

Increased exposure to predators increases both exploration and activity level in *Brachyrhaphis episcopi*

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Two temperament traits, tendency to explore and activity level, were measured in a tropical poeciliid fish, the Panamanian bishop *Brachyrhaphis episcopi*. Open-field arena tests were used to quantify how predation pressure shapes activity levels and exploratory behaviours. Fish behaviour differed between high and low-predation populations. Fish that experienced higher levels of predation were both more explorative and more active. There were also some individual differences within populations; fish varied in their levels of exploration and activity in a novel open arena, but these differences were not related to sex or size. Together with previous studies on this species, these results indicate that there is a behavioural syndrome associated with predation pressure. Fish from high-predation populations are bolder, more explorative and more active than those from low-predation populations.

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INTRODUCTION

The study of animal temperament (or personality) is based on the fact that behavioural variation between individuals is not simply noise around locally adaptive averages (Wilson, 1998; Dall *et al.*, 2004). Instead, behavioural variation is often consistent within individuals over time and across contexts. It has important fitness consequences and is itself under selection (Sih *et al.*, 2004; Réale *et al.*, 2007; Smith & Blumstein, 2008). In addition, temperament can often be at least partially explained by intrinsic factors or states such as reproductive status or nutritional need [*e.g.* in six-lined trumpeters *Pelates sexlineatus* (Quoy & Gaimard); Biro & Booth, 2009], demographic factors such as sex, age or size (*e.g.* in lion-headed cichlids *Steatocranus casuarius* Poll; Budaev *et al.*, 1999) and extrinsic factors such as predation pressure or habitat characteristics (*e.g.* in three-spined sticklebacks *Gasterosteus aculeatus* L.; Dingemanse *et al.*, 2007; Brydges *et al.*, 2008).

Risk of predation is a key selective pressure that affects many aspects of an animal's life (Lima & Dill, 1990). It is often studied using a comparative approach to investigate the effects of predation on behaviours such as mate choice, foraging and

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schooling (e.g. in guppies *Poecilia reticulata* Peters; Endler, 1995). Several studies have investigated population-level effects of predation pressure on fish temperament behaviours (e.g. *G. aculeatus*; Huntingford *et al.*, 1994; Dingemanse *et al.*, 2007).

In the poeciliid the Panamanian bishop *Brachyrhaphis episcopi* (Steindachner), fish from environments with high compared to low levels of predation pressure are bolder, as measured by emergence from a shelter (Brown *et al.*, 2005, 2007a, b). These predation effects have both a heritable and an environmental component (Brown *et al.*, 2007a). There is also evidence that boldness may be linked to fitness because fitness in fishes is directly related to body mass [e.g. mosquitofish *Gambusia affinis* (Baird & Girard); Reznick & Braun, 1987; bluegill sunfish *Lepomis macrochirus* Rafinesque; Neff & Cargnelli, 2004], and *B. episcopi* with heavier mass for a given length are bolder (Brown *et al.*, 2007b). To date, boldness is the only temperament trait that has been measured in *B. episcopi*, thus this study addresses whether predation pressure affects two other axes of temperament variation: exploration and activity level. Boldness is a measure of an individual's reaction to risk in the known environment, such as that imposed by the presence of a predator. In contrast, exploration describes an individual's willingness to explore (or avoid) new situations, including habitats, food items and novel objects. Activity level may affect both boldness and exploration, but is considered a separate temperament axis (Réale *et al.*, 2007).

Behavioural syndromes occur when behaviours are correlated across situations or across contexts (Sih *et al.*, 2004). For example, in *G. aculeatus*, aggression towards conspecifics and heterospecifics is correlated across individuals, and aggressive individuals are also bolder in their antipredator behaviour (Huntingford, 1976). In *L. macrochirus*, boldness, exploration and activity are all positively correlated (Wilson & Godin, 2009). These types of across-context correlations are also seen in other taxa. For example, in great tits *Parus major* boldness and exploration and aggression are all positively correlated (Verbeek *et al.*, 1996). In general, boldness (in the face of risk) and exploration (in novel situations or towards novel objects) are often positively correlated. Both may be affected by activity levels, which may also reflect metabolic constraints (Réale *et al.*, 2007). Thus, the first aim was to determine whether correlations between different temperament traits also occur in *B. episcopi*.

