

# Abiotic environmental factors contribute to spatial variation in boldness and exploration in guppies (*Poecilia reticulata*)

Alexis M. Heckley<sup>1</sup>  | Pierre-Olivier Montiglio<sup>2</sup> | Janay A. Fox<sup>1</sup> | Sarah Sanderson<sup>1</sup> | Alison M. Derry<sup>2</sup> | Kiyoko M. Gotanda<sup>3</sup> | Andrew P. Hendry<sup>1</sup>

<sup>1</sup>Department of Biology, McGill University, Quebec, Canada

<sup>2</sup>Département des sciences biologiques, Université du Québec à Montréal, Quebec, Canada

<sup>3</sup>Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada

## Correspondence

Alexis M. Heckley, Redpath Museum and Department of Biology, McGill University, Montreal, Quebec, Canada.

Email: [alexis.heckley@mail.mcgill.ca](mailto:alexis.heckley@mail.mcgill.ca)

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## Abstract

Research on wild fishes has tended to overlook the role of abiotic factors in shaping behaviours associated with boldness and exploration. This oversight could exist because small-scale variation in the abiotic environment might seem unlikely to influence such behaviours. We challenged this assumption through research in the Trinidadian guppy (*Poecilia reticulata*) system. We started by quantifying how behaviours associated with boldness (time in a shelter and time frozen in an open field) and a behaviour associated with exploration (number of grid squares crossed in an open field) varied for guppies within and among 15 pools across two streams, where all of the pools within a stream were within 150 m of each other. The measured behaviours differed little between streams, yet they varied dramatically among pools within streams and among individuals within pools, thus illustrating how such behaviours can be structured on very small spatial scales. We next assessed how the observed behavioural variation might be explained by individual-level attributes (sex and body mass) and pool-level abiotic factors (e.g. temperature and dissolved oxygen). Individual-level attributes explained little of the behavioural variation, although smaller guppies did display slightly bolder behaviour. Among-pool abiotic factors, however, were quite informative. As a clear example, guppies from pools with less dissolved oxygen displayed bolder behaviour and (possibly) greater exploration. Our results highlight the importance of abiotic factors in shaping behaviour even on small spatial scales.

## KEYWORDS

animal behaviour, conductivity, dissolved oxygen, freshwater, plasticity, spatial heterogeneity

## 1 | INTRODUCTION

With the recognition that intraspecific phenotypic variation can have strong ecological and evolutionary effects (Des Roches et al., 2018; Hendry, 2017), researchers have been increasingly interested in understanding the causes and consequences of among-individual

variation. A large proportion of the research investigating such intra-specific variation has focused on behaviour, with two behaviours, boldness (risk taking) and exploration (movement in a novel environment) (Réale et al., 2007), having received particular attention (e.g. Brown et al., 2007; Chapman et al., 2011; Reader, 2015). These behaviours can affect ecological processes ranging from altered

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acquisition of information and resources (e.g. Patrick et al., 2017) to success in mating interactions (e.g. Ariyomo & Watt, 2013; McCowan et al., 2014) and survival (e.g. Ballew et al., 2017; Rödel et al., 2015). Among individuals, boldness and exploration can be highly variable, particularly among individuals from different populations (e.g. Magnhagen et al., 2012; Rudh et al., 2013). As such, investigating how bold and exploratory behaviours are associated with factors that differ within and among populations can provide insight into the causes of these ecologically and evolutionarily important behaviours.

Nearly all of the work investigating causes of variation in bold and exploratory behaviours has focused on linking these behaviours to individual-level attributes (e.g. sex, size or individual behavioural 'type', that is, each individual's average behavioural trait value) or biotic components of the environment experienced by individuals, such as the number and type of conspecifics or heterospecifics (e.g. Archard & Braithwaite, 2011; Brown & Braithwaite, 2004; Burns et al., 2016; Harris et al., 2010; Ingley et al., 2014; Magnhagen et al., 2012; Moran et al., 2016; Piyapong et al., 2010; Rasmussen & Belk, 2017; Ward et al., 2007). The consensus from this large body of work is that individual-level attributes and biotic environmental factors can strongly shape bold and exploratory behaviours within and among natural populations. In every such study, however, a considerable amount of unexplained behavioural differences exist among different populations in similar biotic environments (e.g. Bell et al., 2010; Magurran & Seghers, 1994). For instance, Trinidadian guppies (*Poecilia reticulata*) collected from sites with similar predation and parasitism regimes show substantial between-river behavioural variation, including in boldness (Jacquin et al., 2016). This unexplained variation suggests that other factors, beyond the measured biotic components, are important contributors to behaviour. Perhaps we should be taking a closer look at abiotic factors.

A few studies have analysed associations between abiotic factors and boldness or exploratory behaviours, and some of those studies have found strong effects (e.g. temperature, sulfide, dissolved oxygen, water flow velocity; Culumber, 2020; Riesch et al., 2009; Sommer-Trembo et al., 2017; Tang & Fu, 2021). Abiotic environmental factors thus could contribute to unexplained variation within biotically similar environments. Of particular interest, abiotic factors can vary at spatial scales that range from small (e.g. two pools separated by a shallow riffle within a stream) to large (e.g. two different streams), yet relevant studies from nature typically focus on larger scales only (e.g. among populations: Sommer-Trembo et al., 2017; Tang & Fu, 2021) or deliberately exaggerate abiotic factors to exceed the conditions of typical undisturbed environments (e.g. thermal or salinity stress; Culumber, 2020; Leite et al., 2019, 2022). As a result, we still have little understanding of how natural abiotic variation at small spatial scales might contribute to variation in behaviour. To address this research gap, we sought to explain behavioural variation through measures of abiotic factors within biotically similar environments. That is, we deliberately controlled for some 'known-to-be-important' biotic sources of variation to assess whether abiotic factors could address the otherwise unexplained variation.

## 1.1 | Our study

We focused on bold and exploratory behaviours of Trinidadian guppies *Poecilia reticulata* (Peters 1859) revealed through observations in an open-field test, a well-established assay for quantifying variation in these behaviours in guppies (Burns, 2008) and other fish species (Toms et al., 2010). Some studies that measure bold and exploratory behaviours do so with the intent of studying repeatable behavioural differences, which are often viewed as 'personality' or 'temperament'; Réale et al., 2007). Although previous studies of these behaviours in guppies often do find they are repeatable (Table S1), our interest was explaining variation in point estimates of 'behaviour' not in attempting to infer variation in 'personality'. That is, our measures of bold and exploratory behaviours probably include effects of among-individual behavioural differences, as well as within-individual plastic responses to the environment from which they were collected, presumably then also influenced by the experimental process (e.g. stress and handling).

