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Boldness and Reproductive Fitness Correlates in the Eastern Mosquitofish, *Gambusia holbrooki*

Alexander D. M. Wilson*, Jean-Guy J. Godin* & Ashley J. W. Ward†

* Department of Biology, Carleton University, Ottawa, ON, Canada

† School of Biological Sciences, University of Sydney, Sydney, NSW, Australia

Correspondence

Alexander D. M. Wilson, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.
E-mail: alexander.wilson@ymail.com

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Abstract

Animals are known to exhibit 'personality'; that is, individual differences in behaviour that are consistent across time and/or situations. One axis of personality of particular importance for behavioural ecology is boldness, which can be defined as the tendency of an individual to take risks. The relationship between individual personality and fitness correlates, particularly those involved in reproduction, remains largely unexplored. In the current study, we used female Eastern mosquitofish (*Gambusia holbrooki*) to ascertain whether certain reproductive traits (e.g. stage of pregnancy, fecundity) are correlated with individual personality in two wild populations in New South Wales, Australia. To quantitatively assess this relationship, we tested individual fish for their level of boldness, as measured by their latency to exit a refuge and tendency to shoal in a novel environment. We also quantified individual differences in general activity and tendency to swim near the water surface and substratum. For both populations taken together, bolder individuals tended to be smaller, relatively less fecund (when taking body size into account), and spent more time near the water surface than near the substratum compared with timid individuals. Individual boldness was not correlated with either general activity or stage of pregnancy. To our knowledge, our study characterizes for the first time a relationship between an individual personality trait (boldness) and a reproductive fitness correlate (fecundity) in fishes.

Introduction

The concept of animal 'personality', or individual level differences in behaviour that are consistent across time and/or situations, has become a subject of much research in recent years (Réale et al. 2007). This is particularly true in terms of understanding the mechanisms underlying the origin, evolution and fitness consequences of animal personality (Gosling 2001; Sih et al. 2004; Dingemanse & Réale 2005; Wolf et al. 2007; Smith & Blumstein 2008). However, many questions remain unanswered, including whether or not individual differences in

morphological, physiological or life-history traits can be predicted by individual differences in personality or vice versa. Further, individual differences in personality may have profound implications for behavioural ecologists as they have been documented to be associated with a broad range of ecologically relevant behaviours (e.g. mating, foraging, habitat use, exploration, antipredator behaviour; Dingemanse & Réale 2005; Réale et al. 2007) and because their importance is often ignored when conducting field research (Biro & Dingemanse 2009).

One axis of personality of particular relevance to behavioural ecology is that of 'shyness' and

'boldness'. Boldness can be defined as the tendency of an individual to take risks (Wilson et al. 1994). Along the shy–bold axis (Wilson et al. 1994), individuals vary from being extremely bold (reacting to novel stimuli by becoming actively exploratory) to extremely shy or timid (reacting to novel stimuli by retreating or becoming vigilant). The ecological importance of boldness is apparent in its broad taxonomic expression and the range of other behaviours it is associated with. Boldness has been widely documented in fishes (Ward et al. 2004; Webster et al. 2007; Wilson & Godin 2009), birds (Dingemanse et al. 2003; Carere & van Oers 2004; Both et al. 2005), mammals (Réale & Festa-Bianchet 2003; Martin & Réale 2008), reptiles (Lopez et al. 2005), insects (Kortet et al. 2007) and cephalopods (Sinn et al. 2006, 2008). Further, boldness has also been shown to be associated with aggression (Johnson & Sih 2005), mate selection (Godin & Dugatkin 1996), dispersal (Fraser et al. 2001), invasiveness (Rehage & Sih 2004), general activity, exploration and space use (Wilson & Godin 2009), as well as foraging and antipredator behaviour (Wilson & Stevens 2005). Boldness has also been posited to be associated with traits that are fitness-related (Dingemanse et al. 2004; Sinn et al. 2006), heritable (Dingemanse et al. 2002; Brown et al. 2007a) and subject to natural selection (Réale & Festa-Bianchet 2003). Thus, investigating the ecological and evolutionary implications of shy–bold behaviour, as well as its mechanistic bases, may provide important information on how these individual differences arise and are maintained in natural populations.

