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Repeatability in Boldness and Aggression Among Wild Zebrafish (*Danio rerio*) From Two Differing Predation and Flow Regimes

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Although flexibility in behavior is adaptive, this flexibility is limited, and the extent of variation and consistency of a trait could depend on the environment. In this study, we investigated repeatability in risk-taking during feeding among individuals and agonistic interactions among dyads of wild zebrafish, *Danio rerio*, collected from two habitats that differed in predation and flow regimes. We measured boldness as the latency to emerge from a shelter and feed in the presence of predators. We tested this for each individual from the two populations repeatedly across seven trials. We assessed aggression by subjecting size- and sex-matched pairs of fish to dyadic contests repeatedly across seven trials. Individuals from the high-predation stream population were bolder than individuals from the low-predation stagnant water population. Males were bolder than females, and in the low-predation population, larger individuals took greater risks to feed than smaller individuals. The high-predation stream population showed lower inter- and intraindividual variation in boldness than the low-predation stagnant water population. Further, both populations showed significant repeatability in risk-taking during feeding. The high-predation stream habitat fish were more aggressive than low-predation stagnant-water fish. Male dyads from the low-predation stagnant-water population were significantly more aggressive than female dyads. Most fish from the low-predation stagnant water population did not show aggressive behavior, resulting in low between-dyad but high within-dyad variation in aggression. The difference in behavioral responses between the populations and consistency in these traits within individuals is discussed in the light of confounding role of ecological and state-dependent factors.

Keywords: inter- and intraindividual variation, risk-taking, aggression, predation, flow

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The ability of organisms to alter their behavior in response to challenges in their environment plays a crucial role in the adaptive evolution of populations. However, although individuals can adjust their behavior to prevailing environmental conditions, not all behaviors can vary with unlimited flexibility (Réale, Reader, Sol, McDougall, & Dingemanse, 2007). Some behaviors are more plastic and vary across environments, whereas other behaviors are constrained by morphology or physiology of the organism and remain relatively stable across environments (Bell, Hankison, & Laskowski, 2009; Bhat, Greulich, & Martins, 2015). For instance, among male collared fly catchers, *Ficedula albicollis*, consistently higher within-year repeatability was observed for risk-taking compared with aggression (Garamszegi et al., 2015). The magnitude of variation observed in a trait and the degree of repeatability, however, differs among life-history stages and populations and depends on the environment in which the trait has been measured (Bell et al., 2009; Nespolo & Franco, 2007). For example, latency to emerge from a safe refuge (boldness) was found to be repeatable across metamorphosis in female but not in male field crickets, *Gryllus integer* (Hedrick & Kortet, 2012). Repeatability in risk-taking during feeding across four contexts were found to vary among four populations of wild zebrafish, *Danio rerio* (Roy, Shukla, & Bhat, 2017). Latency to resume feeding in response to a startling stimulus was repeatable across social and asocial contexts in great tits, *Parus major* (van Oers, Klunder, & Drent, 2005). Studies on boldness and aggression have attracted considerable attention in recent years, as they form an important behavioral axis that determines the ability of an organism to survive in potentially risky environments (Sih & Bell, 2008) and to access resources in the face of intraspecific competition. Furthermore, relationships between boldness and aggression have been linked to shared

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physiological mechanisms or environmental effects (Coppens, de Boer, & Koolhaas, 2010). With a few recent exceptions (Roy et al., 2017), most studies on repeatability have revolved around a single population and have been compared across contexts. However, among species that occur across wide geographic ranges, populations can occur in various ecological contexts, which often change seasonally. Therefore, as expected, different selective forces in these habitats could result in the evolution of interpopulation differences in behavior (Martins & Bhat, 2014). Consistency within populations and repeatability within individuals can be determined by environmental contexts (Roy et al., 2017). This study, therefore, investigates temporal repeatability in boldness measured by risk-taking during feeding and aggressive interactions among two zebrafish populations from widely different habitat regimes.

Temporal consistency in behavioral responses within or across contexts defines personality (Réale et al., 2007; Stamps & Groothuis, 2010). Studies have shown that the propensity to take risks, deemed boldness, may be consistent within and across-contexts. This means, individuals would be repeatedly willing to take risks or shy away when faced with a dangerous situation; e.g., in gray mouse lemurs, *Microcebus murinus*, individual differences in risk-taking during feeding were repeatable and repeatability increased with predation risk (Dammhahn & Almeling, 2012). Startled great tits were also repeatable in their latencies to resume feeding in response to a startling stimulus, across social and asocial contexts (van Oers et al., 2005). Coleman and Wilson (1998) found that individual differences in risk-taking behavior was consistent across but not within contexts in a natural population of juvenile pumpkinseed sunfish, *Lepomis gibbosus*. These studies have typically used a correlation-based approach to explore consistency in behaviors between two contexts. However, few studies have investigated within-context repeatability of risk-taking (Wilson & Godin, 2009) and have rarely considered the effects of individual factors like sex and body size on behavioral repeatability. Here, we investigated temporal repeatability in risk-taking during feeding. We estimated repeatability in boldness across multiple trials to obtain a measure of individual consistency in behavior over time. We also measured between-individual variation in boldness, taking into consideration the sex and body size of individuals.