Behavioural syndromes may result from simultaneous adaptation of multiple behaviours or from underlying physiological or genetic constraints that link behaviours (Sih *et al.*, 2004; Réale *et al.*, 2007). Extending the concept to how individuals differ between different populations inherently involves investigating an adaptationist explanation of overall levels of behaviour. Furthermore, high-predation populations are situated in larger, downstream habitats, which are physically and biologically more complex: they contain a wider range of microhabitats and substrata, as well as more conspecifics, a greater number and diversity of heterospecifics (including predators) and a wider range of prey types (Angermeier & Karr, 1983). All these factors suggest that *B. episcopi* in high-predation populations will experience more novelty than those in low-predation populations, suggesting that they should be more explorative and active in order to find, assess and utilize the best resources. This does not rule out, however, the possibility of underlying physiological or genetic constraints that also affect temperament differences and the relationships between temperament traits. Either way, *B. episcopi* from high-predation populations are predicted to be more explorative and have higher activity rates than those from low

predation populations. The second aim, therefore, was to test for this difference using two high and two low-predation populations. A third, methodological, aim was to demonstrate that open-field trials are a useful way to assess temperament variation, in the form of both exploration and activity levels, in this species.

MATERIALS AND METHODS

Brachyrhaphis episcopi are small, live-bearing fish found in freshwater streams in the Republic of Panama. On multiple rivers, populations are separated by waterfalls, which prevent the upstream migration of predatory fishes. Thus, upstream populations live with only one minor predator (the killifish *Rivulus brunneus* Meek & Hildebrand), whereas downstream populations live with a number of cichlid and characin predators (Brown & Braithwaite, 2004; Brown *et al.*, 2005). Fish were captured using dip-nets and seines from one upstream, low-predation population and one downstream, high-predation population on both the Rio Limbo (upper: 09° 09.837' N; 079° 44.487' W and lower: 09° 09.739' N; 079° 44.498' W) and the Rio Macho (upper: 09° 10.972' N; 079° 45.611' W and lower: 09° 10.932' N; 079° 45.674' W) in August 2008. After capture, fish were housed in the laboratory at Gamboa field station of the Smithsonian Tropical Research Institute.

The laboratory had a 12L:12D cycle, with lower light levels at the start and end of the day to mimic dawn and dusk. Fish were kept as separate populations in 50 l plastic tanks with power filtration and aeration, and water depth 280 mm, maintained at 25–26° C. Gravel from each site was collected with the fish and placed in the corresponding tank to provide natural stimuli. Fish were fed flake food twice daily, supplemented every 2 days with newly hatched brine shrimp *Artemia* sp. Fish were housed for 4–9 days in the laboratory prior to use in tests to allow them to settle. Food was withheld the morning before trials. To prevent pseudoreplication, fish that had completed trials were held temporarily (keeping populations separate) in a second set of smaller tanks until the experiment was finished.

Exploration and activity levels were tested in open-field trials. Open-field trials can occur in many forms, but they are all based on placing an animal into a novel open space, from which escape is prevented by a barrier, and monitoring subsequent behaviour (Walsh & Cummins, 1976). Specifics of the arena design vary widely, depending on the taxa being tested, the behaviours being monitored and the scientific questions being asked. In the current study, the test arena was a clear, plastic tank (width 300 mm × length 400 mm × height 240 mm) with water depth 100 mm covered on all sides with black plastic. Light levels were constant between all trials and no direct light fell on the arena. There were no shadows created by the walls of the arena and no side biases in light intensity. Trials commenced by placing a fish in a clear, colourless plastic cylinder of 65 mm diameter in the centre of the arena. The fish was left to settle for 2 min before the cylinder was remotely raised *via* a pulley. Behaviour was recorded for 8 min using a video camera positioned above the tank. The base of the arena was marked with a line 50 mm in from the perimeter of the tank. It also had two lines (crossing at 90° to one another in the centre of the box) to divide the base into quarters. At the end of trials, fish were anaesthetized in buffered 2 g l⁻¹ MS-222, and standard length (L_S) was determined to the nearest 0.5 mm.

Videos were analysed, using Etholog v2.2.5 (Ottoni, 2000), to determine when fish were moving or frozen, and when they moved between the different areas of the arena. These data were used to calculate the latency to first reach the edge zone after settling, the latency to then return into the centre zone, the proportion of time spent in the centre zone of the arena (not counting the time taken to first reach the edge zone), the mean duration of visits to the centre zone, the proportion of time spent frozen and the rate of movement between the four quarters.

