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Environmental habituation and sexual composition affect juveniles' shoaling activity in a cichlid fish (*Pelvicachromis taeniatus*)

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ABSTRACT

Group living is widespread in animals, and many fishes form shoals. Examining within-group interactions in fishes may contribute to the general understanding of dynamic social structures in animals. The sex ratio of a group has been shown to influence grouping decisions of fishes and can be expected to affect behaviour at group level. Behavioural experiments usually involve relatively short acclimatisation times, although the establishment of environmental habituation in fishes is understudied. This study tests whether the sex ratio and long-term habituation to experimental conditions influence general shoal performance (activity parameters, density) and responses of shoals to an acoustic-mechanical disturbance cue in juveniles of the cichlid fish *Pelvicachromis taeniatus via* individual tracking. The disturbance consisted of a defined hit against the experimental tank, which caused sudden noise and water movement. We found that a higher proportion of females increases shoal activity (swimming speed and distance covered), suggesting that female *P. taeniatus* are more active than males. Furthermore, shoal activity declined when shoals habituated to the experimental settings and with the time that the shoals were grouped together, which may reflect intensified group member familiarity. Moreover, behavioural changes after disturbance were weaker when individuals were kept with their group longer and more familiar to the experimental conditions. For prey species, lower activity might be beneficial under natural conditions due to lower conspicuousness of the group. We did not find any significant effects of the investigated factors on shoal density (mean interindividual distance) and speed synchronisation. The results indicate that sexual composition, familiarity between shoal members and habituation to the experimental environment affect shoal performance in a cichlid fish.

KEYWORDS
cichlid fishes, collective animal behaviour, group motion, shoaling behaviour, social behaviour, sociality

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1 | INTRODUCTION

Group living is widespread in animals (reviewed in Ward & Webster, 2016). Fishes often live at least a part of their life in groups, *i.e*., shoals or schools, and represent a model taxon to study the ultimate and proximate causes of social behaviour (Krause & Ruxton, 2002). In shoals, the predation risk is reduced because of dilution and confusion effects: grouping makes it less likely for each individual to be preyed on (Delm, 1990), and predators may not be able to focus on specific individuals in homogenous groups (Pitcher, 1986). The "many-eyes" effect increases the probability of predator detection in a group by the cumulative vigilance of its members (Siegfried & Underhill, 1975). Furthermore, collective behaviours such as predator inspection, consensus decision-making and social foraging make up significant advantages of shoaling (Hesse *et al*., 2015; Magurran, 1990; Sullivan, 1984; Sumpter *et al*., 2008). Accordingly, juvenile fish in groups grow faster than isolated conspecifics (Hesse & Thünken, 2014), which in turn decreases the predation risk because smaller individuals are preyed on by a broader spectrum of predatory species (reviewed in Sogard, 1997). Nonetheless, shoaling also bears costs in terms of intraspecific competition for food or mating partners (Krause & Ruxton, 2002) and may increase conspicuousness to predators (reviewed in Ioannou, 2017). On balance, the advantages often exceed the disadvantages, especially at juvenile stage, when predation risk and mortality rate are highest (reviewed in Sogard, 1997).

Shoal composition and performance are assumed to affect individuals' fitness (Croft *et al*., 2003b). Therefore, association decisions in fishes are not random (reviewed in Krause & Ruxton, 2002) but often based on individual recognition of conspecifics and discrimination between familiar and unfamiliar individuals. Familiarity has been shown to affect several aspects of shoaling behaviour in various fish species (Cattelan & Griggio, 2020; Frommen *et al*., 2007; Magurran *et al*., 1994; Metcalfe & Thomson, 1995). For example, associating with familiar conspecifics can improve antipredator functions, because familiar shoal members swim more cohesively (Chivers *et al*., 1995). Familiarity can also improve foraging success (Atton *et al*., 2014), facilitate social learning (Swaney *et al*., 2001) and reduce the aggression in a group (Edenbrow & Croft, 2012). Although the effects of general familiarity on grouping decisions and shoal performance have been investigated intensively, little is known about the influence of varying degrees of familiarity among shoal mates on these aspects. When the degree of familiarity within shoals increases, enhanced shoal performance may be expected. This might be reflected by denser, more synchronised shoaling, which might ultimately enhance the confusion effect (Chivers *et al*., 1995) and facilitate communication *via* chemical cues or signals. Furthermore, reduced activity might decrease the predation risk by minimising conspicuousness and encounter rates with predators; nonetheless, it also decreases encounter rates with food (Balaban-Feld *et al*., 2019; Lima, 1998). A moderate activity level might therefore ideally cope with the trade-off between foraging and cautiousness.

