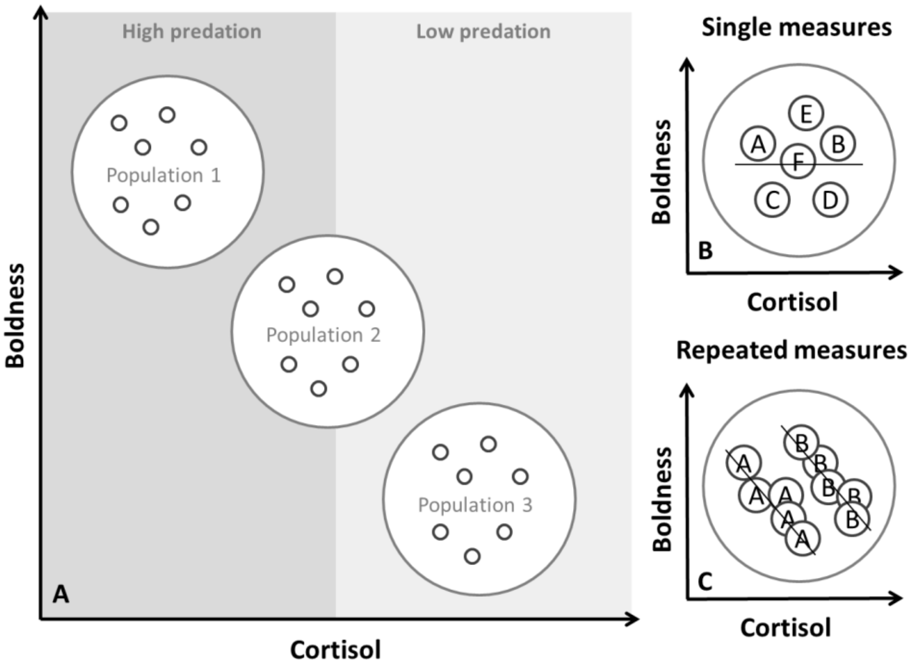


Figure 2:

Figure 3:

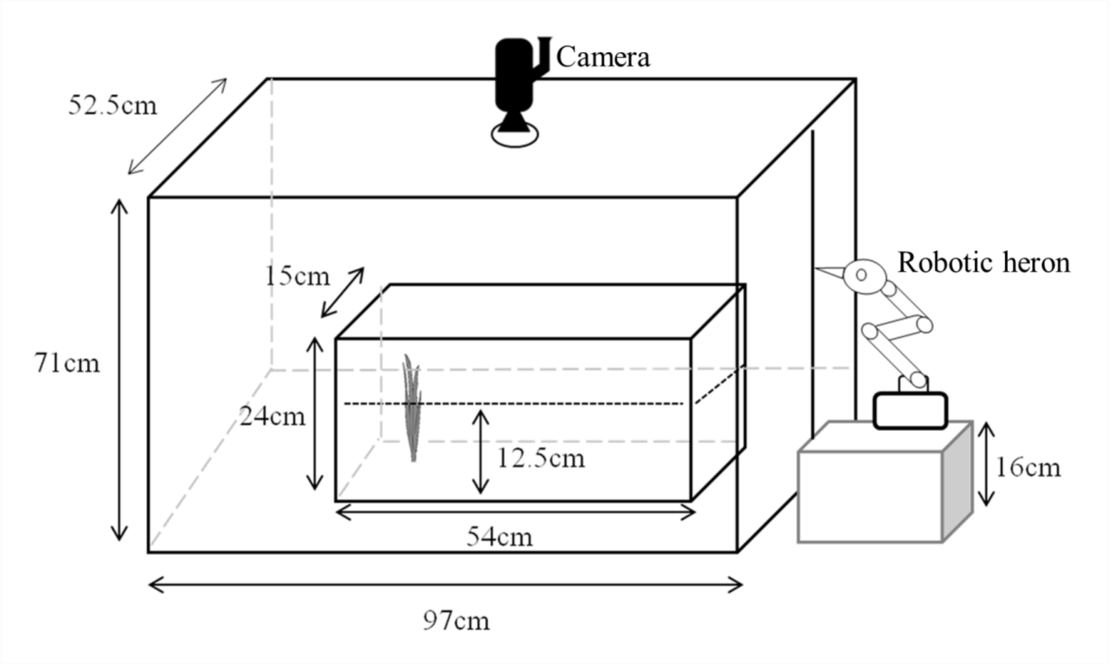

Camera
52.5cm
Robotic heron
15cm
71cm
24cm
12.5cm
16cm
54cm
97cm

Figure 4:

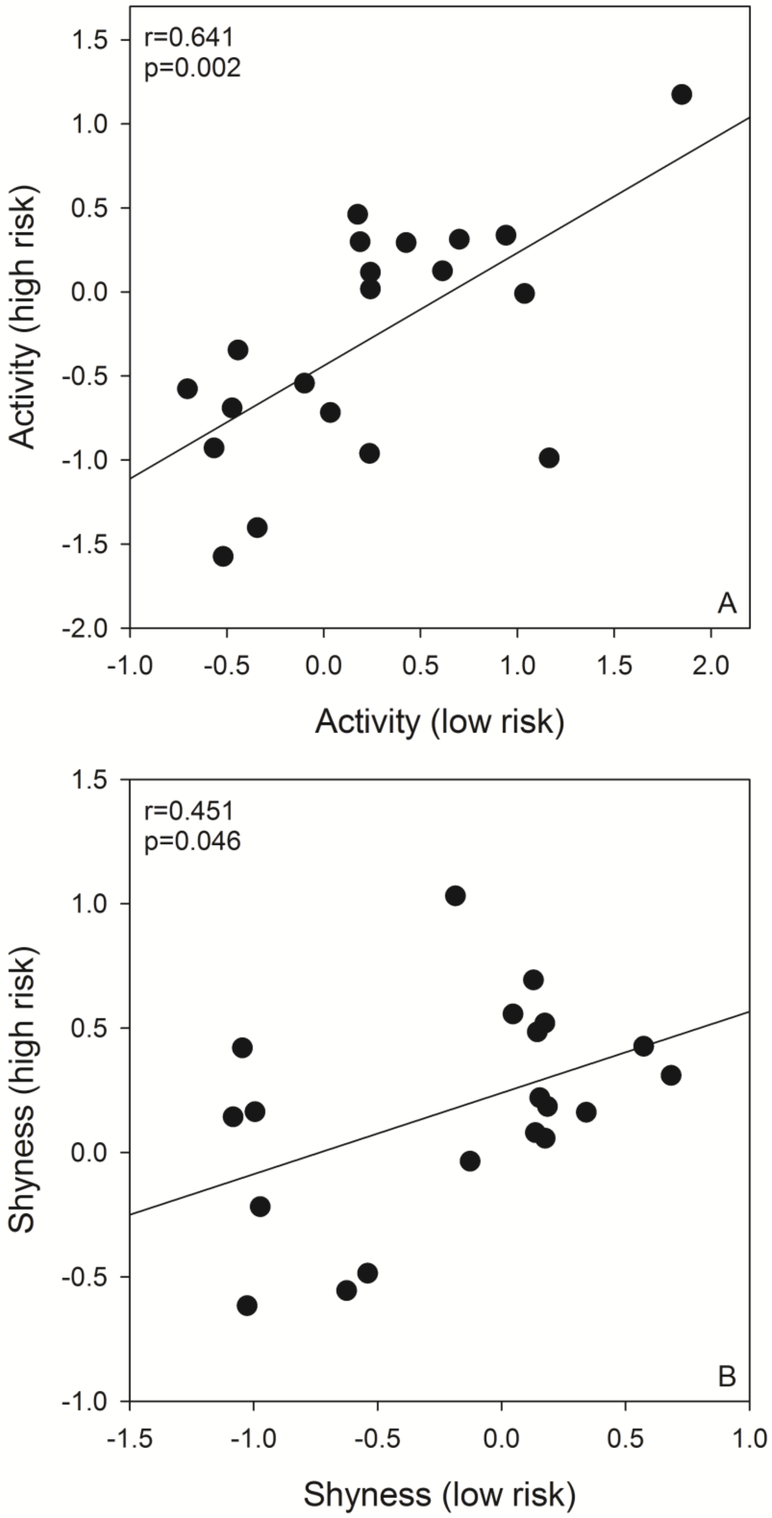