Here, we used female Eastern mosquitofish (*Gambusia holbrooki*) to ascertain whether certain reproductive traits (e.g. reproductive stage, fecundity) are correlated with boldness and other behavioural (general activity, water column use) and morphological (e.g. body size) traits in two wild populations in New South Wales, Australia. Mosquitofish are particularly amenable to this type of study as they practise internal fertilization, are livebearing and exhibit variation in boldness (Rehage & Sih 2004; Pyke 2005; Rehage et al. 2005). Furthermore, mosquitofish (*Gambusia* sp.) are considered to be particularly invasive when introduced into novel habitats and, as such, populations have come to be established in many areas of the world by both natural and anthropogenic means (Rehage & Sih 2004; Pyke 2005).

To quantitatively assess the relationship between reproductive traits and personality (i.e. boldness) in individuals, we captured adult female mosquitofish

from two populations and tested them for individual differences in risk-taking behaviour and general exploratory activity under standardized laboratory conditions. Following behavioural trials, we measured morphological traits (e.g. total length, wet weight) and dissected each fish to ascertain the stage of development and number of embryos each individual was carrying. Should fecundity/pregnancy be correlated with boldness, we predicted that females carrying more embryos and/or embryos closer to parturition (later stage of development) would be bolder than females with fewer embryos or embryos earlier in development due to greater associated metabolic requirements and/or greater embryonic investment (Koch & Wieser 1983; Marsh-Matthews et al. 2001). Characterizing the relationship between reproductive status/traits and boldness should contribute to furthering our understanding of the evolution of personality in animals.

Methods

Subjects

On 7–8 and 17–18 April 2008, we used two large (1 m diam.) dip nets to capture adult female (total length: 2.8–4.4 cm; wet weight 0.14–1.02 g) mosquitofish from the littoral zone of two temperate lakes in New South Wales, Australia. Our first capture site, Lake Northam, is a small (≈ 550 m² surface area) body of water in Victoria Park, Sydney (33°53'6"S; 151°11'35"E). Our second capture site was a small bay in the comparatively larger (≈ 260 000 m² surface area) Manly Dam Reservoir (33°46'38"S; 151°15'3"E). At the time of capture, both field sites possessed a variety of submergent and emergent vegetation and high densities of mosquitofish were observed.

All captured females were placed in buckets containing lake water and transported to our laboratory at the University of Sydney (maximum transit time < 30 min). On each of the 2-d periods above, one day was spent collecting at Lake Northam and the second day was spent collecting at the Manly Dam Reservoir (hereafter referred to as Manly Dam).

Experimental Holding Conditions

On arrival at the laboratory, all fish caught from a given location and on a given day were housed communally, but separate from those captured on different days, in one of two large glass aquaria

(95 l; 60 cm × 45 cm × 35 cm). Each aquarium contained aerated and filtered dechlorinated tap water maintained at $25 \pm 1^\circ\text{C}$, and illuminated by overhead fluorescent lights on a 12 h L:12 h D cycle. In addition, each aquarium contained a 2 cm layer of natural coloured aquarium gravel as a substratum. During the holding period, fish were fed commercial tropical fish flakes *ad libitum* twice daily.

All fish were held for 2–3 wk prior to the onset of behavioural testing to allow for a broad range of pregnancy stages to arise. During testing, individuals were selected from one of the two holding tanks under a standardized protocol, such that the same number of females from each population was tested alternately every other day (e.g. day 1 = 10 individuals from Lake Northam, day 2 = 10 individuals from Manly Dam, etc.), beginning 2 wk after capture. In total, 100 fish (50 from each population) were tested individually and sequentially in both Experiments 1 and 2.

Quantifying Boldness Behaviour and Exploratory Activity

We characterized boldness in individual fish using two behavioural measures (latency to exit a refuge and tendency to shoal with conspecifics) in two separate corollary experiments, as described below. In addition, we concurrently quantified the general exploratory activity of focal fish in a novel environment (as time spent near the water surface or near the substratum, and time spent active).