Moreover, social interactions of shoaling fish can be affected by body size (Marlin *et al*., 2019) or the sex ratio (Etinger *et al*., 2009). High body size heterogeneity is assumed to be disadvantageous for shoaling individuals as it can increase predation risk (Krause *et al*., 2000). Furthermore, synchronous swimming activity maximises energy-saving hydrodynamic advantages (Ashraf *et al*., 2016). Because the optimal swimming speed depends on individuals' body length, hydrodynamic advantages can be maximised when fish swim near to similar-sized shoal mates (Noda *et al*., 2016), which may be more costly to achieve in heterogeneous groups. Thus, body size variation within groups may affect interindividual distances (see also Meuthen *et al*., 2016) and swimming synchrony.

Diverging association preferences have repeatedly been shown between male and female zebrafish, *Danio rerio* (Etinger *et al*., 2009; Ruhl & McRobert, 2005). Whereas most studies investigate sex effects concerning shoal choice by individuals, few studies consider shoal members' behaviour, *i.e*., actual shoaling performance. Yet female guppies, *Poecilia reticulata*, were shown to spend more time shoaling and to swim more cohesively than male conspecifics (Richards *et al*., 2010), whereas non-reproductive male three-spined sticklebacks, *Gasterosteus aculeatus*, shoal more actively than females (Rystrom *et al*., 2018). Sex-specific energy requirements (Blumer, 1986) may have implications for shoaling variables in species that forage in shoals. For example, Horppila *et al*. (2011) observed different feeding strategies between male and female perch, *Perca fluviatilis*, likely affecting activity rates. Furthermore, dispersal-related behaviours may affect shoaling activity because dispersing fish may migrate in shoals to minimise predation risk (van Dongen *et al*., 2014). Nonetheless, little is known about underlying sex-dependent activity patterns in shoaling contexts unaffected by dispersal or feeding.

Shoaling fishes are sensitive to changes in their natural or experimental environment. Therefore, acclimatisation of individuals to experimental surroundings is required to study natural behaviour, and suitable acclimatisation protocols are essential in laboratory behavioural experiments (Milinski, 1997). Familiarity to the environment has already been shown to affect animal behaviour: rats, *e.g*., increase interaction with novel objects when they are familiar to their surroundings (Besheer & Bevins, 2000; Bevins *et al*., 2001). In shoaling experiments with fishes, acclimatisation times between 2 and 10 min seem widely accepted (*e.g*., Mehlis *et al*., 2015; Moss *et al*., 2015; Schaerf *et al*., 2017). Nonetheless, knowledge of long-term effects of habituation to experimental procedures and surroundings on behaviour in fishes is scarce (but see Finger *et al*., 2016), although this may strongly affect shoaling variables. Fish that are repeatedly used in an experiment and familiar with experimental conditions may be less active due to reduced exploration or inspection, even if the experimental setting is unstructured.