Total sample size for analyses was 79 fish: 10 fish of each sex were tested from each population, but one Lower Limbo female was excluded from the analyses because she tried repeatedly to jump out of the arena. Data were analysed using SPSS v17.0 (www.spss.com). First, the behavioural variables were summarized using principal component analysis (PCA). PCA does not require that variables show multivariate normality (Timm, 2002), so variables

were not transformed. After PCA, the PC coefficient scores were analysed using MANCOVA, after confirmation of assumptions of normality of residuals and homogeneity of variance. Predation regime, river of origin and sex were between-subjects factors. The L_S was a covariate because it is known to affect another temperament measure, *i.e.* boldness in this species (Brown & Braithwaite, 2004; Brown *et al.*, 2005, 2007b). Data are presented as means \pm s.e.

RESULTS

Females were larger than males (ANOVA, d.f. = 1, 71, $P < 0.001$; mean \pm s.e. L_S of females = 28.06 ± 0.95 mm and males = 22.99 ± 0.50 mm). There was no difference in L_S between predation regimes (d.f. = 1, 71, $P > 0.05$) or rivers (d.f. = 1, 71, $P > 0.05$), and none of the interaction terms were significant (all d.f. = 1, 71, $P > 0.05$).

Principal component analysis (PCA) on the six untransformed behavioural variables (see Table I) from the videos gave two principal components (PC) that explained more variance than one of the original variables, and hence had an eigenvalue of >1 . PC1 had an eigenvalue of 2.28 and explained 37.97% of the variance in the data set. PC2 had an eigenvalue of 1.36 and explained a further 22.60% of the variance, giving a cumulative variance of 60.57%.

The component score coefficients show that the proportion of time frozen, the latency to reach the edge of the open-field arena and the rate of movement had the most effect on the ordination of the PC1 axis (Table I). Rate of movement covaried negatively with both the other variables. In contrast, rate of movement was the only variable that had no strong effect on the axis ordination of PC2. The other five variables were split into two groups. The proportion of time spent frozen, the latency to reach the edge and the latency to return to the centre all covaried positively together. The proportion of time spent in the centre and the mean duration of visits made to the centre covaried positively together but negatively with the other group of variables (Table I).

MANCOVA on the factor scores of these two principal components shows that there were significant overall effects of predation regime (Wilk's lambda, d.f. = 2, 69, $P < 0.001$) and river of origin (Wilk's lambda, d.f. = 2, 69, $P < 0.001$). There was no overall effect of L_S (Wilk's lambda, d.f. = 2, 69, $P > 0.05$) or sex (Wilk's lambda, d.f. = 2, 69, $P > 0.05$), and all the interaction terms were non-significant (all d.f. = 2, 69, $P > 0.05$).

TABLE I. Component score coefficients from the principal component analysis on behaviours of *Brachyrhaphis episcopi* in open-field arena trials

Variable	PC1	PC2
Proportion of time frozen	0.35	0.33
Latency to reach the edge	0.37	0.30
Latency to return to the centre	-0.13	0.35
Proportion of time spent in the centre	0.23	-0.38
Mean duration spent in the centre	0.15	-0.52
Rate of movement	-0.30	0.08

Principal component coefficients >0.30 are described in the text and given in bold.

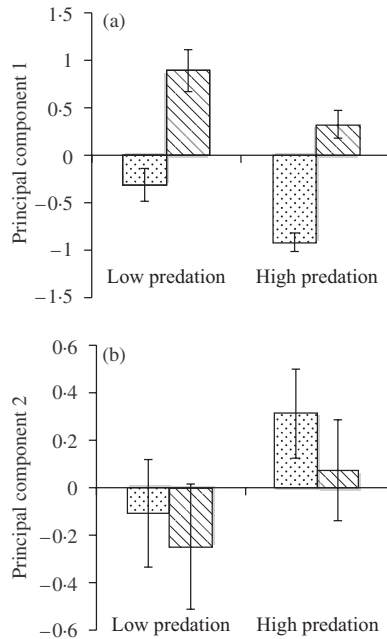


FIG. 1. Effect of predation regime and river of origin on the principal component coefficient scores of *B. episcopi* monitored in open-field arena trials. Data are shown as means \pm S.E. (a) Fish with higher PC1 scores froze more, took longer to reach the edge and had slower rates of movement. (b) Fish with higher PC2 scores were generally less explorative. \square , River Limbo; \square , Rio Macho.