Experiment 1: latency to exit a refuge and explore a novel environment

The experimental arena used for this experiment consisted of a rectangular aquarium (30 l, 40 × 25 × 30 cm) that had been 'divided' into four equal zones (two vertically and two horizontally) using lines drawn on the front and back walls to facilitate the observation of fish water column use. The tank was covered with opaque black plastic on the back and sides to prevent any outside stimulus from disturbing the fish. The aquarium was placed behind an opaque plastic blind equipped with a small window (20 cm × 20 cm) for observing fish behaviour remotely. The experimental tank contained a mix of new aged water and water from the appropriate holding tank, which was replaced between trials. Located at one end of the experimental arena was a 'refuge' that consisted of an upright PVC cylindrical

tube (inside diameter: 7.5 cm, 0.25 cm thick) with a rectangular door (3.5 × 4.5 cm) cut into the bottom portion. This tube was fixed to the bottom substratum, which consisted of 2 cm of natural coloured aquarium gravel. This first tube was placed inside a second larger PVC tube (inside diameter: 8.5 cm; 0.25 cm thick) that also had a rectangular door (4 × 5 cm) cut into its bottom portion. Prior to the onset of testing, the doors of each tube were kept offset to each other, such that the inner opening was held closed and the focal fish could not exit. At the onset of a trial, the outer tube was turned clockwise such that the two doors lined up and the fish could exit the refuge area. Just outside the tube, and visible from the refuge, was a plastic aquarium plant (20 cm tall) anchored into the substratum. The planted area comprised approx. 10% of the open arena.

Just prior to the beginning of a behavioural trial, a focal fish was haphazardly dipnetted from the appropriate holding aquarium, placed in a small container (1 l) containing water from its holding tank, and promptly transported to the novel experimental aquarium. The focal fish was slowly poured into the refuge within the aquarium. The fish was then given 10 min to acclimatize to its surroundings, prior to the onset of testing for its willingness to explore the novel environment. After this period, the refuge door was opened and the focal fish was given a further 10 min to exit the refuge and explore the open arena. Where the fish exited completely within 10 min, we recorded the latency time to exit and subsequently quantified several behavioural measures for a further 10 min. These variables were: (1) total time spent in the upper (15 cm nearest surface) and lower (15 cm near substratum) zones of the water column, and (2) total time spent being active in the open arena. Fish that did not exit the refuge were assigned a maximal latency-to-exit score of 10 min and no further behavioural measures were recorded for those individuals. Activity was defined as the total time spent swimming (vs. resting on the bottom or holding position in the water column) in the open arena during the 10-min observation period. Because we were interested primarily in exploration and risk-taking tendencies, only behaviour exhibited in the open arena, and not inside the refuge (prior to or upon volitional re-entry during the course of a trial), was quantified. All behavioural variables were quantified by a single observer (A.D.M.W.) using the behavioural analysis software JWatcher (v1.0) (Blumstein & Daniel 2007).

Following the 10-min behavioural observation period, the focal fish was removed (by dipnetting) from the experimental aquarium and immediately carried over to the second phase of experimentation (Experiment 2). Approximately 10 fish were similarly tested on each day of experimentation (for a total of 100 fish).

Experiment 2: shoaling tendency

Immediately following the conclusion of Experiment 1, each focal female ($n = 100$; same individuals as in Experiment 1) was placed separately and singly into a second novel experimental arena, given 5 min to acclimatize, and then tested individually for their shoaling tendency. As for Experiment 1, this experimental aquarium (15 l, $36 \times 20 \times 20$ cm) was covered on the sides and back with opaque black plastic and was located behind an observation blind. The aquarium was divided into two adjacent compartments by a transparent, perforated partition, which allowed both visual and olfactory cues to be transmitted between compartments. The smaller of the two compartments ($8.5 \times 20 \times 20$ cm) contained a stimulus shoal consisting of four females (two from Lake Northam and two from Manly Dam) matching the size range of focal fish being tested. Throughout all behavioural trials, the same four fish were used as a stimulus shoal and were held separately from the main holding tanks, though under similar holding conditions. The second, larger compartment ($27.5 \times 20 \times 20$ cm) was 'divided' lengthwise into nine distinct zones by vertical lines drawn every 3 cm (approximately equivalent to 1 body length) on the front wall. This compartment contained the focal test fish. The bottom of the experimental arena was uniformly covered with 2 cm of natural coloured aquarium gravel.