In addition to boldness, individuals differ in interactive behaviors such as aggression, and population level responses are driven by ecological factors such as predation and food availability (Archard & Braithwaite, 2011a). However, although aggression can benefit an individual in terms of providing greater access to food resources or mates, there are costs associated with these interactions. Indulging in fights consistently could be costly due to injuries, depletion of energy reserves, or a change in endocrine status of the contesting animals, along with circumstantial costs such as loss of time available for other activities or increased risk of predation (Briffa & Sneddon, 2007). If these costs are high enough, then contesting animals may restrict the intensity of agonistic behavior. Previous studies have investigated consistency in aggressive behavior. For example, individuals within a free-ranging population of the Australian lizard, *Egernia whitii*, exhibited temporal repeatability in their aggressive response toward conspecific intruders (While et al., 2010). Aggressiveness toward a same-sex conspecific was highly repeatable in male and female spiders, *Larinioides sclopetarius*, and heritable (Kralj-Fiser & Schneider, 2012). McGhee and Travis (2010) reported repeatability in male aggression toward males and females within the context of mating among bluefin killifish, *Lucania goodei*. On the other hand, plasticity in aggression was reported among three-spined sticklebacks, *Gasterosteus aculeatus*, which was influenced by social environment, perceived predation risk, and sex (Herczeg, Ghani, & Merilä, 2016). Furthermore, aggressive monopolization of food and mate resources was also found to be inconsistent among wild zebrafish (Roy & Bhat, 2015). However, repeated measurements of aggressive interactions between dyads would be needed to understand if individuals reduced aggressiveness because they anticipated costs related to fights. In this study, we measure agonistic interactions repeatedly over multiple trials among same-sex dyads across two populations of zebrafish.

Zebrafish is a popular model system in many fields of biology and has been used in recent years for behavioral studies. However, there have been relatively few studies on wild populations of this species. Zebrafish is a small, group-living cyprinid found across the Indian subcontinent (Roy & Bhat, 2015, 2016, 2017, 2018a, 2018b; Roy et al., 2017). This wide distribution across a range of ecological habitat conditions makes it suitable for comparative studies of behavior. Here, we first aimed to estimate between- and within-individual variation and the repeatability in boldness within populations. Individuals from two wild populations of zebrafish (one from a high-predation and high-flow stream habitat and the other from low-predation stagnant habitat) were subjected to measurements of latencies to emerge from shelter and feed in the presence of predators across seven trials. Repeatability estimates are not affected by the number of observations per individual (see review by Bell et al., 2009). Our previous study on boldness responses across contexts revealed that fish from high-predation, high-flow habitats were consistently bold and expressed low between- and within-individual variation in boldness compared with low-predation, low-flow/stagnant-water populations (Roy et al., 2017, Roy & Bhat, 2018a). Fish from high-predation areas would be under a stronger selection pressure to take risks even in the event of threat from predators (Lima & Bednekoff, 1999). Again, because fish from high-flow habitats have higher metabolic costs for maintaining their position against the current, they would be more inclined to maximize energy intake through feeding (Hill & Grossman, 1993). We, therefore, hypothesized that within a context, fish from high-predation, high-flow population would show greater consistency in risk-taking during feeding compared with the low-predation stagnant water population. Furthermore, based on findings from earlier studies, we expected males to be bolder (Roy et al., 2017) and to show greater consistency in behavior (Bell et al., 2009) than females. Because reproductive investment in females is higher than in males, we expected that females would be more risk-averse.

Following this, we tested consistency in aggression among dyads of wild zebrafish. We subjected size- and sex-matched pairs to dyadic contests (Roy & Bhat, 2015) across seven trials. We hypothesized that the high-predation, high-flow population would be more aggressive than the low-predation stagnant-water population. A positive relationship is often observed between aggression and boldness within fish populations (nine-spined sticklebacks: Herczeg, Gonda, & Merilä, 2009). Therefore, individuals from high-predation, high-flow habitat that exhibited "bold" phenotype (Roy et al., 2017) could be expected to be more aggressive.

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Further, we expected aggression among dyads to decrease across trials for both populations. As testosterone could be a cause for higher aggression and consistency in behavioral responses among males (Wingfield, 1994), we hypothesized that males would be consistently more aggressive than females.

Materials and Method

Populations

Two populations of zebrafish, Kaushalya (from Panchkula district of Haryana; Roy, et al., 2017; Roy & Bhat, 2018a; Roy & Bhat, 2018b) and Wahsympoh (from Jaintia hills district of Meghalaya; Roy, & Bhat, 2018a), were collected during the premonsoon season (March–May) of 2013. Cast nets and drag nets were used for fish collections. Similar sampling efforts were applied (approximately 15–20 cast nets and drag nets each) across a 100-m stretch at each location. The fish species captured in the samples were identified using identification keys. The relative abundance of zebrafish was estimated as the proportion of zebrafish to the total number of fishes sampled (Roy et al., 2017; Roy & Bhat, 2018a). Simultaneously, the piscivorous fish species co-occurring in the habitat with zebrafish were identified. The relative abundance of each species of predatory fish was calculated, and summation of all the values for relative abundances gave an estimate of the total relative abundance of predatory fish (Roy et al., 2017; Roy & Bhat, 2018a; Roy & Bhat, 2018b). Although inherent differences in attack mode and frequency among predator species would determine the actual predation pressure in the environment, this measure was used as a general indicator for predation pressure in habitats. Kaushalya is a large river, and the microhabitat of zebrafish constitutes a fast-flowing stream with rocky substratum and large amount of submerged and floating vegetation. Zebrafish was the most abundant species recorded (relative abundance = 0.56), and the total relative abundance of piscivorous fish was 0.04. The predatory fishes sampled included *Channa orientalis*, *Amblyceps mangois*, and *Mastacembelus armatus* (Roy & Bhat, 2018b). Wahsympoh is a stagnant agricultural drain located between paddy fields, with mud and silt as substratum, and is devoid of vegetation. Zebrafish was the most abundant species recorded (relative abundance = 0.62), and the total relative abundance of piscivorous fish was 0.007. *Channa punctatus* was the only predatory fish co-occurring with zebrafish in the habitat. In the rest of this article, high-predation, high-flow Kaushalya population is referred to as "high" population, and low-predation, low-flow Wahsympoh population is referred to as "low" population in the subsequent sections.

Maintenance of Wild Populations

Approximately 150 individuals were collected for each of the two populations and transported to the laboratory in oxygen bags. Fish were maintained in the laboratory in bare aquaria (45.7 × 25.4 × 25.4 cm) with standard corner filters. For both populations, approximately 30 individuals (mixed sex) were maintained per aquarium. Holding room temperature was kept constant at 25 °C, and lighting was set at 14:10 hr light:dark cycle. The fish were alternately fed freeze-dried bloodworms or *Artemia spp.* every morning. To ensure that only adults were used for the study, fishes were maintained for at least 8 months before the commencement of experiments.