This study investigated the influence of these aspects on the shoaling behaviour of juvenile *Pelvicachromis taeniatus*, a monogamous, bi-parental cichlid fish from Western Africa. In this species, parents care for their offspring for several weeks (Thünken *et al*., 2007), but subsequently, juveniles live in shoals until they become sexually mature (Hesse & Thünken, 2014). Shoal choice in *P. taeniatus* is affected by several aspects, *e.g*., kinship (Thünken *et al*., 2015) or

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competition (Thünken *et al*., 2020), and shoals respond to sudden environmental changes (Meuthen *et al*., 2016). We individually tracked shoal members, and aimed to examine the effects of (a) the sexual composition by varying the sex ratio of shoals, (b) habituation to the shoal mates by repeatedly testing individual shoals (4 days in 1 week) and (c) long-term habituation to experimental conditions by re-mixing the same experimental fish to generate differently composed shoals every week. Computer-based tracking of individuals is a highly adequate method, because it produces higher resolution data than analysing videos manually, and as marking of individuals is not necessary, it reduces handling of animals during experiments (*e.g*., MacGregor *et al*., 2020). We examined baseline shoaling behaviour as well as reactions of shoals to a mechanical disturbance cue ("shock stimulus") and predicted that shoaling activity will decline with long-term habituation to experimental conditions (repeated use of test fish over a period of 3 weeks) due to reduced exploration behaviour. As shoal mates familiarise with each other, we expected them to increase cohesion and synchrony, while reducing overall activity and reactions to the disturbance to a moderate level, which may minimise predation risk. Furthermore, we assumed the sex ratio to influence group-level activity, which may reflect sex-specific differences in dispersal, mating or foraging behaviours.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Experimental fish were juvenile F4 offspring of wild-caught *P. taeniatus* from the Moliwe River in Cameroon. They were bred in the laboratory at the Institute for Evolutionary Biology and Ecology in Bonn, Germany. At the beginning of the experimental phase, individuals were aged 282 ± 38 days (mean ± s.d.), and standard length (SL) measured 3.09 ± 0.30 cm (mean ± s.d.). Although fish were juvenile, sexes could be visually determined by the emerging sex-specific body colouration. In total, 16 individuals were used (9 males and 7 females). Preceding the experiment, fish were kept together in a group of 34 fish, originating from three different families. They were kept in one tank (60 × 35 × 30 cm, l × w × h), equipped with sand and a filter ("Gully-Filter" by Hobby). Out of this group, 19 randomly chosen individuals were kept in small groups (one group of four and three groups of five) for 2 weeks preceding the first trial.

2.2 | Shoal composition

To build up the first four shoals for the experiment, one randomly chosen fish out of each of the four pre-existing groups was taken into one new tank (tank A). This was repeated thrice (tanks B–D), resulting in four shoals of four fish. Each shoal was then housed in a separate tank (A–D, each measuring 30 × 20 × 20 cm, l × w × h) for 1 week. Shoals were re-mixed weekly by randomly catching one fish out of each existing group (one fish out of tanks A–D, respectively) and putting them together in one new tank. Again, this was done four times to generate four re-mixed shoals. Thus, 16 individuals were tested repeatedly in differently composed shoals over a period of 3 weeks to investigate the effects of environmental habituation. It is possible that individuals that belonged to one shoal in the first week were put together in the third week again. Nonetheless, the probability of two shoals, consisting of the same four individuals, being generated in weeks 1 and 3 is negligible (0.39%), and investigating only weeks 1 and 2 revealed similar results to those of the analysis of 3 week trial time (results not shown). Holding tanks were equipped with sand and an airstone for oxygen supply. They were separated by opaque grey PVC plates to avoid visual contact between shoals. Water temperature was maintained at 25 ± 1°C, and the light–dark cycle was set to 12:12 h. A mixture of defrosted *Chironomus* larvae and *Artemia* served as food and was provided *ad libitum* at the end of the day, following the last experiment, to ensure an equal hunger status throughout all trials.