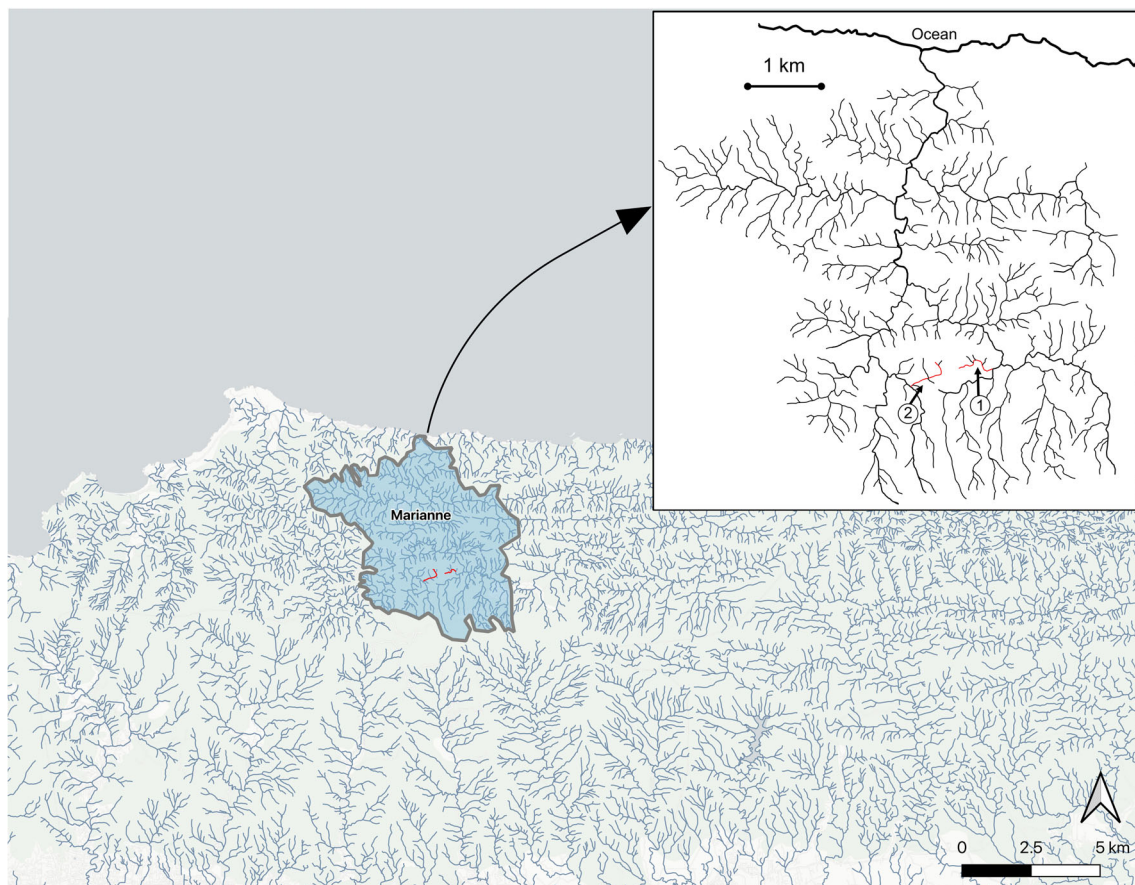
Behavioural assays were conducted on all individuals collected from 15 pools (i.e. microhabitats with seasonally restricted gene flow) distributed across two streams. Within a given stream, the pools were separated by as little as 0.75 m and by no more than 150 m. The two streams are within the same watershed (Figure 1), and so are geographically close yet contain guppy populations that are phenotypically and genetically distinct (Blondel et al., 2019). To focus on biotically similar environments, both streams contained 'low-predation' guppy populations, where the main predator is the gape-limited *Anablepsoides hartii* that only eats small guppies (Endler, 1978; McKellar et al., 2009; Seghers, 1973). Additionally, similar streams in the same river can have very low levels of the guppy parasite *Gyrodactylus* spp. (Gotanda et al., 2013; Pérez-Jvostov et al., 2012).

Analytically, we first established how bold and exploratory behaviours varied at three spatial scales. First, we investigated variation among individuals within a given pool. Second, we investigated variation among pools within a given stream. Third, we investigated variation between the two streams. We next examined factors that were associated with behavioural variation at these spatial scales. Within pools, we focused on sex and size, two individual-level attributes that could contribute to behavioural variation among individuals. Among pools, we focused on several abiotic environmental factors, such as dissolved oxygen (mg/L) and temperature (°C), that were plausibly associated with the measured behaviours (e.g. Culumber, 2020; Sommer-Trembo et al., 2017).

## 2 | MATERIALS AND METHODS

### 2.1 | Pool selection and ecological data collection

Our study focused on a total of 15 pools selected across two streams (stream 1,  $n = 7$ ; stream 2,  $n = 8$ ) in the Marianne River in Trinidad (Figure 1). These two streams represent distinct genetic clusters (Blondel et al., 2019), although they are still part of the same river.



**FIGURE 1** Map of the Northern Mountain Range in Trinidad. The Marianne River, where our study was conducted, is shaded in blue and shown zoomed-in with the inlay map. The specific streams that we studied are coloured red, and the approximate locations of stream 1 and stream 2 are labelled on the inlay map (1, stream 1; 2, stream 2). The map of the Northern Mountain Range was modified from Heckley et al. (2022).

Our work was conducted during low-water/dry-season (February 2020), which ensured that the pools were mostly or entirely isolated from each other. Before collecting guppies, we recorded pool-level ecological variables that could affect their behaviour. First, for each pool we used a tape measure or marked PVC pipe to measure the maximum width (m), maximum length (m), maximum depth (m) and mean depth (m); the last of these measures was obtained by taking depth measurements every 50 cm along the longest and widest sections of the pool. These linear dimensions were used to then estimate the surface area (length  $\times$  width in  $m^2$ ) and volume (surface area  $\times$  mean depth in  $m^3$ ) of each pool. Second, we used a Yellow Springs Instrument (YSI) probe (model 10,102,030; Yellow Springs Inc.) to record pH, temperature ( $^{\circ}C$ ) and dissolved oxygen concentration (mg/L) of the water. We also recorded specific conductance ( $\mu S/cm$ ) with the YSI, which provides conductivity standardized by temperature. Finally, we used a concave spherical densimeter to record canopy cover above each pool.

We returned to the pools 72 h after the above physical and ecological variables were measured to collect guppies. Using butterfly nets, we attempted to capture all guppies from each pool, which is usually possible because capture rates are extremely high in small

pools in the dry season, as shown by numerous mark-recapture studies (e.g. Bryant & Reznick, 2004; Reznick et al., 1996; Weese et al., 2010). To ensure a high capture rate in our study, we waited for at least 2 min following the last captured guppy. If another guppy was spotted, it was captured and the 2-min timer was reset. This process was repeated until no more guppies were seen. Although it is possible that we failed to capture some resident guppies, we at least captured a very high proportion of them, yielding a total of 303 adult guppies (stream 1,  $n = 126$ ; stream 2,  $n = 177$ ) (see Table S2 for pool-specific sample sizes).