Tests of the between-subject effects for predation regime and river reveal that both predation (d.f. = 1, 70, $P < 0.001$) and river (d.f. = 1, 70, $P < 0.001$) had a significant effect on PC1. While PC1 was lower for fish from the Rio Limbo than those from the Rio Macho, it was also lower for fish from high predation compared to low predation [Fig. 1(a)]. Thus, fish from high-predation regimes moved faster around the arena than those from low-predation regimes, but spent less time frozen and had shorter latencies to reach the edge of the arena. Similarly, fish from the Rio Limbo were faster, spent less time frozen and had shorter latency to edge times than those from the Rio Macho.

Neither predation regime nor river had a significant effect on the component scores of PC2 [predation: d.f. = 1, 70, $P > 0.05$; river: d.f. = 1, 70, $P > 0.05$; Fig. 1(b)]. Thus, while this axis describes variation between the different behaviours measured, this variation did not differ significantly between treatments.

DISCUSSION

Different populations of wild *B. episcopi* behaved in different ways in open-field trials. Those from high-predation environments spent less time frozen, had shorter latencies to reach the edge zone at the start of trials and moved faster than those from low-predation environments. This suggests that fish from high-predation environments are more willing to explore novel areas, and that they have higher activity

levels, than fish from low-predation environments. These results extend previous observations that found *B. episcopi* from high-predation environments to be bolder than fish from low-predation environments (Brown *et al.*, 2005, 2007a, b).

In the current experiments, shorter latencies to reach the edge were linked to time spent frozen because most fish did not explore the centre of the arena immediately after the settling tube was lifted. Instead, they either froze for a time or swam directly towards the edge. Given previous results showing that high-predation site fish tend to be bolder (Brown *et al.*, 2005, 2007a, b), it is surprising that fish from high-predation environments did not spend more time in the centre of the arena. Time spent in the centre of an open-field assay is a standard measurement (Walsh & Cummins, 1976), and it assumes that animals perceive themselves to be safer when adjacent to the wall. It might also be expected that more explorative fish would move more readily throughout the whole arena, but there was no evidence of this.

Overall, the effect of predation regime on temperament concurs with previous studies in other fish species (*e.g.* *G. aculeatus*; Huntingford *et al.*, 1994; Dingemanse *et al.*, 2007), and other taxa (*e.g.* desert spiders, *Agelenopsis aperta*; Riechert & Hall, 2000), which demonstrate differences in temperament between populations experiencing different levels of predation. Brown *et al.* (2005, 2007a, b) describe *B. episcopi* from high-predation environments as being bolder than those from low-predation environments because they emerge sooner from shelter. Given that high-predation environments are by definition more dangerous for prey than low-predation, what could explain the increased risky behaviour, *i.e.* boldness, in these environments? Fish living with predators cannot simply hide from them because they must continue to perform routine behaviours such as foraging, courting and mating. As such, risk of predation is balanced against loss of fitness due to reduced opportunities for maintenance, growth and reproduction when fishes are actively avoiding predators. This trade-off is thoroughly studied in fishes and results in individuals that need to be bolder in the face of risk in order to optimize their lifetime reproductive success (Lima & Dill, 1990).

Rate of movement was higher for fish from high-predation environments. Activity rates may reflect metabolic constraints, but they are also considered an axis of temperament in their own right [Réale *et al.*, 2007; *e.g.* in zebrafish *Danio rerio* (Hamilton); Moretz *et al.*, 2007]. Rate of movement, at least in terms of maximum speed, may be selected to be higher in high-predation environments due to the need to escape predators (*e.g.* in *P. reticulata*; O'Steen *et al.*, 2002). Rate of movement, however, was measured while fish were exploring a novel environment and were not at risk of predation, rather than their maximum speed. Alternative explanations that explain this result could be that fish from high-predation environments are more active to help them locate predators, or permit them to maximize food intake or mating opportunities whilst not directly under threat.