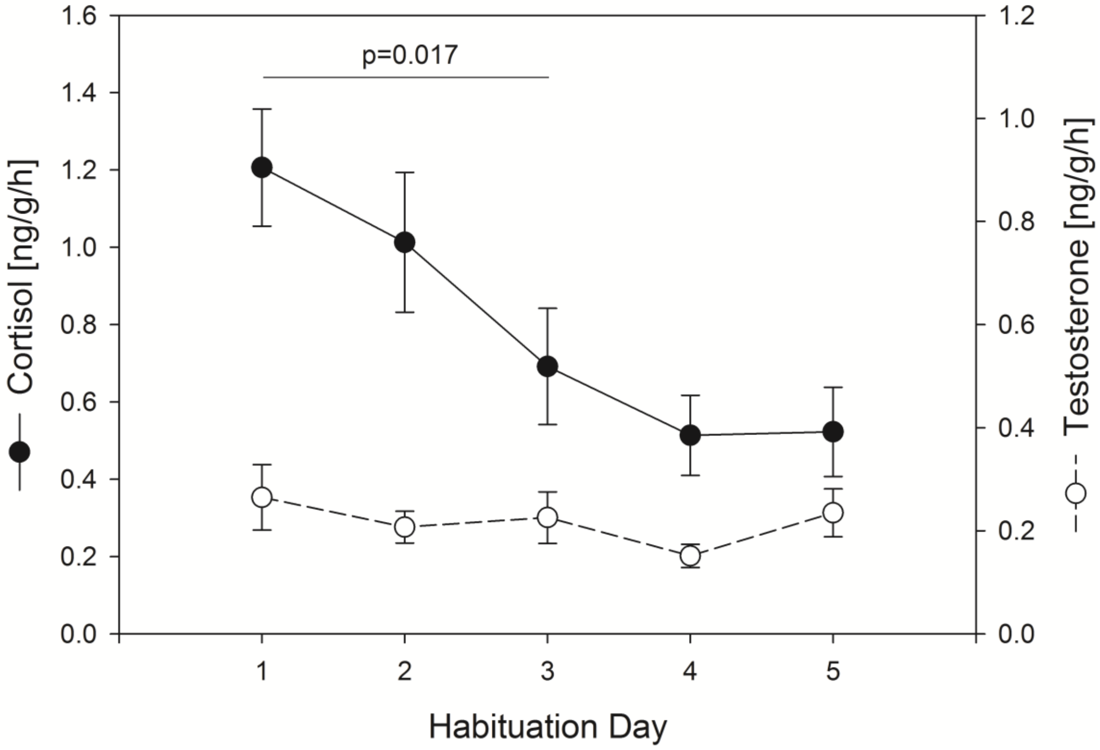