Live predators, *Channa spp.* (snakehead), known to occur commonly in zebrafish habitats across India (Roy & Bhat, 2018a; Roy & Bhat, 2018b; Roy et al., 2017), were used for the experiments. Six snakeheads (approximately 12 cm in body length) were captured using cast nets from local water bodies (of unknown flow-regimes) and transferred in oxygen bags to the laboratory. The snakeheads were kept in three bare glass aquaria similar to the experimental tanks (61 × 30.5 × 30.5 cm), were fed with standard pellet food, and were acclimatized to the laboratory conditions (holding room temperature 25 °C and 14:10 hr light:dark cycle) for at least a month before being used in the experiments.

Experiments

Separate sets of individuals were used for the boldness and aggression experiments. Boldness was tested by latency of fish to emerge from shelter and feed in the presence of predators (high, $n = 30$ [17 males and 13 females]; low, $n = 21$[eight males and 13 females]), and aggression was measured through dyadic contests between conspecifics (high, $n = 15$ dyads [eight male and seven female dyads]; low, $n = 15$ dyads [five male and 10 female dyads]). Individual fish were repeatedly assayed for boldness over seven trials, every alternate day for 14 days. A gap of 1 day was allowed between assays to minimize stress and chances of habituation to the setup. Similarly, pairs of fish were repeatedly subjected to seven dyadic contests, every alternate day over 14 days.

Fish were kept individually to facilitate repeated measurements for boldness and aggression. A bare glass tank (60 × 29.4 × 20.6 cm) was divided into 10 compartments, each measuring 14 × 11 × 15 cm, using nylon mesh. The use of mesh allowed for visual and chemical exchange between the physically isolated fish. Replicates of similar holding tanks were set depending on the number of fish that were used for the experiments. A constant water level of 12 cm was maintained for the holding tanks. All experiments were conducted at the same time of the day. To maintain uniform hunger levels, all fish were fed only at the end of their trials for the day.

Boldness assay. Test fish were sorted from the stock population and were kept individually in the holding tanks (described earlier). The compartments in the holding tanks were labeled for identifying individual fish. The fish were maintained individually in the holding tanks throughout the period of experiments (14 days). The setup and the protocol used for the experiment were similar to those used in our previous study (Roy et al., 2017). A bare glass aquarium (61 × 30.5 × 30.5 cm) was used for the experiments. The aquarium was divided into three compartments with removable fiberglass barriers (Figure 1). One of the compartments with an opaque partition, measuring 10.2 × 30.5 × 30.5 cm, was meant for housing the test fish. Plastic plants were randomly placed, and a cover-shed was provided in this compartment to be used as refuge by the fish. The compartment in the middle (35.6 × 30.5 × 30.5 cm) was the testing arena. The compartment at the other end (15.2 × 30.5 × 30.5 cm) with a transparent perforated fiberglass window screen was meant to hold two snakeheads. The transparent perforated screen allowed for both visual and chemical cues to be perceived by the test fish. The sides of the tank were lined with brown cardboard paper to avoid disturbances.

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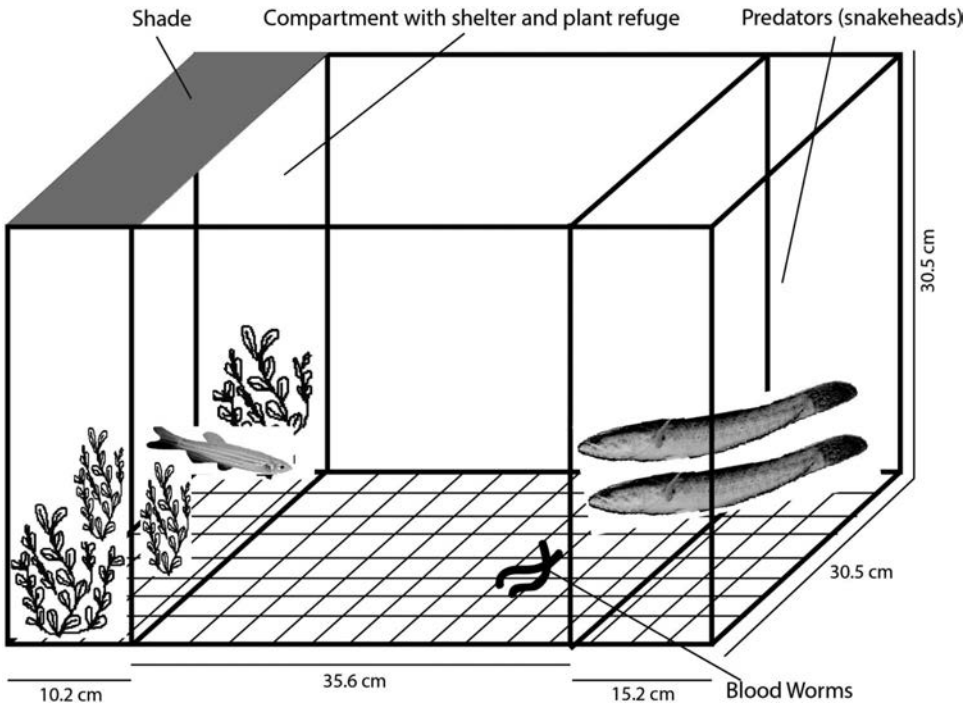


Figure 1. Experimental setup for boldness assay testing latency to emerge from shelter and feed in presence of predators (side-view).