2.3 | Shoaling assays

The experimental tank consisted of a plastic tank (38.5 × 30 × 24.5 cm, l × w × h) with the sides and bottom covered by opaque, white adhesive foil, which ensured visual separation from the surroundings and a uniform illumination. Before each trial, the test tank was rinsed with tap water. Subsequently, it was filled to a height of 5 cm with 1 day old tap water (water temperature: 25 ± 1°C). *P. taeniatus* is a species that moves and feeds mainly benthically, especially juveniles barely swim in the pelagic or on the surface; thus, the chosen water level enabled natural swimming behaviour but minimised vertical swimming, which could not be measured due to 2D recordings. A camera (HD Pro C920 Webcam, Logitech, Lausanne, Switzerland), connected to a laptop (Lifebook S Series SH531, Futjitsu, Tokyo, Japan), was installed centrally above the tank at 50 cm distance to the water surface. Shoals were carefully caught out of the holding tank by hand and immediately transferred into a small plastic box (Karlie Smart Keeper, 18.5 × 11.5 × 13.5 cm, l × w × h) filled with old tap water (water temperature: 25 ± 1°C). Individuals were then carefully placed into a small dip-net and released simultaneously into the centre of the test tank. The shoals were video recorded for 40 min using the software VirtualDub (version 1.10.4). Videos were taken at an HD resolution (1920 × 1080 pixels) with 30 frames per second. After 20 min of recording, a shock stimulus was created by letting a laboratory bottle (6 × 5 × 11 cm, l × w × h, filled with 200 ml of water) hit one side of the tank. The bottle was fixed to the ceiling of the room with a string and hit the middle of the tank wall from 67 cm at an angle of 28°. Fish were carefully returned to their holding tanks using a dip-net directly after each trial. Shoaling behaviour was analysed 15 min before and after the shock stimulus, respectively. The first 5 min of recording served as acclimatisation phase and were not used for analyses, neither were the last 5 min.

Each shoal was tested four times within 1 week. Shoals were tested once per day, starting after the shoal composition was completed. The second trial was followed by a 2 day break (trials on days

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1, 2, 5 and 6 that shoals were kept together). One day following the last trial, sex was determined visually, and SL was measured to the nearest millimetre using graph paper. The variance of SL within shoals (*SL variance*) was calculated using the software Microsoft Excel 2010. Individuals' weight was determined to the nearest milligram using a digital scale (LC221S-00MS, Sartorius, Goettingen, Germany). The next day, four new shoals were composed by re-mixing the fish of the existing shoals, as described earlier, and again tested for 1 week. The total experimental phase took 3 weeks, with 12 differently composed shoals tested four times each, resulting in 48 trials.

2.4 | Tracking

The videos were analysed using the software idTracker (Pérez-Escudero *et al*., 2014). This software automatically generates a "fingerprint" of each individual to identify them in each frame and uses an additional algorithm to estimate the position of crossing or occluded individuals (for details, see Pérez-Escudero *et al*., 2014). Thus, each fish could be tracked individually throughout a video. The applied settings in idTracker were *intensity threshold*: 0.75; *minimum size*: 250 pixels; and *number of frames for references*: 6000. The software created a data set including coordinates for each individual in each video frame. It estimates the reliability of the output coordinates, based on the probability of correctness of each assigned identity in each frame (Pérez-Escudero *et al*., 2014). The reliability of the results was 88.8% ± 8.1% (mean ± S.D.). Due to technical issues during the recording, one video had to be excluded from the analysis.

2.5 | Analysis

The exact time point (the particular frame) at which the shock stimulus occurred was identified for each video using the software VirtualDub (version 1.10.4). Subsequently, data were split into two sub-sets, one consisting of all frames before the stimulus occurred ("before") and the other containing all frames afterwards, including the frame in which the stimulus occurred as the first frame of this data set ("afterwards"). Using the software ImageJ (version 1.51h), the conversion factor from pixels to centimetres was determined (for details, see Audira *et al*., 2018). The distances between coordinates were then converted from pixels to centimetres, and shoaling-related variables [*total distance travelled*, *speed*, *inter-individual distance* (IID), *synchronisation of speed* (S_v), *freezing*, Table 1] were calculated for each individual (*total distance travelled*, *speed*, *freezing*) or each possible combination of two individuals in a shoal (IID, S_v) using Microsoft Excel 2010.