r=0.641
p=0.002
A
Activity (high risk)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Activity (low risk)
r=0.451
p=0.046
B
Shyness (high risk)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
Shyness (low risk)

Figure 5:



p=0.017
Cortisol [ng/g/h]
Testosterone [ng/g/h]
Habituation Day
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5

Figure 6:

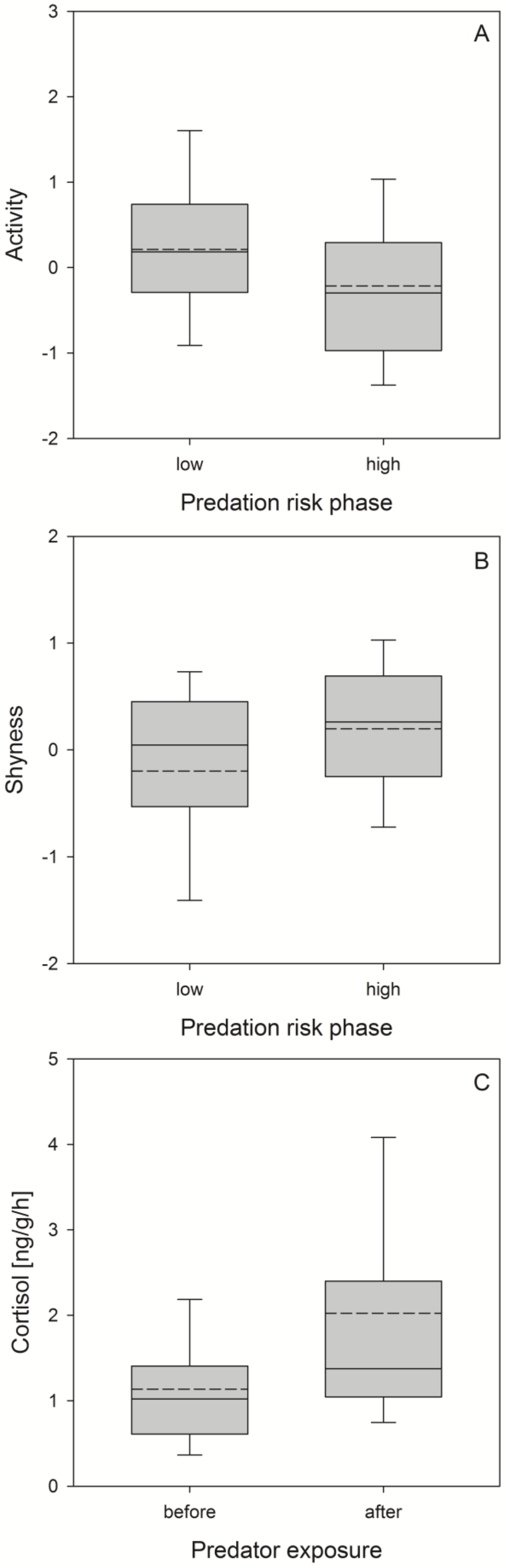

A
Activity
3
2
1
0
-1
-2
low
high
Predation risk phase
B
Shyness
2
1
0
-1
-2
low
high
Predation risk phase
C
Cortisol [ng/g/h]
5
4
3
2
1
0
before
after
Predator exposure

Figure Legends

Figure 1: Schematic representations of potential relationships between cortisol levels and boldness. (A) Across populations, those populations exposed to high predation pressure are often bolder and exhibit lower cortisol levels (Population 1) compared to populations exposed to low predation pressure (Population 3), representative of ‘stress coping styles’, i.e. consistent sets of behavioural and physiological stress responses. (B) Within populations, studies are usually based on single measurements per subject (A-F), and a relationship between cortisol and boldness is often lacking. (C) A repeated measures design, i.e. multiple measurements per subject (A, B) may reveal a within-subject correlation between cortisol and boldness.

Figure 2: Adult female *Gasterosteus aculeatus* in tank © the authors.

Figure 3: Test setup. Test tank and arena, containing a green plastic plant to provide cover. The ‘robotic heron’, made of polymer clay and attached to robotic arm with a USB PC interface, was hidden behind a curtain before the simulated attack. The behaviour of the fish during low and high predation risk phases was filmed using a Panasonic HDC-SD60 high definition video camera, mounted above the test tank.

Figure 4. Correlation in behaviour before and after predator exposure. Plots show significant and positive correlation between mean average (A) activity and (B) shyness during low and high predation risk phases In female sticklebacks (n=20). Values were averaged across 5 weeks.

Figure 5: Habituation to beaker confinement. Waterborne cortisol (solid line, filled circles) and testosterone (dashed line, open circles) concentrations (mean±SE) during five consecutive beaker habituation days in female three-spined stickleback fish (n=20). Cortisol concentrations decreased significantly from day 1 to day 3 (p=0.017), whereas testosterone concentrations remained constant (p=0.721).

Figure 6: Population-level effect of changes in perceived predation risk. Data are shown for the (A) activity and (B) shyness of n=20 female three-spined sticklebacks during a low and high predation risk phase, and the same fishes' (C) cortisol concentrations before and after exposure to a model predator (total n=186). The boxes indicate means (dashed line), medians (solid line) and upper and lower quartiles. The whiskers indicate the 90th and 10th percentiles.