On the same day that guppies were collected (stream 1, 17 February 2020; stream 2, 18 February 2020), all the fish were transported in 2-L cleaned plastic bottles by car to the William Beebe Tropical Research Station in the Arima Valley, where they were housed in pool-specific aquaria filled with rainwater collected at the research station. While in the laboratory, guppies were fed fish flakes (TetraMin Tropical Flakes) daily, the aquarium water was treated with API Stresscoat and API Quick Start, and water changes were conducted every few days. The guppies were held under these conditions for at least 2 days, with a maximum holding time of 18 days, prior to being used in the behavioural assays. The length of time varied in this

manner owing to the total processing time needed for all fish before subsequent simultaneous release back into the stream.

## 2.2 | Behavioural assays

The behavioural assays were conducted in 72-L aquaria (30.5 cm wide, 38.5 cm deep, 61 cm long) with a 5 × 5 cm grid drawn across the bottom. Red-brown and yellow aquarium gravel was lightly spread across the bottom to approximate some natural substrate colours in Trinidad streams. The water was maintained at 6 cm deep and was changed twice per day: before the first trial and after approximately half the trials were completed. Tanks were illuminated with both natural light from a large window and indoor lighting at the field station. An artificial plant was placed in the corner of each aquarium to provide an opportunity for the fish to seek refuge during the trial. Behaviours (details below) were scored live by an observer. An opaque plastic blue sheet was placed between the tank and the body of the observer to minimize disturbance to the fish while allowing the observer to still see the fish from above the sheet. Assays were conducted between sunrise and sunset to correspond to the diurnal activity period.

For each trial, an individual fish was placed in the experimental arena inside a clear plastic holding container, which was constructed by gluing two clear mini food storage containers together. The holding container was placed in the corner of the aquarium on the same short side as the plant-refuge (the refuge was in one corner and the holding container was placed in the other corner). The fish were left in the holding cylinder for a 3-min acclimation period, after which the holding cylinder was gently lifted by hand by the observer and the trial immediately began. Each individual trial then ran for 5 min, during which time the four behaviours were recorded. Our measures of bold behaviour were refuge use (time, in seconds, that the fish spent in an artificial plant refuge), freezing instances (number of instances where the fish was moving and then stopped) and total freezing time (time, in seconds, that the fish was not moving). Our measure of exploratory behaviour was number of squares crossed in a novel open field. These proxies were chosen because previous studies confirmed their use for studying variation in bold and exploratory behaviours (e.g. Carlson & Langkilde, 2013; Diaz Pauli et al., 2019). We used the behaviour software BORIS v 7.9.7 (Friard & Gamba, 2016) to live-record the time spent in the refuge, the total freezing time, the number of freezing instances and the number of grid squares crossed. On completing the behavioural trials, the fish were lightly anaesthetized using tricaine methanesulfonate (MS-222) and visually scanned for ectoparasites under a Zeiss dissecting microscope (we did not find any, although *Gyrodactylus* can sometimes be dislodged during fish capture and transport and other studies have reported *Gyrodactylus* in the Marianne; Gotanda et al., 2013). Sex and mass (g) were recorded. Only data for adult guppies are included in the present manuscript.

To determine the validity of our behavioural proxies, we used the 'factoextra' package v.1.0.7 in the R environment (Kassambara & Mundt, 2020; R Core Team, 2024 v 4.3.0) to conduct a principal

component analysis (PCA) on a correlation matrix of the behavioural data and the four behavioural proxies (refuge use, number of freezing instances, total freezing time and number of squares crossed). The first principal component (PC1) explained 41% of the total variance and was associated with refuge use (38% contribution to PC1), number of freezing instances (31% contribution to PC1) and total freezing time (31% contribution to PC1), indicating that this PC consisted of boldness behaviour. PC2 explained 35% of the total variance and was mostly associated with the number of squares crossed (64% contribution to PC2), indicating that this PC consisted of exploratory behaviour.

In the following analyses, rather than use the two PCs, we instead used the raw behavioural scores as proxies for bold behaviour and exploratory behaviour. As a proxy for bold behaviour, we created a new variable that represented the sum of the time spent frozen in the open field and the time spent underneath the refuge. This new variable was necessary because these two behavioural measures are not independent. For instance, if a fish spent most of the trial under the refuge, indicative of a shyer behaviour, that same individual would likely have a lower time frozen score (because they are spending less time in the open field for that behaviour to be observed), indicative of a bolder behaviour. As a proxy for exploratory behaviour, we used the number of squares crossed in the open field. We selected these behaviours as they were easily transformed to satisfy the assumptions of linear models (see below), whereas the PCs were not easily transformed to meet assumptions of linearity and their use in the models could therefore risk inflating type 1 errors. The Pearson correlation between these two behavioural proxies was  $r = -0.67$ . The correlation is negative because a lower value for our boldness proxy reflects bolder behaviour (i.e. less time frozen and under shelter is indicative of bolder behaviour). Therefore, bolder individuals were also more exploratory.

## 2.3 | Statistical analysis

We started our investigation of the spatial structure of bold behaviour (the sum of time spent frozen in the open field and under the plant refuge) and exploratory behaviour (number of squares crossed in the open field) by using nested ANOVA (SPSS v.29.0.0.0) to partition the total variation in bold and exploratory behaviours among streams and pools nested within the streams. Estimates of the partial eta-squared (proportion of the total variance,  $\eta_p^2$ ) explained by each random effect allow us to answer how bold and exploratory behaviours are structured at these scales. We next investigated how the spatial structure of bold and exploratory behaviours might be explained by the individual-level and pool-level variables.

Individual-level and pool-level variables were analysed with linear mixed-effects models (LMMs) using the 'lme4' R package (Bates et al., 2015), with either bold or exploratory behaviours as the dependent variables. Fixed effects in these models included sex and mass, as well as the independent effects of several ecological variables (outlined below). We also included time of day (am/pm) of behavioural

assay as a covariate to account for variation in behaviour that could emerge throughout the day because time of day can affect some guppy behaviours (e.g. O'Neill et al., 2019). We included random effects for a pool nested within a stream to account for non-independent spatial structuring. Initially, because male and female guppies differ in size, we included a sex-by-mass interaction, but the bold behaviour model did not converge with this interaction included and the interaction was not significant in the exploratory behaviour model ( $\chi^2 = 0.76$ ,  $df = 1$ ,  $p = 0.38$ ) so it was removed from both models to improve model fit (we instead considered the independent effects of sex and mass) (Engqvist, 2005). The abiotic environmental variables in the models were dissolved oxygen (mg/L), specific conductance ( $\mu\text{S}/\text{cm}$ ), surface area ( $\text{m}^2$ ), temperature ( $^{\circ}\text{C}$ ), canopy cover (squares covered on densiometer) and pool volume ( $\text{m}^3$ ). We excluded mean depth (m) and pH owing to high correlations (i.e.  $r > 0.8$ ) with volume ( $r = 0.93$ ) and specific conductance ( $r = 0.83$ ), respectively (Table S3). We also excluded maximum depth (m) because its inclusion resulted in an estimate of zero variance for the pool level random effect (i.e. effectively only considering the effect of stream). All continuous predictors (in these models and those described below) were standardized via conversion to z-scores prior to their inclusion in the models. Model diagnostics were assessed using the 'DHARMA' R package (Hartig, 2022). Bold behaviour was cube-root transformed and exploratory behaviour was square-root transformed to meet assumptions of linearity. No other assumptions were violated. Using the 'car' R package, we calculated  $p$  values for each LMM with a type II sum of squares.