Considered together with previous work on *B. episcopi* (Brown *et al.*, 2005, 2007a, b), the current study reveals that individuals that are more explorative also have higher activity levels, and that fish from high-predation environments in addition to being bolder, are more explorative and more active than those from low-predation environments. In addition, when fish are tested twice in the open-field arena, all behaviours except the mean duration of visits to the centre are significantly repeatable (G. A. Archard, V. A. Braithwaite and N. Colegrave, unpubl.

data), definitively demonstrating consistent behavioural variation within individuals. Together, these results suggest the presence of a behavioural syndrome in wild *B. episcopi*, where behaviours are correlated across contexts (Sih *et al.*, 2004). It is unclear, however, whether predation in this case acts as a selective force driving all these behaviours separately (an adaptation hypothesis) or whether the behaviours are linked, for example, *via* underlying physiological or genetic mechanism (a constraint hypothesis).

If a physiological or genetic constraint causes boldness, exploration and activity levels to covary, then the accepted explanation for differences in boldness is sufficient to explain the differences in exploration and activity. If selection acts on these traits independently, however, then there must be other explanations for the variance in exploration and activity. The physical and biological differences found between the two environments (Angermeier & Karr, 1983) provide a strong suggestion of why fish may be more explorative as well as bolder in high-predation environments. Only breeding experiments under different conditions, however, could determine whether this is actually the case and whether selection is acting on boldness and exploration independently.

It is also possible that some of the predation-related differences are a result of non-genetic factors or gene-by-environment interactions (Merilä & Sheldon, 1999). The development of adult behavioural phenotype can be affected by conditions during ontogeny including maternal effects. In addition, environmental effects and learning also affect behaviour (Falconer & Mackay, 1996). In *B. episcopi*, laboratory-reared offspring showed similar boldness to their wild-caught parents, demonstrating that boldness has a heritable component. Offspring that were repeatedly chased with a net during a 2 week period prior to temperament testing as adults also showed increased boldness, demonstrating an environmental and learned component to adult boldness (Brown *et al.*, 2007a).

Nonetheless, predation pressure is a key selection force driving the evolution of multiple morphological and behavioural traits of animals (Lima & Dill, 1990), and fish studies have provided classic examples of predation effects using the comparative approach to compare evolutionary changes between populations living with and without predators (*e.g.* *P. reticulata*, Endler, 1995; *G. aculeatus*, Huntingford *et al.*, 1994). In *B. episcopi*, boldness is heritable (Brown *et al.*, 2007a) and it is likely that other temperament traits will also show genetic differences between populations.

In addition to predation effects, there were differences in temperament between rivers; Rio Limbo fish spent less time frozen, they had shorter latencies to reach the edge zone at the start of trials and they moved faster than fish from the Rio Macho. Thus, Rio Limbo fish were more explorative and more active than those from the Rio Macho. Differences between rivers are not surprising and are also observed in emergence behaviour (Brown & Braithwaite, 2004; Brown *et al.*, 2005). *Brachyrhaphis episcopi* populations from different rivers are isolated geographically because this species does not occur further downstream, or in the Panama Canal, into which both rivers drain. Additionally, the two rivers are ecologically distinct, differing in topography, rainfall and substratum (Angermeier & Karr, 1983). It is therefore likely that both genetic drift and differential selection pressures will occur between the two river populations generating river-specific differences.

Previous work has shown that smaller *B. episcopi* emerge more rapidly from a shelter, demonstrating a size (and therefore age) dependent effect on temperament.

Brown & Braithwaite (2004) postulate that this difference is best explained by a metabolic-cost hypothesis whereby smaller fish, which have higher metabolic rates per unit mass (Krause *et al.*, 2000), have to resume normal behaviours sooner after interruption. Despite this, and the fact that male and female poeciliids have very different life-history priorities (Magurran & Garcia, 2000), there was no evidence that body size or sex affected exploratory behaviour or activity levels.

Nevertheless, the results demonstrate that there was variation in exploration between individuals within populations, as well as between individuals from different populations. For example, PC2 (which was not significantly affected by any of the independent factors) describes how fish that had longer freezing times, longer latencies to reach the edge zone and that took longer to initially move into the centre of the arena also made shorter visits to the centre and spent a shorter total time in the centre zone. Thus, there is temperament variation, but this is not all due to differences in predation regime because it also occurs within populations.

Open-field trials are an ideal tool with which to study temperament in *B. episcopi*. As is in many other taxa (Walsh & Cummins, 1976), they can be used to quantify a range of behaviours that together summarize exploration and activity. These measurements differ between fish from high and low-predation environments, but also between individuals within populations. It appears that open-field trials will be an important technique for future studies in which temperament and other behaviours are integrated to determine how they relate to fitness.

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