Table 1: Loadings of the six behavioural variables extracted from video on the Principal Components (PCs) “activity” and “shyness”, Eigenvalue, and percent variance explained.

Behavioural parameter	**PC 1 "Activity"**	**PC 2 "Shyness"**
Distance	.846	-.247
Time spent in cover	-.028	.896
Immobility	-.594	.682
High mobilility	.731	-.356
Speed	.347	-.735
Transitions out of cover	.893	-.055
Eigenvalue	3.5	1
% variance explained	58.2	17.2

Table 2: Individual repeatability of hormones and behaviour in female sticklebacks (n=20) across five weeks.

Variable	**ICC**	**95% CI**		**$F_{17,72}$**	**P**
Cortisol before	0.11	-0.04	0.36	1.60	0.087
Cortisol after	**0.34**	0.14	0.60	3.52	**<0.001**
Testosterone	-0.01	-0.12	0.20	0.96	0.515
Activity low risk	**0.26**	0.13	0.60	3.50	**0.001**
Activity high risk	**0.36**	0.16	0.62	3.87	**<0.001**
Shyness low risk	0.08	-0.06	0.32	1.41	0.157
Shyness high risk	0.11	-0.04	0.36	1.64	0.076

Table 3: Comparisons of models with different fixed and random effects using log-likelihood ratio tests (n=186 observations). “Date” was included as random effect in all models.

Comparison	**Model**	**Models compared**	**Log-likelihood**	**df**	**χ^2**	**P**
	Activity					
E	M1: predator as fixed effect	M1 vs M2	-259.05	4	8.75	**0.003**
	M2: without predator		-263.42	3		
I	M3: ID as random intercept	M3 vs M4	-239.75	5	38.59	**<0.001**
	M4: without ID		-259.05	4		
I x E	M5: ID as random intercept; predator as random slope	M5 vs M6	-239.74	7	0.03	0.987
	M6: without predator as random slope		-239.75	5		
	Shyness					
E	M1: predator as fixed effect	M1 vs M2	-256.90	4	8.27	**0.004**
	M2: without predator		-261.03	3		
I	M3: ID as random intercept	M3 vs M4	-256.31	5	1.17	0.279
	M4: without ID		-256.90	4		
I x E	M5: ID as random intercept; predator as random slope	M5 vs M6	-255.55	7	1.52	0.468
	M6: without predator as random slope		-256.31	5		
	Cortisol					
E	M1: predator as fixed effect	M1 vs M2	-204.91	4	31.32	**<0.001**
	M2: without predator		-220.57	3		
I	M3: ID as random intercept	M3 vs M4	-201.08	5	7.66	**0.006**
	M4: without ID		-204.91	4		
I x E	M5: ID as random intercept; predator as random slope	M5 vs M6	-199.47	7	3.21	0.201
	M6: without predator as random slope		-201.08	5		

Table 4: Results from LMMs testing the effects of endocrine variables on activity and shyness in female three-spined sticklebacks (n=20). “Predation risk” (low/high) was included as fixed effect, and “ID” and “Date” were included as random effects in all models. To account for between versus within subject effects of hormones on behaviour, daily hormone levels per subject as well as the mean hormone levels per subject were included (see text for details).

Response variable	**Predictor variable**	**estimate±se**	**t value**	**p-value**
Activity	mean testosterone	-0.17±1.71	-0.10	0.921
	within-subject testosterone	0.13±0.37	0.34	0.735
	mean basal cortisol	-0.14±0.35	-0.40	0.693
	within-subject basal cortisol	-0.03±0.11	-0.24	0.820
	mean post-predator exposure cortisol	0.02±0.12	-0.13	0.901
	within-subject post-predator exposure cortisol	0.02±0.10	0.38	0.711
Shyness	mean testosterone	0.83±1.15	0.72	0.478
	within-subject testosterone	-0.04±0.49	-0.09	0.932
	mean basal cortisol	-0.06±0.25	-0.24	0.816
	within-subject basal cortisol	0.23±0.11	2.10	**0.038**
	mean post-predator exposure cortisol	0.01±0.09	0.08	0.937
	within-subject post-predator exposure cortisol	0.09±0.10	1.29	0.242