The night before the experiment was conducted, the experimental tank was filled with well-aerated water to a depth of 12.7 cm. Two snakeheads (predators), approximately 12 cm in size, were released into the predator compartment and allowed to settle overnight. The assay in presence of predators was carried out the following day. A test fish was gently transferred to the compartment with vegetation refuge (Figure 1) and allowed to acclimatize for 2 min. After that, the opaque fiberglass partition between the compartment with refuge and the rest of the arena was removed. The test fish, when emerged from the refuge, could see the predators through the transparent window screen but could not pass through to their compartment. A piece of freeze-dried bloodworm was dropped near the far end of the testing arena close to the predator compartment, and the fish behavior in presence of predators was filmed for 10 min using a high-definition camcorder (Canon LEGRIA HF R306, Canon, China) placed overhead of the experimental setup. After the experiment, the individual was put back into the holding tank. From the videos, the latency of the fish to emerge out of the shelter and feed in the presence of predators was noted. Fish across the two populations were tested repeatedly over seven trials in the same way. After the experiments with each population, sex and length of all individuals were recorded. The individuals were stored separately and were not mixed with the stock until the aggression assay began.

Aggression assay. Prior to the start of the behavioral assay, 40 individuals were sorted from the stock population. Of these individuals, 15 dyads (pairs) were constructed, and the paired conspecifics were size- and sex-matched. The sexes of the paired individuals was recorded. The compartments in the holding tanks that were described previously were labeled for identifying the dyads, and the fish were held individually throughout the period of experiments (14 days). The setup and the protocol for testing aggression between dyads were based on our previous study (Roy & Bhat, 2015). A bare glass aquarium (30.5 × 20.3 × 20.3 cm) was divided into three compartments using two opaque removable partitions (Figure 2). The compartment with dimension 20 × 6 cm had an air stone, and the opaque partition that separated it from the rest of the arena had perforations to allow for aeration in the entire tank. The broad compartment (23.5 × 20 cm) was further divided using opaque partitions into two chambers (23.5 × 10 cm), which were perpendicular to the end compartment (Figure 2). The tank was covered with brown cardboard paper on three sides except the front side to avoid external cues from affecting fish behavior.

The experimental tank was filled with well-aerated water to a depth of 13 cm and included an air stone for constant aeration. Water temperature was maintained at 27 °C throughout the experiment. The two individuals that formed a dyad were introduced in the two adjacent chambers in the experimental tank and allowed 2 min for acclimatization. After this, the opaque partition separating the two individuals was gently raised, and the behavior of the fish was video-recorded on a high-definition camcorder (Canon LEGRIA HF R306, Canon, China). After the experiment, the individuals were put back into their respective labeled compartments in the holding tanks. From the video recordings, the number of chases and bites by the two individuals of a dyad was counted. Because it was not possible to tell the two individuals of a dyad apart, we considered the total count of agonistic interactions between the two individuals and estimated the repeatability within each dyad over trials. The dyads were tested repeatedly over seven trials in a similar way.

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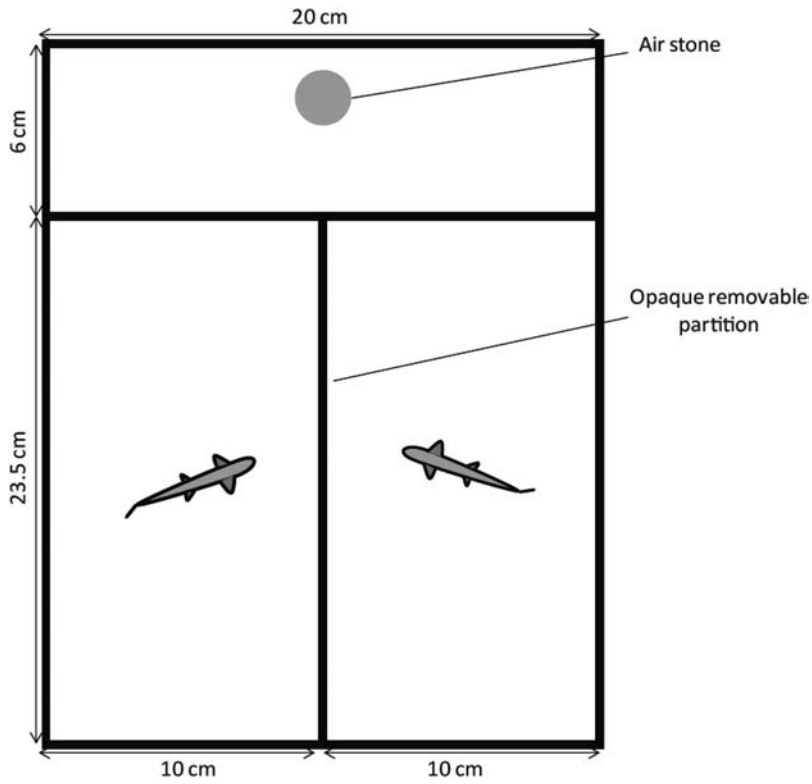


Figure 2. Experimental setup for the aggression assay (top-view).

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All studies complied with the existing rules and guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The studies also adhered to the Institutional Animal Ethics Committee rules and guidelines of Indian Institutes of Science Education and Research, Kolkata. Zebrafish (*Danio rerio*) and *Channa spp.* are designated as a species of least concern by International Union for Conservation of Nature's Red List of threatened species. The community sampling, zebrafish collections, and the collection of snakeheads were not made inside any reserved forest or protected area. The fishes caught during community sampling were identified, recorded, and put back into the water bodies. No animals were euthanized or sacrificed during any part of the study, and behavioral observations were conducted without any chemical treatment on individuals. At the end of the study, no individuals were sacrificed, and all zebrafish were returned to the stock tanks and continued to be maintained in the laboratory. The snakeheads were also returned to the respective stock tanks in laboratory and continued to be maintained.