Shoaling tendency was quantified by recording the amount of time the focal fish spent in each of the nine zones of the larger compartment over a

period of 3 min. Individuals that spent more time swimming at least three body lengths (>3 zones) away from the stimulus shoal were considered to be bolder (i.e. taking greater risks) than those individuals that spent more time closer to the partition/stimulus shoal (≤ 3 zones). In nature, members of fish shoals are typically within three body lengths of their nearest neighbour (Pitcher & Parrish 1993). Shoals offer safety from predation, and an individual that moves away from a shoal exposes itself to a greater risk of predation than otherwise (Krause & Ruxton 2002). All behavioural variables were quantified by a single observer (A.D.M.W.) using JWatcher (v1.0). Immediately following the 3-min trial period, the focal fish was captured, euthanized using clove oil, and stored in 95% ethanol for later morphometric measurements and dissection.

Quantifying Morphological and Reproductive Traits

Following the completion of the behavioural trials, all fish were individually weighed and standard morphological measurements (e.g. total length) were taken. Each female was then dissected and the number and developmental stage of her embryos were quantified. The different stages of embryonic development were categorized based on a classification scheme (Table 1) modified from the one described by Reznick (1981).

Data Analyses

In the current study, both boldness and water column use each comprised two independent but correlated behavioural variables. Consequently, latency to exit the refuge and time spent away from a shoal were entered into a Principal Components Analysis and the first principal component (PC1) was used as a single composite score to characterize the boldness level of individual female mosquitofish, for both populations collectively. In addition, time spent near

Table 1: A comparison of the stages of classification for Eastern mosquitofish embryonic development, as described by Reznick (1981) and used in the current study

Current study (stage no.)	Reznick (1981)	Description
1	No development	No development readily discernible under a dissecting microscope
2	Uneyed	Embryo is seen as a 'pale ribbon' on the surface of the ovum
3	Early-eyed	Some retinal pigment, little or no body pigment
4	Mid-eyed	Melanophores appear on the dorsum, fin rudiments present and the yolk is reduced in size
5	Late-eyed	Melanophores present over the surface of the body, fin rays present, yolk size substantially reduced
6	Very late-eyed	Fins are fully formed and yolk is virtually absent

Table 2: Principal Components Analysis loadings of behavioural variables used to generate a principal component score (PC1) to assess correlations between boldness, water column use, and other behavioural, morphological and reproductive traits in female mosquitofish

Trait	Behavioural variables	Loadings for PC1	% variation explained
Boldness	Latency to emerge from refuge	-0.7071	50.6
	Time spent away from shoal (>3 zones)	0.7071	
Water column use	Time spent in upper zone	-0.7071	99.9
	Time spent in lower zone	0.7071	

the water surface (upper water column zone) and time spent near the substratum (lower zone) were collapsed into a separate single composite score (PC1) to characterize water-column-use behaviour of individual females (see Table 2). Time spent in both the upper and lower zones were used in the principal component (PC1) score for water-column-use as individuals could re-enter the refuge on their own volition during trials, and this refuging behaviour was not quantified. Therefore, time spent in each zone may not be the exact inverse of each other for all individuals.

These two principal component scores and a general activity score (time spent active in the open arena) for each fish were then separately compared with their morphological and reproductive traits using nonparametric Spearman's rank correlation tests (df = 97 for each test, sequential Bonferroni correction applied; Rice 1989). Because the relationship between absolute fecundity and female body

length overall was positive and linear ($r^2 = 0.63$, $n = 100$, $p < 0.0001$) in the current study (more specifically, Lake Northam population, $r_s = 0.72$, $df = 48$, $p < 0.0001$; Manly Dam population, $r_s = 0.90$, $df = 48$, $p < 0.0001$), we calculated 'relative fecundity' for each female as the ratio of the number of eggs/embryos she carried to her total body length to control for differences in body size between individuals and populations.

Two fish (of 100 tested) were identified as statistical outliers using the extreme studentized deviate method and were thus removed from our statistical analyses. All statistical tests were carried out using JMP (v. 4.0.4).