To calculate effect sizes for sex and mass, we used the 'r2glmm' package (Jaeger, 2017) to extract semi-partial  $R^2$  values for each fixed effect from the LMMs. Much like partial eta-squares that come from ANOVAs or linear models (LMs), semi-partial  $R^2$  estimates the variance explained for each predictor in LMMs (Jaeger et al., 2017), providing insight into the amount of variation in the response variable (i.e. behaviour) that is explained by the fixed effects (i.e. individual-level attributes).

We used a different approach to calculate effect sizes for the ecological variables. Because the level at which we collected our ecological data (i.e. the pool level) differs from the level at which we collected our behavioural data (i.e. the individual level), calculating  $R^2$  for ecological variables using the LMMs above generates very low effect sizes (see the supplementary materials for effect size estimates and plots built using the LMM results; Figures S1 and S2). Indeed, a single value as an independent variable cannot explain variation in the dependent variable; to have assessed  $R^2$  values from the LMMs in a biologically meaningful way would have required finer-scale sampling to collect micro-environmental data for each individual. Therefore, rather than assess how much of the variation in *individual* behaviour can be explained by the fixed effects (as above), we instead assessed how much of the variation in *pool-level* behaviour can be explained by the fixed effects. To calculate these effect sizes, we built two linear models (LMs). The response variable in these models was the average score for bold or exploratory behaviour for a given pool ( $n = 15$ ). The fixed effects were the independent effects of all abiotic

environmental variables included in the above models. To control for between-stream variation, we also included stream as an additional co-variate in these models. We extracted partial eta-squared using the 'effectsize' R package with a type 2 sum of squares (Ben-Shachar et al., 2020). Exploratory behaviour was log transformed to meet assumptions of linearity.

Although sex and mass are individual-level attributes, and estimating  $R^2$  from the LMMs is therefore appropriate, we conducted a sensitivity analysis to ensure that the effect sizes calculated from LMs do not differ substantially from those estimated from the LMMs. Therefore, we also built two additional LMs, with bold or exploratory behaviours as the response variable ( $n = 303$ ), where sex, mass and stream were the only fixed effects. Bold behaviour was cube root transformed to meet assumptions of normality and linearity. We again extracted partial eta-squared using the 'effectsize' R package with a type II sum of squares. The effect sizes from these LMs do not qualitatively differ from those extracted from the LMMs above, and so only the effect sizes from the LMMs are presented in the text (Table S4).

## 2.4 | Ethics statement

Permission to carry out this work came from McGill University Animal Care (AUP 8058).

## 3 | RESULTS

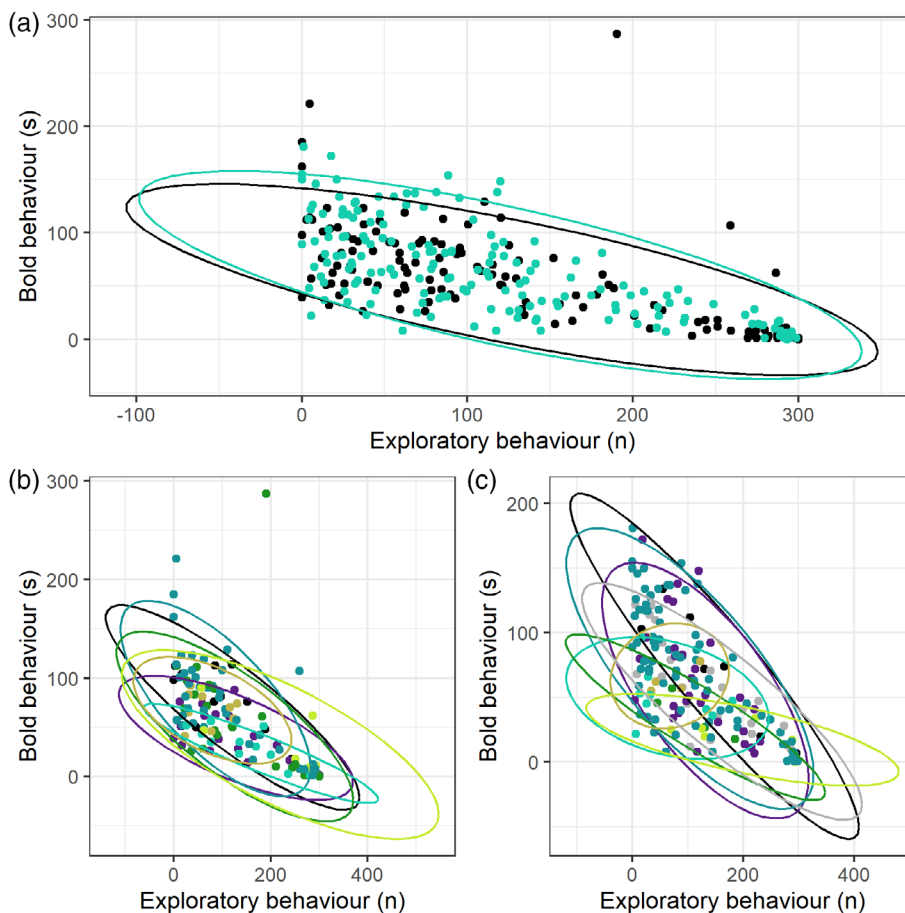
When examining the spatial structure of bold and exploratory behaviours, considerable variation was evident among pools within streams, but not between streams, for both behaviours ( $\eta_p^2$  for pools within streams: bold = 9.0%,  $p = 0.01$ ; exploratory = 12%,  $p < 0.001$ ;  $\eta_p^2$  for streams: bold = 2.0%,  $p = 0.50$ ; exploratory = 0%,  $p = 0.99$ ) (Table 1 and Figure 2).

For individual-level variables, sex explained 0% of variance in both behaviours (Figure 3). Whereas mass also explained 0% of variance in exploratory behaviour, mass explained 3% (95% CI 0.00–0.08) of variance in bold behaviour and smaller individuals displayed bolder behaviours ( $\chi^2_1 = 9.3$ ,  $p < 0.01$ ) (Table 2 and Figure 3).