Analysis

We used lme4 (Bates, Maechler, & Bolker, 2012) to fit linear mixed models in R Version 3.3.1. We square-root transformed the behavioral measures (feeding latencies) to normalize them. All individual measures of latency to feed across populations were fitted as dependent variable and "population Identity" and "individual Identity" were fitted as random effects (to control for nonindependence of data). Variance explained by population ID informed about between-population variations in behavior, whereas residual variance (remaining unexplained variance) informed about within-population variance. The significance of the random effects (i.e., between-individual variation) was assessed using likelihood ratio tests where models with and without the random factor were compared (Fish Identity; Bolker et al., 2009). We compared the linear mixed model having population and individual ID as random effects and trials as fixed effect with the model having only individuals as random effects. This comparison showed no significant difference using the likelihood ratio test ($p > .05$), and hence, we carried out further model building with individual ID as random effect. The Satterthwaite method was used to approximate degrees of freedom. The significance level was set at $p < .05$. The full model consisted the fixed factors Trial, Population, Sex, Size, and their interactions (Trial × Sex, Trial × Size, Trial × Population, Sex × Population, and Size × Population) as fixed effects. Any interactions between fixed factors that were not significant ($p > .05$) were removed from the final model. The data on aggression were based on counts and indicated a Poisson distribution (Lo & Andrews, 2015). The significance level for random effects for the linear mixed models for aggression was assessed using the Laplace approximation method (significance set at $p < .05$). For the full model, Trial, Population, Sex, and their interactions (Trial × Sex, Trial × Population, and Sex × Population) were included as fixed effects. Because Trial × Sex did not show a significant effect, this interaction was removed, and a reduced model with interactions Trial × Population and Sex × Population was used. $R^2_{(m)}$, the proportion of variance explained by

the fixed predictors, was used to measure effect size and their confidence intervals using the r2glmm package (Nakagawa & Schielzeth, 2013).

To estimate behavioral repeatability and consistency within populations, we used separate linear mixed models for boldness and aggression within each population. Repeatability is defined as the proportion of the behavioral variation attributable to differences between individuals, and the repeatability estimate "R" is formally expressed as between-individual variance/(between-individual variance + residual variance) (Boake, 1989). For estimating repeatability in boldness within populations, we ran linear mixed models separately with the individual ID (individual identities) as a random effect and Trials, Sex, Size, and the interactions, Trial × Sex and Trial × Size, as fixed factors. For aggression, we ran linear mixed models with the dyad identities (dyad ID) as a random effect and Trials, Sex, and the interaction Trial × Sex as fixed factors within populations. No aggressive interactions were recorded across trials for most dyads of "low" population (overdispersion), and therefore, a log-link function was added to the code specifying the distribution. The model revealed the repeatability estimates for aggression within "low" population for the dyads ID and overdispersion separately. The repeatability was calculated using rpt.remlLMM function from the rptR package (Betini & Norris, 2012; Nakagawa & Schielzeth, 2010), and the 95% confidence intervals (CIs) were used as estimates of uncertainty (Betini & Norris, 2012).

Results

Boldness

The linear mixed model for boldness (Model $R^2_m = 0.32$, 95% CI [0.41, 0.27]) showed a significant effect of Population ($F_{1,45} = 4.28$; $p = .04$), Sex ($F_{1,45} = 13.69$, $p < .001$), Size ($F_{1,45} = 8.02$, $p = .007$), and Population × Size ($F_{1,45} = 4.48$, $p = .04$) on feeding latencies. Individuals of "low" population had significantly higher feeding latencies (lower boldness) than individuals of "high" population ($t = 2.21$, $df = 45$, $p = .03$; Figure 3A). Details of the parameter estimates and their confidence intervals are provided in file S1a in the online supplemental materials. Although there was no significant effect of trials on boldness, there was a slight Population × Trial effect indicated with fluctuating feeding latencies observed for "low" population between Trial 1 to Trial 3. Feeding latencies in the "high" population, however, were steadier across subsequent trials. Males ($n = 25$, $M = 25.25$ s ± 9.18) had significantly lower feeding latencies than females ($n = 26$, $M = 131.25$ s ± 35.09; $t = -2.79$, $df = 28$, $p = .009$). A significant negative interaction between body size and latency to emerge from shelter and feed in presence of predators was observed for the "low" population ($t = -2.12$, $df = 45$, $p = .04$). This is also indicated by a slightly significant Population × Size interaction, with a negative effect of size on feeding latencies among the "low" population in the model (File 1 in the online supplemental materials).

Aggression

The results of the linear mixed model (Model $R^2_m = 0.08$, 95% CI [0.22, 0.07]) for aggression showed a significant effect of Population ($\chi^2_1 = 7.55$; $p = .006$), Sex ($\chi^2_1 = 12.71$; $p < .001$), Trials ($\chi^2_6 = 611.38$; $p < .001$), Population × Sex ($\chi^2_1 = 14.52$; $p < .001$) and Population × Trial ($\chi^2_1 = 296.77$; $p < .001$). Dyads of "low" population showed significantly lower aggression than "high" population dyads (*Z* test: *z* score = −6.9, $p < .001$; Figure 3B). A significant effect of trials implied that the number of aggressive interactions decreased significantly across repeated trials. This was indicated by a significant decline in interactions from the first trial to the subsequent ones for "high" population and an initial increase in Trial 2, followed by a decrease in aggressive interactions for "low" population (see Figure 3B; see File S1b in the online supplemental materials for details of parameter estimates and their confidence intervals). Differences in aggressive interactions were significant between populations for the first ($t = 2.36$, $p < .001$, $df = 14$) and the fifth ($t = 2.31$, $p = .01$, $df = 14$) trials. Further, the change of aggressive interactions across trials was significantly different between the populations "high" and "low" as indicated by a significant interaction Population × Trial. Although there was a steady decrease in aggression in the "high" population, aggressive interactions in the "low" population fluctuated, indicated by an initial increase in aggression in Trial 2, and then a steady decrease. Within the "low" population, male dyads were significantly more aggressive than the female dyads ($Z = 3.81$, $p < .001$).