A mean value for the 15 min of analysis before as well as after the stimulus occurred was calculated for each individual (*total distance travelled*, *mean speed*, *freezing*) or each possible combination of two individuals, respectively (IID, S_v). Based on these values, a mean value for the shoals was calculated for each variable. All variables were calculated in both data sets "before" and "afterwards." The data set "before" was assumed to represent baseline shoaling behaviour. To examine the response to the shock stimulus, the relative change in behavioural variables was calculated [data set "change," *i.e*., the difference ("afterwards" minus "before") divided by the sum ("afterwards" plus "before")]. The "before" and "change" data were used for statistical analyses.

2.6 | Statistical analyses

Statistical analyses were performed using R 3.4.4 (R Core Team, 2018). Data were tested for normal distribution using Kolmogorov–Smirnov test with Lilliefors correction. Data significantly deviating from normal distribution were transformed after Box and Cox (1964) using the "bcpower" function of the "car" package (Fox & Weisberg, 2019) to meet the assumptions of normality. Transformation was applied for IID (data set "before") and the relative changes in *total distance travelled*, S_v and *freezing* (data set "change"). Linear mixed effect (LME) models were fitted for the dependent variables *total distance travelled*, *mean speed*, S_v, *freezing* and IID in the data set "before" as well as for the relative changes after the shock stimulus in *total distance travelled*, *mean speed* and IID in the data set "change," using the "lme" function of the "nlme" package (Pinheiro *et al*., 2009). The *day* (days 1–6 since the shoal was created), *week* (weeks 1–3 that fish were involved in the experiment, habituation to experimental design test), *sex ratio* and *SL variance* were chosen as explanatory variables. The shoal ID was included as a random factor to correct for repeatedly tested shoals. Backward stepwise model reduction was performed to remove non-significant explanatory variables from the models in the order of their statistical relevance (*e.g*., Rystrom *et al*., 2019; Vitt *et al*., 2020). The significance was calculated for each variable by comparing models with and without the variable of interest using ANOVA tests. Tests of significance were based on likelihood-ratio tests, which follow a χ^2 distribution. Normal distribution of the residuals obtained from the best explaining models was confirmed by Shapiro–Wilk tests.

The change in S_v and *freezing* could not be successfully transformed to conform to normal distribution, and the residuals of the best-explaining model were not normally distributed. Therefore, Spearman's rank correlation tests were used to examine the relations with the *sex ratio*, *week* and *SL variance*. For these tests, the mean value for the week for each shoal was used to exclude non-independence of data due to repeated testing. To analyse the effects of the *day* on the change in S_v and *freezing*, Friedman's tests were applied.

2.7 | Ethical statement

The present study follows the Animal Behaviour Society guidelines for the use of animals in research, as well as the legal requirements of Germany, and was conducted in accordance with German laws for animal experiments.

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TABLE 1 The variables calculated to score the behaviour of shoals of juvenile *Pelvicachromis taeniatus*

Variable	Calculation	Reference
Distance travelled	$D(t)=\sqrt{(x(t)-x(t-1))^2+(y(t)-y(t-1))^2}$	Audira *et al.* (2018)
Total distance travelled	Sum of *distance travelled*	
Speed (*v*)	$v(t)=\sqrt{(x(t)-x(t-1))^2+\ (y(t)-y(t-1))^2}/d$	Fu (2016)
Interindividual distance (IID)	$\mathrm{IID}(t)=\sqrt{(x_1(t)-x_2(t))^2+(y_1(t)-y_2(t))^2}$	Fu (2016)
Synchronisation of speed (S_v)	$S_v=\lvert\frac{v_1-v_2}{v_1+v_2}\rvert$	Fu (2016)
Freezing	Total time when $v<1$ cm s^{-1}	Audira *et al.* (2018)

Notes. $x(t)$ and $y(t)$ are the coordinates of the fish at time t, and d is the length of the time interval between two frames, *i.e.*, 1/30 s. IID and S_v were first calculated for each possible combination of two individuals within a shoal and, thereafter, shoals' mean values were calculated from the pair-wise values. The subscripts (1 and 2) refer to any possible combination of two individuals within a shoal.