For the abiotic pool-level variables, first concerning bold behaviour, individuals in pools with higher specific conductance ( $\chi^2_1 = 4.84$ ,  $p = 0.03$ ) and in pools with lower dissolved oxygen ( $\chi^2_1 = 4.73$ ,  $p = 0.03$ ) displayed bolder behaviour. We found 22% of the variation in pool-level mean bold behaviour was attributed to specific conductance and 16% was attributed to dissolved oxygen (Figure 4). Although not statistically significant in the LMMs, volume ( $\text{m}^3$ ) also had small effects on pool-level mean bold behaviour ( $\eta_p^2 = 0.05$ ). Stream explained 11% of the variance in bold behaviour when included as a covariate in these models. Concerning exploratory behaviour, although only marginally significant, the trend suggests that individuals explore more in environments with less dissolved oxygen ( $\chi^2_1 = 3.55$ ,  $p = 0.06$ ) and, consistent with this finding, dissolved oxygen explained 27% of the variance in pool-level mean exploratory

			<i>df</i>	<i>f</i>	<i>p</i>	$\eta_p^2$
Bold behaviour	Intercept	Hypothesis	1.00	360.64	0.03	1.00
		Error	1.00			
	Stream	Hypothesis	1.00	0.48	0.50	0.02
		Error	21.16			
	Pool (stream)	Hypothesis	13.00	2.24	0.01	0.09
		Error	288.00			
Exploratory behaviour	Intercept	Hypothesis	1.00	186,140.64	<0.001	1.00
		Error	2.44			
	Stream	Hypothesis	1.00	0.00	0.99	0.00
		Error	19.06			
	Pool (stream)	Hypothesis	13.00	2.94	<0.001	0.12
		Error	288.00			

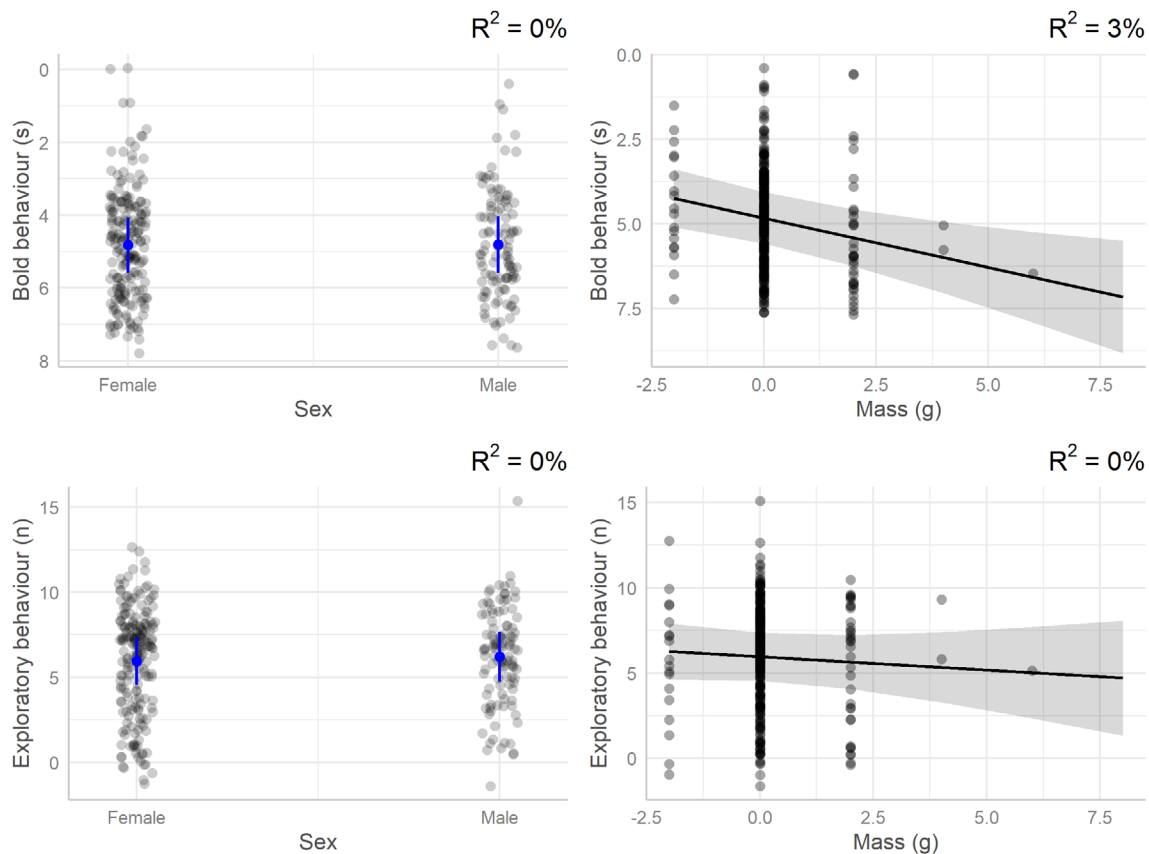
Note: Pool nested in stream was included as a random effect.



**FIGURE 2** Variation in bold behaviour (the sum of time spent frozen in the open field and under the plant refuge) and exploratory behaviour (number of squares crossed in the open field). The panels show (a) all data, with the ellipses around each stream, (b) data for stream 1 only, with the ellipses around each pool, and (c) data for stream 2 only, with ellipses around each pool. (a) is coloured by stream and (b and c) are coloured by different pools within each stream.

behaviour (Figure 5). Temperature and specific conductance also had small effects on pool-level mean exploratory behaviour ( $\eta_p^2 = 0.08$  and  $0.06$ , respectively). Stream explained 14% of the variance in pool-level mean exploratory behaviour. All other ecological variables had small ( $\eta_p^2 < 0.02$ ) effects on pool-level mean behaviour and no other pool-level variable had a statistically significant effect on behaviour (Table 2). Among all pools in a stream, the mean  $\pm$  standard deviation for specific conductance ( $\mu\text{S}/\text{cm}$ ) was  $106.03 \pm 9.48$  in stream 1 and

$84.61 \pm 18.30$  in stream 2, the mean dissolved oxygen (mg/L) was  $4.16 \pm 0.82$  in stream 1 and  $4.06 \pm 0.86$  in stream 2, the mean temperature ( $^{\circ}\text{C}$ ) was  $22.52 \pm 0.17$  in stream 1 and  $22.89 \pm 0.13$  in stream 2, and the mean volume ( $\text{m}^3$ ) was  $0.20 \pm 0.25$  in stream 1 and  $0.31 \pm 0.33$  in stream 2 (Table 3). Finally, time of day was also significant in both models, confirming the value of including this term as a covariate, with individuals exhibiting bolder behaviour in the morning and more exploratory behaviour in the afternoon (Table 2).



**FIGURE 3** The effects of sex and mass on bold behaviour (the sum of time spent frozen in the open field and under the plant refuge) and exploratory behaviour (number of squares crossed in the open field). Data points are the partial model residuals and are alongside trend lines predicted by the linear mixed models. Mass is z-score standardized, so values on the x axis do not correspond to the literal measured mass. Bold and exploratory behaviours were cube root and square root transformed, respectively, to meet assumptions of linearity. The y axis is inverted in the bold behaviour plots (but not the exploratory behaviour plots) for more intuitive interpretation: bold behaviour increases as the y axis approaches zero, towards the top of the plot.