Repeatability

"High" population showed lower between-individual variance and lower within-individual variance (intraindividual variation; IIV; Stamps, Briffa, & Biro, 2012) in boldness than the "low" population (Table 1). Significant repeatability in boldness was observed for "high" and "low" populations. The confidence intervals for the "high" and "low" populations overlap, indicating that repeatability estimates for boldness for the two populations are not very different. No significant effects of Trial × Sex and Trial × Size on boldness were observed for either population. "High" population showed higher between-dyad variance and lower within-dyad variance in aggression than "low" population (Table 1). Significant repeatability in aggression was observed for "high" population dyads. Within "low" population, repeatability for overdispersion (indicating data with mostly "0" values) was significant ($r = .98$, $SE = 0.07$, CI [0.81, 1], $p < .001$; Table 1) because most dyads of "low" population showed no aggression across most trials. The overall repeatability in aggression of "low" population dyads was not significant ($r = 0$, $SE = 0.03$, CI [0, 0.08], $p = .5$); possibly there were only a few individuals that were aggressive. The confidence intervals of the repeatability estimates do not overlap, indicating repeatability of aggression for "high" and "low" populations are different for the two populations. There was no significant effect of Trial × Sex for either of the populations.

Discussion

Interpopulation Variation and Repeatability in Boldness

Our results showed that fish from a high-predation, high-flow habitat were bolder than fish from a low-predation, stagnant habitat (Figure 3A). Risk-taking during foraging is usually a trade-off

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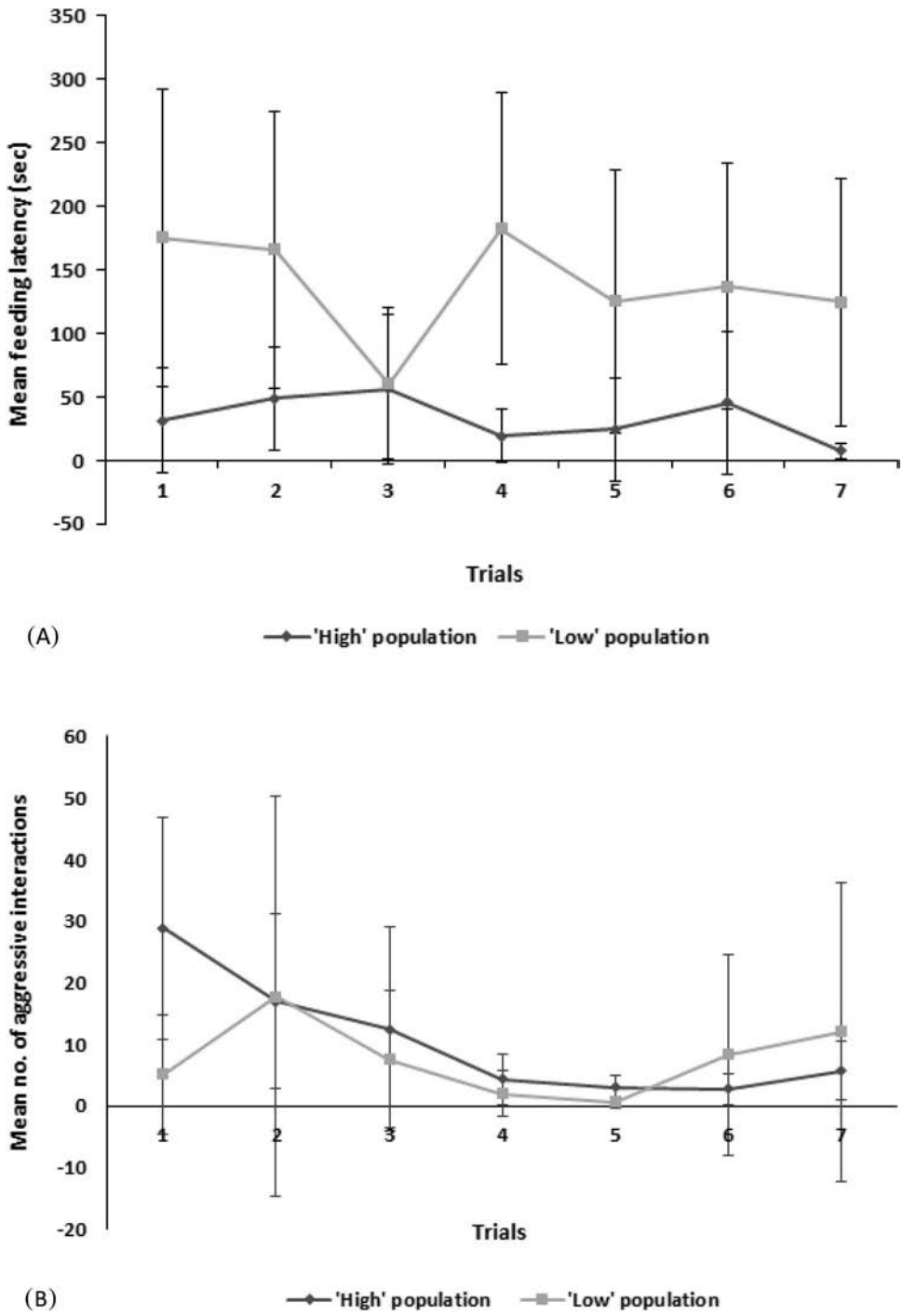


Figure 3. Comparisons of repeatability of boldness and aggression between the "high" and "low" populations. (A) Mean feeding latencies of individuals. (B) Mean number of aggressive interactions among dyads across repeated trials. The error bars represent the 95% confidence intervals.

between feeding and antipredatory behavior and could be influenced by environmental factors like predation (Dowling & Godin, 2002; Herczeg & Välimäki, 2011; Sih, 1997) and state-dependent factors like metabolic rate (Killen, Adriaenssens, Marras, Claireaux, & Cooke, 2016; Metcalfe, Van Leeuwen, & Killen, 2016). Previous studies on guppies, *Poecilia reticulata* (Pitcher & Parrish, 1993), and nine-spined sticklebacks, *Pungitius* (Herczeg, Gonda, Kuparinen, & Merilä, 2012), showed predator-sympatric populations to act more cautiously than predator-allopatric populations. Our results agree with other studies conducted on guppies (Harris,

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Table 1
Repeatability Estimates of Each Population With 95% Confidence Intervals and a Significance Level of 5%. Within-Population Repeatability Was Calculated as the Ratio of Between-Individual Variance and the Sum of Between-Individual and Residual Variance

Population	Between-individual variance	Within-individual variance	Repeatability	*SE*	95% CI	*p*
Boldness						
"High" population	3,741	8,753	.39	.08	[.25, .56]	<.001
"Low" population	21,005	17,439	.54	.1	[.36, .73]	<.001
Aggression						
"High" population	101.1	295.7	.39	.14	[.1, .66]	<.001
"Low" population	54.82	840.30	.98[a]	.07	[.81, 1]	<.001

Note. CI = confidence interval.
[a] Estimate for overdispersion.