Results

Overall, the boldness (PC1) score of individual females was negatively correlated with both their body length ($r_s = -0.37$, $p < 0.001$; Fig. 1a) and

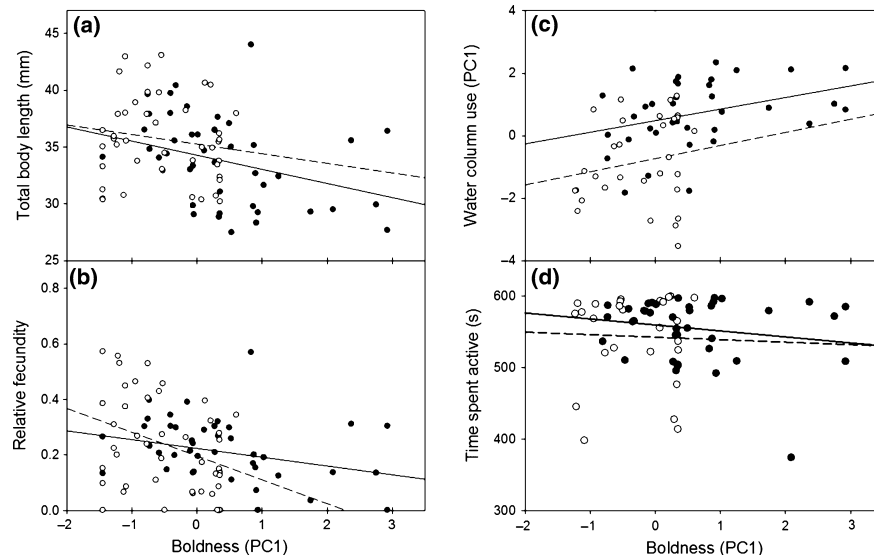


Fig. 1: Observed individual differences in (a) total body length, (b) relative fecundity (=number of eggs or embryos/total body length), (c) water column use and (d) time spent active in relation to boldness in female mosquitofish. Each data point represents an individual fish ($n = 98$). Open circles (○) and dashed lines represent values for individuals collected from Lake Northam. Closed circles (●) and solid lines represent values for individuals collected from Manly Dam. Each line denotes a line-of-best-fit through the relevant data obtained from a least-square regression to illustrate the trend in the data.

relative fecundity ($r_s = -0.31$, $p = 0.003$; Fig. 1b), and strongly positively correlated with their water-column-use behaviour ($r_s = 0.43$, $p = 0.0003$; Fig. 1c). Boldness was not, however, correlated with either activity ($r_s = -0.04$, $p = 0.76$; Fig. 1d) or stage of pregnancy ($r_s = 0.05$, $p = 0.68$). These trends were consistent across the two study populations (Fig. 1). So, for both populations taken together, bolder individuals tended to be smaller, relatively less fecund (when taking body size into account), and spent more time near the water surface than near the substratum compared with timid individuals.

Discussion

Our study addresses two current areas of interest in behavioural ecology, namely, animal personality and reproductive/fitness correlates in wild animal populations. Here, we sought to ascertain the nature of the relationship between these two types of trait in mosquitofish originating from two populations. Most importantly, we found that smaller female mosquitofish tended to be bolder than larger conspecifics under standardized experimental laboratory conditions. When controlling for inter-individual variation in body length, the fecundity (expressed as relative fecundity) of individual females was negatively correlated with their boldness score. Contrary to our expectations, boldness was not correlated with stage of pregnancy nor general activity. That said, the observed relationship between boldness and relative fecundity suggests that reproductive investment influences boldness behaviour, independent of individual body size. However, the direction of this relationship between boldness and fecundity was opposite to the one which we predicted *a priori*. To our knowledge, the current study is the first to have characterized the relationship between a reproductive fitness correlate (fecundity) and boldness in fishes, and among only a few to date to have attempted to describe similar relationships between personality and reproductive fitness in other taxa (e.g. Réale et al. 2000; Stapley & Keogh 2005; Boon et al. 2007).

We also observed that the water-column-use behaviour of female mosquitofish was positively correlated with their boldness score; that is, bolder individuals tended to spend more time near the water surface (than near the substratum) than more timid individuals. Water column use and general activity have been previously recognized as meaningful measures of risk-taking behaviour in fishes (e.g. Wilson & McLaughlin 2007; Wilson & Godin 2009), as presence

near the water surface and increased activity can elevate the risk of predation from both aerial and aquatic predators (Lima & Dill 1990; Godin 1997; Sih et al. 2003). However, in mosquitofish, time spent near the water surface may not reflect a greater willingness to take risks, as this species is generally a surface-dwelling taxon (Pyke 2005). As such, water column use by mosquitofish may be more indicative of foraging or general exploratory tendencies. Both foraging and exploration have been shown to be strongly associated with risk-taking behaviour (see Lima & Dill 1990; Sih et al. 2004; Réale et al. 2007), and our finding of a strong positive correlation between boldness and water column use further supports this association. However, contrary to our initial expectation, individual boldness behaviour and general activity were not significantly correlated in the current study. Based on studies of other species (e.g. Sneddon 2003; Reaney & Backwell 2007; Wilson & Godin 2009), we had expected that bolder individuals would be more active than more timid ones. It is possible that the relationship between boldness and activity may differ between pregnant/reproductive and non-pregnant/non-reproductive individuals, and that this may explain the difference between our finding and those of other studies on the nature of this particular relationship.