## 4 | DISCUSSION

The role played by the abiotic environment in shaping bold and exploratory behaviours—especially at small spatial scales—has been generally overlooked relative to the role played by individual-level attributes and among-site biotic factors. In the present study, we aimed to reduce that knowledge gap. To do so, we first established how guppy behaviour varies at three scales: among individuals, among pools within streams and between streams. We then examined how two individual-level attributes (sex and body size) and a number of abiotic parameters were associated with the behavioural variation. We found that both bold and exploratory behaviours of guppies varied much more among pools within streams than between the two streams. For individual-level attributes, smaller guppies displayed bolder behaviour, although this effect was small (only explaining 3% of variance in behaviour) and sex had no apparent effect on behaviour. In contrast, abiotic pool-level variables were far more important for explaining behavioural variation. For instance, 22% and 16% of the variance in pool-level mean bold behaviour could be attributed to pool-level measures of specific conductance and dissolved oxygen.

Furthermore, 27% of the variance in pool-level mean exploratory behaviour could be attributed to pool-level measures of dissolved oxygen. These results suggest that other studies could benefit from sampling designs that standardize for known-to-be-important biotic variables (e.g. predation) to better understand how abiotic factors can also shape behaviour.

### 4.1 | The spatial structure of bold and exploratory behaviours

Pool and stream collectively explained 12% of the variance in exploratory behaviour and 11% of the variance in boldness. For both behaviours, pool (rather than stream) was by far the most important contributor to the variance, highlighting the very small spatial scale over which environments can influence behaviour. These very small spatial scales tend to be overlooked in many study systems, including in guppies, where behavioural researchers often sample (or report) only a single location from an entire stream. Our work here emphasizes that a high degree of behavioural variation exists within even

**TABLE 2** Results from the linear mixed models investigating the individual-level and pool-level factors.

Behaviour	Variable 'type'	Term	$\chi^2$	df	p
Bold	Individual-level factors	Sex	0.01	1	0.94
		Mass	9.26	1	<0.01
	Pool-level factors	Dissolved oxygen (mg/L)	4.73	1	0.03
		Surface area (m <sup>2</sup> )	0.44	1	0.51
		Temperature (°C)	0.79	1	0.37
		Specific conductance (µS/cm)	4.84	1	0.03
		Volume (m <sup>3</sup> )	0.52	1	0.47
	Co-variate	Canopy cover (squares covered on densiometer)	0.65	1	0.42
		Time of day	4.76	1	0.03
Exploratory	Individual-level factors	Sex	0.43	1	0.51
		Mass	0.64	1	0.42
	Pool-level factors	Dissolved oxygen (mg/L)	3.55	1	0.06
		Surface area (m <sup>2</sup> )	0.56	1	0.45
		Temperature (°C)	0.31	1	0.58
		Specific conductance (µS/cm)	0.05	1	0.82
		Volume (m <sup>3</sup> )	0.04	1	0.84
	Co-variate	Canopy cover (squares covered on densiometer)	0.00	1	0.95
		Time of day	12.23	1	<0.001

Note: Pool nested in stream was included as a random effect. Bold behaviour (the sum of time spent frozen in the open field and under the plant refuge) was cube root transformed and exploratory behaviour (number of squares crossed in the open field) was square root transformed to meet assumptions of linearity.

small stretches of streams, and behavioural studies in nature benefit from this finer-scale sampling and reporting.

The different pools within each stream were isolated from each other at the time we sampled them (the dry season), yet mixing will occur in the wet season, such that guppies from the different pools within each stream still come from a single guppy 'population' (Blondel et al., 2019; Crispo et al., 2006). Conversely, guppy populations in the two streams we studied are isolated from each other and very genetically distinct (Blondel et al., 2019). Given our finding that behaviour varied primarily among pools within streams, rather than between streams, we suggest that the behavioural variation was mostly due to plastic responses rather than genetic differences. Depending on the type of plasticity (e.g. contextual, developmental, transgenerational; Fox et al., 2024) that contributes to behavioural variation at this scale, the observed behavioural variation might, or might not, persist into the wet season or subsequent dry seasons.

## 4.2 | Individual-level factors were not very important

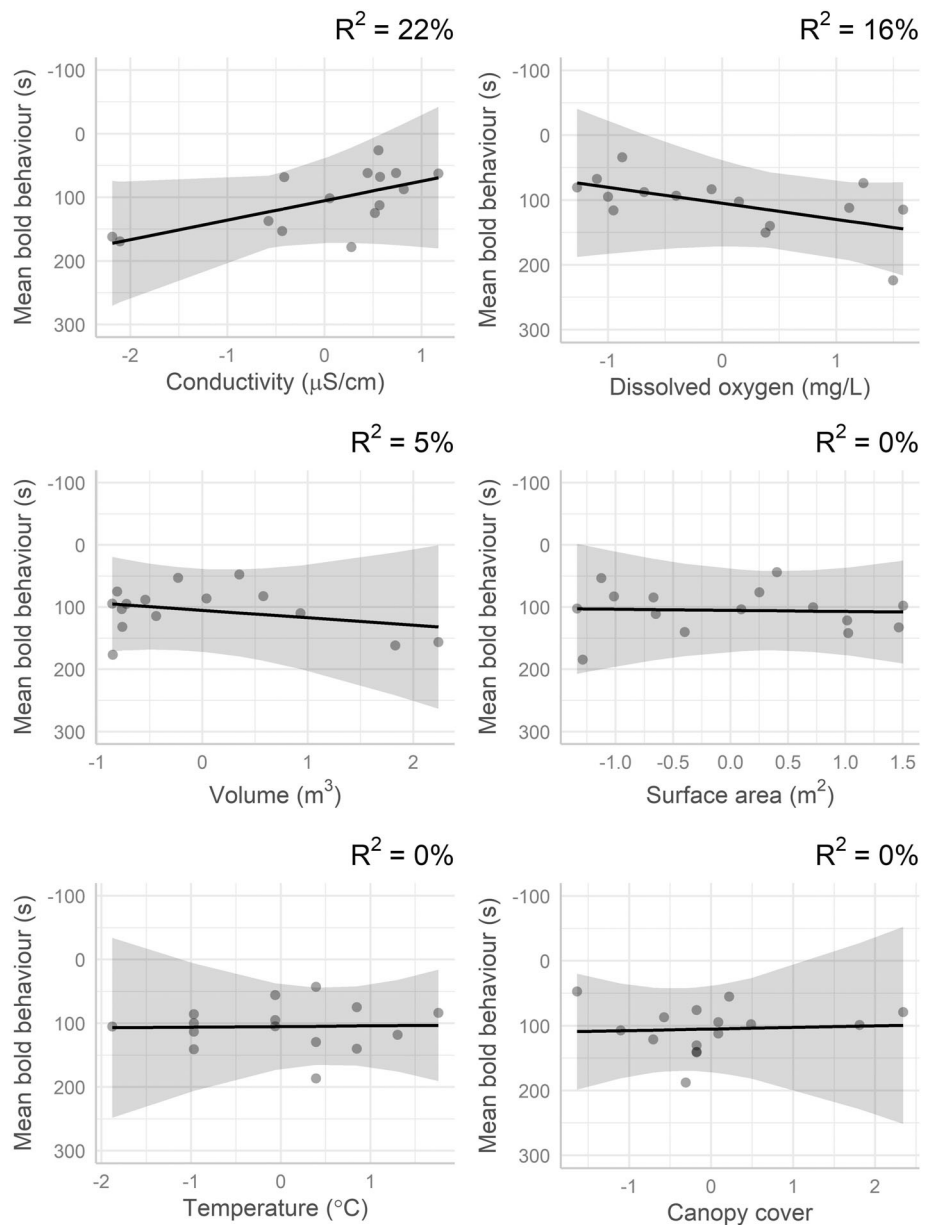
Many individual-level attributes have been investigated as potential contributors to variation in bold and exploratory behaviours, yet researchers often fail to find strong correlations. For instance, although associations between body size and bold behaviour are sometimes observed (e.g. Brown et al., 2005; Brown &