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Ramnarine, Smith, & Pettersson, 2010) and poeciliids, *Brachyraphis episcopi* (Archard & Braithwaite, 2011b; Brown, Jones, & Braithwaite, 2005, 2007), which showed that high-predation populations behaved boldly in comparison with low-predation populations. The results match our previous study in which fish from the high-predation stream population were significantly bolder than fish from low-predation, low-flow/stagnant-water populations across four contexts (Roy et al., 2017). The ability to explore, forage, and reproduce in the face of predation threat has direct fitness consequences for individuals inhabiting high-predation habitats (Brown et al., 2005). Individuals that continue to forage and reproduce in the face of constant predation pressure would have a fitness advantage over those that remain cautious, leading to lost foraging or mating opportunities. Thus, high-predation habitats select bold individuals compared with low-predation habitats (Brown et al., 2005).

Similarly, differences in intrinsic metabolic demand may exert a strong influence on feeding behavior and boldness (Killen, Adriaenssens, et al., 2016; Metcalfe et al., 2016). In flowing water, fish are expected to incur higher metabolic costs with respect to maintaining position and swimming against the flow and may require greater muscle mass and mitochondria even while at rest and not swimming against a current. This, in turn, could increase their foraging requirements and, hence, boldness (Killen, Glazier, et al., 2016; Sinclair, de Souza, Ward, & Seebacher, 2014). Therefore, "high" population fish, which likely experience higher levels of physical exertion in maintaining their position in moving water currents, might be more willing to incur risks during feeding (Roy et al., 2017) compared with fish from the "low" population. Our results also agree with a recent study in which zebrafish from flowing-stream habitats were shown to be bolder and more aggressive than those from slow-moving and stagnant habitats (Martins & Bhat, 2014).

Findings such as these point to the likely role of multiple, often confounding factors such as habitat complexity, food availability, intrinsic metabolic demand, and predation pressure in determining boldness (Biro, Post, & Parkinson, 2003; Brown & Warburton, 1997). Further studies with more replicate populations for each of these factors could help disentangle the seemingly contrasting influences of habitat complexity, flow, and predation pressure on boldness. Studies on replicate populations within each ecological condition (e.g., high vs. low predation and high flow vs. stagnant water), along with experiments in seminatural conditions (e.g., mesocosms) controlling for other possible ecological factors, would be required to concretely confirm the role of each factor in determining behavior.

Males were bolder than females, as reported in our previous study (Roy et al., 2017). Males and females are likely to differ in boldness due to differences in life-history strategies (Magurran, 2005). Among the poeciliid genus *Brachyraphis*, males were found to be bolder than females, measured by the time to emerge from shelter into novel environments (Ingley, Rehm, & Johnson, 2014). According to life-history theory, individuals with high future expectations (that have much to lose) should be more risk-averse than individuals with low expectations (Wolf, van Doorn, Leimar, & Weissing, 2007). Because reproductive investment of females is greater than males, females are expected to be more risk-averse. Further, a significant negative interaction between body size and feeding latency for "low" population fish implied that smaller individuals were less inclined to take risk to emerge from shelter and feed in presence of predators than larger individuals. Previous studies have demonstrated that smaller individuals emerge from shelters sooner than larger fish for feeding (metabolic hypothesis: Krause, Loader, McDermott, & Ruxton, 1998; Skalski & Gilliam, 2002). Our results appear to be in line with the predation hypothesis that states that smaller fish are more prone to predation and would emerge from shelters later than larger fish (reviewed in Sogard, 1997). In a low-predation population like here, smaller fish could be expected to be more wary of novel situations and presence of predators (as in the experiment) than larger ones. As a counterargument, being less bold may also translate to lost foraging opportunities and reduced food intake, which might lead to smaller body size.

"High" population showed significantly low between-individual variance in feeding latencies across trials in comparison to "low" population (Table 1). Trait variation between individuals within population could be limited due to physiological constraints imposed by environmental conditions (Gotthard & Nylin, 1995). The "high" population fish belonged to a high-predation and high-flow habitat. Hence predation pressure, along with higher energy requirements in high-flow conditions (Killen, Adriaenssens, et al., 2016; Sinclair et al., 2014), could limit behavioral variation between individuals of "high" population. This observation is consistent with the results from our previous study in which individuals from "high" population exhibited low between-individual variation in boldness (Roy et al., 2017). Such constraints being

absent in "low" population could mean lower selective forces favoring boldness and therefore possibility of greater inter- and intraindividual variations in behavior. Within-individual variance or IIV (Stamps et al., 2012) in risk-taking during feeding was also lower in "high" (Roy et al., 2017) than "low" population. Because IIV informs about the predictability of behavior (Osborn & Briffa, 2017), lower IIV for "high" population implied higher predictability of the trait than "low" population.

Repeatability demonstrates the strength of individual consistency for a behavioral trait (Boake, 1989). We found latency to emerge from shelter and feed in the presence of predators to be repeatable across multiple trials within both "high" and "low" populations. The "low" population showed higher repeatability in boldness than "high" population. However, the repeatability estimates did not differ significantly as indicated by the CIs (Table 1). Fish could be expected to behave in an equally bold manner when they are subjected to a certain experimental condition repeatedly (Adriaenssens & Johnsson, 2013). Our study supports a previous study on an anadromous population of juvenile three-spined sticklebacks in which foraging and predator inspection behaviors were found to be repeatable (Dzieweczynski & Crovo, 2011). Conditions of low food availability may increase between-individual variation in risk-taking during feeding in fish (Killen, Adriaenssens, et al., 2016). Studies in European sea bass, *Dicentrarchus labrax* (Killen, Marras, & McKenzie, 2011), showed that repeatability in risk-taking behavior with respect to foraging was maintained with a 1-week period of food deprivation. In our study, fish were not fed before the experiments to maintain constant hunger levels. This condition of low food availability could have resulted in increased between-individual variation and significant repeatability in risk-taking during feeding across trials. The variation in repeatability estimates could be due to variation in response of the populations to the unpredictability in food availability (Wang, Hung, & Randall, 2006). Because the zebrafish populations in our study likely differ in ecological and evolutionary histories, they are likely to differ genetically in their response to stress. This could explain the observed differences in responses.