The level of parental investment of resources, both temporal and physiological, in bringing embryos to parturition (Marsh-Matthews et al. 2001) may play an important role in determining the willingness of a female to take risks in novel situations. High fecundity and embryos close to parturition both represent a significant energetic investment by females and may be associated with increased metabolic demand in pregnant individuals, making them more willing to take risks to forage so as to meet those demands. Conversely, high fecundity and later-stage embryos could render pregnant females less willing to take risks (i.e. more cautious) and, in doing so, minimizing their risk of mortality to predation, thereby safeguarding their current and future reproductive investment in offspring. Indeed, our results support this latter proposition as more timid female mosquitofish typically had greater relative fecundity than bolder ones. Thus, individual differences in boldness may be affected differentially depending on proximity to parturition and overall current embryonic investment. More importantly, the association between individual boldness and relative fecundity exists irrespective of body size in mosquitofish. This novel finding further suggests that pregnancy influences an individual female's boldness, which

hitherto has been a largely ignored avenue of animal personality research (e.g. Réale et al. 2000; Stapley & Keogh 2005).

Refuge emergence is also a common measure of boldness in many species, including fishes (e.g. Brown & Braithwaite 2004; Wilson & Godin 2009). In the current study, it constituted one component measure of the composite boldness score for individual fish. Poor or uncertain information about the environment outside a refuge can pose a significant risk with regard to predation and also poses a constraint on 'prey' to respond adaptively to ambient conditions (Sih 1992, 1997). As such, the decision of an individual to emerge from a refuge involves the assessment of a number of habitat variables (e.g. food availability, predation threat) and should be fairly robust across populations (Brown & Braithwaite 2004). In our study, female refuge-emergence behaviour was strongly correlated with both relative fecundity and body size. Larger female mosquitofish took longer to emerge from a refuge than smaller individuals, and more timid individuals tended to have higher fecundity independent of overall body size than larger conspecifics. These findings corroborate those of recent studies on refuge-emergence behaviour in another poeciliid fish (Brown & Braithwaite 2004; Brown et al. 2005, 2007b), wherein smaller individuals tended to be bolder. Brown & Braithwaite (2004) hypothesized that this trend towards smaller fish leaving refuge sooner may be a result of energetic factors associated with metabolic demands and life-history stage. Alternatively, individual differences in female boldness may be related to pregnancy, with more timid fish behaving as such due to greater embryonic investment, as suggested by our current results.

Although we found that the nature (direction) of the relationships between individual boldness and certain behavioural and reproductive traits were consistent across our two study populations (Fig. 1), the strength of some of these relationships varied between them. The factors potentially generating such variation between our two study populations are unknown. Dingemanse et al. (2007) postulated that correlations between particular behavioural traits may vary with ambient conditions at particular locales, such that few overarching trends may be revealed across populations and taxa. That said, certain selective agents (e.g. predation) do appear to shape behavioural correlations in a predictable manner (Bell 2005). Indeed, the strong relationships between boldness, water column use and fecundity observed across and within populations in the

current study are consistent with other within-population studies on behavioural correlations (Ward et al. 2004; Wilson & McLaughlin 2007; Wilson & Godin 2009) and suggest that such relationships between behavioural and morphological/reproductive traits may be subject to selection in nature.

In conclusion, our study characterizes for the first time a relationship between an individual personality trait (boldness) and a reproductive fitness correlate (fecundity) in fishes. Given such a relationship, further examination of the potential influence of pregnancy and other reproductive traits on boldness may offer important insights into the underlying mechanistic bases and ecological relevance of this and other personality traits. However, the exact nature of this relationship and its utility as a predictor of boldness in other populations remain a subject for further investigation.

Acknowledgements

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