Braithwaite, 2004), even more studies have found no association (e.g. Archard & Braithwaite, 2011; Bell, 2005; Fraser et al., 2001; Wilson et al., 1993). Our results are more consistent with the latter outcomes: specifically, sex and body mass were of remarkably little importance in explaining variation in bold and exploratory behaviours (Figure 3).

At the outset, it seemed reasonable to expect that body size would be associated with behavioural variation and, indeed, associations are sometimes found in guppies (e.g. Anderson Berdal et al., 2018). Consistent with the lack of effect in our study, however, most work on guppies reports that bold and exploratory behaviours do not differ much between guppies of different sizes (e.g. Diaz Pauli et al., 2015; Kemp & Lynch, 2022), including for wild caught guppies in Trinidad (e.g. Harris et al., 2010). The reasons for an absence of (or only very weak) body size effects are not known. At the same time, we feel it is valuable to at least note our finding of a weak trend for smaller guppies to display bolder behaviour. If this trend is real—most obviously through confirmation in further studies—it would be interesting to test various causal hypotheses. Perhaps most obviously, predation risk from the 'weak' predators in low predation environments is size dependent (Mattingly & Butler, 1994; McKellar et al., 2009; Reznick et al., 1996) and could thereby shape behavioural variation.

We might also have expected sex differences in behaviour—given that such differences have been reported in fishes (e.g. Harris et al., 2010; Ingleby et al., 2014; Kemp & Lynch, 2022). In guppies specifically, males have sometimes been reported to be bolder than

**FIGURE 4** Effects of ecological variables on mean pool-level bold behaviour (the sum of time spent frozen in the open field and under the plant refuge). Data points are the partial model residuals and are alongside trend lines predicted by the linear model. The units for canopy cover are the number of grid sections crossed on a densiometer. Conductivity was temperature standardized (specific conductance). Abiotic factors are z-score standardized. The y axis is inverted for more intuitive interpretation: bold behaviour increases as the y axis approaches zero, towards the top of the plot.

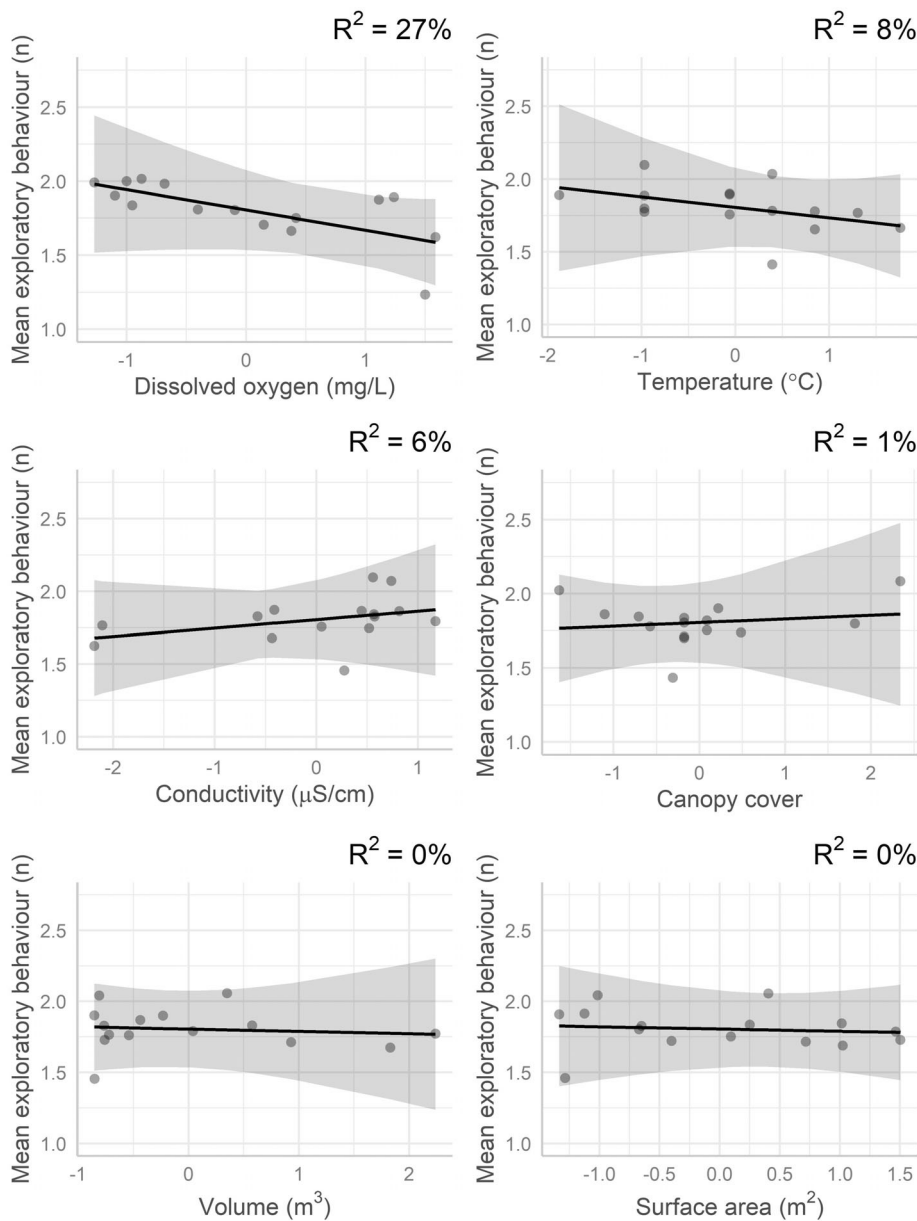


females (Harris et al., 2010; Kemp & Lynch, 2022). Yet, overall, behaviours are often quite similar between males and females within a species. Indeed, a meta-analysis that included data from over 200 studies did not find sex differences in the mean or variance of repeatable behaviours across five taxonomic groups (Harrison et al., 2022). In cases where sex differences in behaviours do emerge, perhaps the effects are mostly due to other factors or dynamics that generate context-dependent variation in behaviour. For instance, female guppies are bolder in male-biased social groups than in female-biased social groups, possibly to reduce male harassment (Piyapong et al., 2010). Such context-dependencies for the role of sex in contributing to bold behaviour will require additional focused studies. Indeed, the above-mentioned size-dependent predation risk in low-predation environments is also sex dependent (Mattingly & Butler, 1994; McKellar et al., 2009), again providing a logical target for future work.