Population Differences in Aggression Across Trials

"High" population fish that belonged to a high-predation and high-flow habitat demonstrated higher levels of aggression than fish from "low" population that belonged to a low-predation stagnant habitat (Figure 3B). A previous study on nine-spined sticklebacks showed that low-predation pond fish were more aggressive than high-predation marine fish (Herczeg & Välimäki, 2011). However, a positive relationship between aggression and boldness within populations has been observed in other studies, in which bolder fish have been found to exhibit higher levels of aggression than shy fish (Archard & Braithwaite, 2011a; Herczeg et al., 2009; Roy & Bhat, 2015). Therefore, "high" population fish that also showed higher levels of boldness could be expected to be more aggressive than fish from "low" population. However, because we did not use the same fish in the studies of boldness and aggression, we cannot draw conclusive correlations between them. Previous studies have shown that fish with higher metabolic rate are more aggressive than fish with lower metabolic rate (Cutts, Betcalfe, & Caylor, 1998). As argued previously, "high" population fish likely have a higher metabolic rate than "low" population, and this may also account for differences in aggression. Another factor that could explain population variation in aggression is food availability (Magurran & Seghers, 1991), and future field studies measuring the productivity of these sites could help understand this relation. We reiterate the need for extensive studies with replicate populations and controlled experiments to understand the individual role of each factor in influencing behavioral responses.

The between-dyad variation in aggression was lower in "low" compared with "high" population, as indicated by the confidence intervals (Table 1). The low variation for "low" population could be primarily because no aggression was observed during most trials for the dyads. The within-dyad variation in aggression was lower in "high" than "low" population, as also indicated by the repeatability estimates and confidence intervals (Table 1; Figure 3b). The high value for within-dyad variation in "low" (Table 1) could be because the dyads displayed aggression only during some trials while being mostly nonaggressive in others. The individuals comprising dyads were matched for sex and size, and the fish were maintained in a controlled feeding regime. However, there might be differences in physiological state (fat/thin; IIVs in metabolic rates) that could not be controlled for. Decreased food availability and increased hunger reduce aggressiveness in juvenile Atlantic salmon, *Salmo salar* (Vehanen, 2003). Therefore different fish might respond to hunger stress in different ways. Furthermore, differences in hormonal profiles could also influence aggression among individuals in a pair.

Aggressive interactions among dyads differed across trials for both "high" and "low" population fish (Figure 3B). Mean aggression in "high" population decreased across trials, and this observation was supported by the significantly low repeatability (0.39). This agreed with our hypothesis that temporal consistency in aggression among dyads of wild zebrafish would be low. Engaging consistently in fights may lead to injuries, exhaustion, or a change in the endocrine status (Briffa & Sneddon, 2007). Establishment of dominant–subordinate relationships between fighting individuals could reduce aggression levels (Liley, 1966). In our study, a likely establishment of dominance by an individual over a less aggressive individual across repeated trials could result in a reduction in aggression over time. This could be tested by changing any one of the individuals of the dyad and assaying them over trials because in the wild, the probability for a fish to repeatedly engage in a dyadic combat with the same conspecific would be low. Also, acclimating the fish with the setup before the start of the experiments would help rule out the possibility of novelty of the arena in influencing behavioral responses. The significantly high repeatability for overdispersion within "low" population (0.98) indicated that most dyads consistently showed no aggression across most trials.

Male dyads of the "low" population were significantly more aggressive than female dyads. Previous studies have documented sex differences in aggression in fish. Agonistic interactions in dyadic combats between size-matched rainbow trout showed males to be more aggressive than females (Johnsson & Akerman, 1998). Aggressive interactions were more frequent between males than between females in Japanese *medaka*, *Oryzias latipes* (Grant & Foam, 2002). However, in contrast to these studies and our results, female aggression rates in *Brachyraphis episcopi* were higher than male aggression rates in low predation populations (Archard & Braithwaite, 2011a). Sex-specific life-histories would possibly ac-

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count for the intersexual differences in aggression. In our previous study, we have demonstrated that agonistic interactions in male dyads were related to monopolization of food, as well as females (Roy & Bhat, 2015). However, aggression in females would be linked to only gaining access to food resources (Archard & Braithwaite, 2011a), and therefore we can expect them to be less aggressive than males. It must also be noted that learning and habituation may affect the novelty aspect of a test replicated several times (Greenberg & Mettke-hofmann, 2001; Wahlsten, 2001). This problem can be controlled by testing behavior in different contexts on alternate days, which would integrate environmental and temporal variation (Réale et al., 2007; Seferta, Guay, Marzinotto, & Lefebvre, 2001).

Conclusion

Recent theoretical and empirical studies indicate that temperament may influence the stability of biological communities, both as a source of intraspecific trait variability (Bolnick et al., 2011) and through its effects on an individual's or population's ability to adapt to environmental conditions (Sih et al., 2015). Predictive theories can help in determining variations in individual responses to environmental change and predict differences in stress responsiveness among individuals (Bensky, Paitz, Pereira, & Bell, 2017). Future investigations on long-term repeatability of boldness and aggression within and across contexts, and over generations are required. Studies need to be conducted across replicate populations from different habitat regimes to understand the specific roles of environmental factors. Importantly, the present findings of population divergences in behavior underline the need for controlled experiments to disentangle the confounding roles of each factor.

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