### 4.3 | Pool-level abiotic factors were sometimes quite important

Our study appears to be the first to measure the contributions of *abiotic* environmental factors to variation in bold and exploratory behaviours across multiple wild guppy populations, an effort that seems long overdue. Moreover, we found that some abiotic environmental factors show strong correlations with some behaviours. Our study therefore points to the value of additional studies conducting multi-scale assessments of the abiotic environmental contributors to behaviour in guppies and other fish species.

One association in our study was that guppies displayed bolder behaviours in environments with higher specific conductance. Higher specific conductance can be indicative of agricultural runoff (Drerup & Vadeboncoeur, 2016; Sohoulane et al., 2022) and, in fact, both streams receive periodic runoff from up-slope small-scale agriculture,



**FIGURE 5** Effects of ecological variables on mean pool-level exploratory behaviour (number of squares crossed in the open field). Data points are the partial model residuals and are alongside trend lines predicted by the linear model. The units for canopy cover are the number of grid sections crossed on a densiometer. Exploratory behaviour values were log transformed to meet assumptions of linearity. Conductivity was temperature standardized (specific conductance). Abiotic factors are z-score standardized.

**TABLE 3** Mean  $\pm$  standard deviation for ecological variables from both streams.

Ecological variable	Stream 1	Stream 2
Temperature (°C)	22.52 $\pm$ 0.17	22.89 $\pm$ 0.13
Dissolved oxygen (mg/L)	4.16 $\pm$ 0.82	4.06 $\pm$ 0.86
Specific conductance (µS/cm)	106.03 $\pm$ 9.48	84.61 $\pm$ 18.30
pH	8.32 $\pm$ 0.57	6.79 $\pm$ 0.40
Surface area (m <sup>2</sup> )	0.57 $\pm$ 0.19	0.89 $\pm$ 0.23
Volume (m <sup>3</sup> )	0.20 $\pm$ 0.25	0.31 $\pm$ 0.33
Mean depth (m)	0.30 $\pm$ 0.30	0.34 $\pm$ 0.32
Max depth (m)	0.19 $\pm$ 0.03	0.25 $\pm$ 0.12
Canopy cover (squares covered on densiometer)	4.36 $\pm$ 1.67	2.16 $\pm$ 0.76

Note: See Table S2 for pool-specific ecological measurements and sample sizes.

which is common throughout the area (Northern Range Assessment, 2005). Such runoff generates higher dissolved nutrients that can generate more—and more nutritious—epilithon (biofilm layer on rocks, which can be composed of algae, fungi and bacteria, among other organisms) in streams in Trinidad, and so fertilizer runoff likely generates variation in food resource quality between pools (Kohler et al., 2012). Such resource differences could plausibly affect boldness. For instance, higher-quality resources could enable guppies to grow faster or larger, thus reducing overall predation risk (see above) and thereby decreasing risk-averse behaviours. Given that we did not directly test for nutrients, concrete assertions on why specific conductance impacts guppy behaviours would require future work.

Another association was that guppies displayed bolder behaviour and (possibly) greater exploratory behaviour in environments with lower dissolved oxygen (the latter association lacked statistical significance). Associations between dissolved oxygen and exploratory

behaviour echo past research on another species of poeciliid fish (*Poecilia vivipara*), where fish from lagoons with low dissolved oxygen moved more in an open field (Sommer-Trembo et al., 2017). Those authors speculated that such effects could be due to fish having to move greater distances to forage in low oxygen environments that have scarcer food resources. Such energetic demands could result in resource acquisition being favoured at the cost of decreased anti-predator behaviour, perhaps resulting in increased boldness. Similar hypotheses could be made about canopy cover or temperature, which are also associated with primary productivity (Grether et al., 2001; Lewandowska et al., 2012; McKellar et al., 2009). In our study, however, temperature only had a small effect on exploratory behaviour (explaining 8% of the variance) and the effects of canopy cover were even weaker (2% of the variance) and far from significant.

#### 4.4 | Future directions

The scarcity of research investigating contributors to variation in bold and exploratory behaviours across multiple wild populations, coupled with the importance of abiotic pool-level variables in the present study, underscores several opportunities for research into the causes of behavioural variation.

First, our study design focused in a small area (Figure 1) in a deliberate effort to minimize variation in key biotic factors (e.g. predators and parasites), thus allowing us to focus on the importance of variation in abiotic variables. Yet the small spatial scale meant that variation in abiotic variables was also rather low (Table 3). As such, our finding of correlations between behaviour and abiotic variables points to the value of now scaling up to consider greater abiotic variation across more distant locations. Such a design would also allow the exploration of interactions between biotic and abiotic factors shaping behavioural variation.

Second, our finding that behaviour varied more among pools within streams than between streams hinted at the importance of plasticity, rather than genetic differences. Multiple sampling events through time, or reciprocal transplant experiments between pools, could provide insight into how different types of plasticity (e.g. contextual, developmental, transgenerational) shape these behaviours in wild guppies (Fox et al., 2024). Such work could also test how pool-level population dynamics contribute to variation in behaviour, such as through frequency dependence (Wolf & McNamara, 2012) or density dependence (Travis et al., 2023). The guppy system is particularly well suited for such small-scale (pool-level) manipulations (e.g. Bassar et al., 2013; Olendorf et al., 2006), and such manipulative experiments could thereby test hypotheses generated in less manipulable study systems.

Finally, our finding that some abiotic variables were strongly associated with some behaviours logically calls for the investigation of more variables and more behaviours. Potentially important abiotic variables could include nutrients (phosphorus and nitrogen), dissolved organic carbon, periphyton biomass or the macroinvertebrate community. Potentially interesting additional behaviours could include social

or foraging behaviours. Overall, our findings suggest several new profitable avenues for investigation that were perhaps not so obvious before we focused on abiotic effects within a single predation regime.

#### AUTHOR CONTRIBUTIONS

A.M.H., P.-O.M. and A.P.H. conceived the initial idea. A.M.H., S.S. and J.A.F. collected data. A.M.H. and A.P.H. performed data analysis. A.M.H. wrote the initial draft. All authors oversaw draft preparation and contributed to revisions and edits. P.-O.M. and A.P.H. supervised the work.

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#### ORCID

Alexis M. Heckley  <https://orcid.org/0000-0002-5644-3535>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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