3 | RESULTS

3.1 | Baseline shoaling behaviour

For 15 min without disturbance, the experimental shoals covered 1433.5 ± 514.4 cm (mean ± S.D.) at a speed of 1.6 ± 0.6 cm s^{-1} (mean ± S.D.). They swam at 51.1 ± 2.8% synchronisation of speed (mean ± S.D.) with 9.8 ± 3.7 cm IID (mean ± S.D.). Experimental shoals spent 570.6 ± 126.1 s (mean ± S.D.) freezing.

Before the shock stimulus occurred, the *mean speed* and *total distance travelled* were significantly reduced over time, *i.e.*, *days* and *weeks* (Table 2; Figure 1). Both *mean speed* and *total distance travelled* significantly increased with the relative number of females, whereas they were not significantly affected by *SL variance* (Table 2; Figure 2). With an increasing number of *weeks* that fish were involved in the experiment, shoals spent significantly more time *freezing* (Table 2). The relative number of females had a significant negative effect on time spent *freezing* (Table 2). *Freezing* was not significantly affected by the *day* nor *SL variance* (Table 2). IID and S_v could not be explained significantly by any of the tested variables (Table 2).

3.2 | Response to shock stimulus

After the shock stimulus occurred, the experimental shoals covered 906.0 ± 395.4 cm (mean ± S.D.) at 1.6 ± 2.1 cm s^{-1} (mean ± S.D.). Their synchronisation of speed was 47.9 ± 8.5% (mean ± S.D.), and IIDs were 13.4 ± 6.1 cm (mean ± S.D.). Experimental shoals spent 614.1 ± 178.1 s (mean ± S.D.) freezing after the stimulus.

Both *day* and *week* had significant positive effects on the change in *total distance travelled* after the shock stimulus (Table 3; Figure 3). It was not significantly affected by the *sex ratio* nor *SL variance* (Table 3). The *week* had a significant positive effect on the change in *mean speed* of shoals and so had the number of females (Table 2; Figure 4). Neither the *day* nor *SL variance* significantly affected the change in *mean speed* (Table 3). The change in *IID* could not significantly be explained by any of the examined variables (Table 2). The change in S_v did not significantly correlate with the *week*, *sex ratio* or *SL variance* (Spearman's rank correlation tests, $N = 12$, all $P \geq 0.140$). The change in *freezing* significantly declined in the course of weeks (Spearman's rank correlation test, $N = 12$, $P = -0.710$, $P = 0.010$) but did not

TABLE 2 Results of linear mixed-effects models examining the impact of shoal members' size variance (SL variance), familiarity to shoal members (day), sex ratio of shoals (sex ratio) and habituation to experimental conditions (week) on baseline shoal performance [(data set "before"); total distance travelled, average speed, *etc.*] of juvenile *Pelvicachromis taeniatus*

Dependent variable	Explanatory variable	χ^2	*P*
Total distance travelled	SL variance	0.887	0.346
	Day	4.685	**0.030**
	Sex ratio	6.520	**0.011**
	Week	24.302	**<0.001**
Mean speed	SL variance	0.597	0.440
	Day	5.746	**0.017**
	Sex ratio	6.542	**0.011**
	Week	23.820	**<0.001**
Synchronisation of speed	Sex ratio	0.222	0.638
	Day	0.637	0.425
	SL variance	3.582	0.058
	Week	3.620	0.057
Interindividual distance	Sex ratio	0.042	0.941
	SL variance	0.371	0.668
	Day	1.145	0.191
	Week	2.706	0.116
Freezing	SL variance	1.682	0.195
	Day	2.315	0.128
	Sex ratio	7.703	**0.006**
	Week	20.227	**<0.001**

Notes. Shoal identity was included as a random factor in each model (all variance and S.D. of shoal identity ≤ 0.001). The degrees of freedom always differed by 1 during stepwise model reduction. *P*-values refer to ANOVA tests comparing the model including the respective explanatory variable to the model excluding the variable of interest. Significant results are in bold ($P < 0.05$). SL: standard length.

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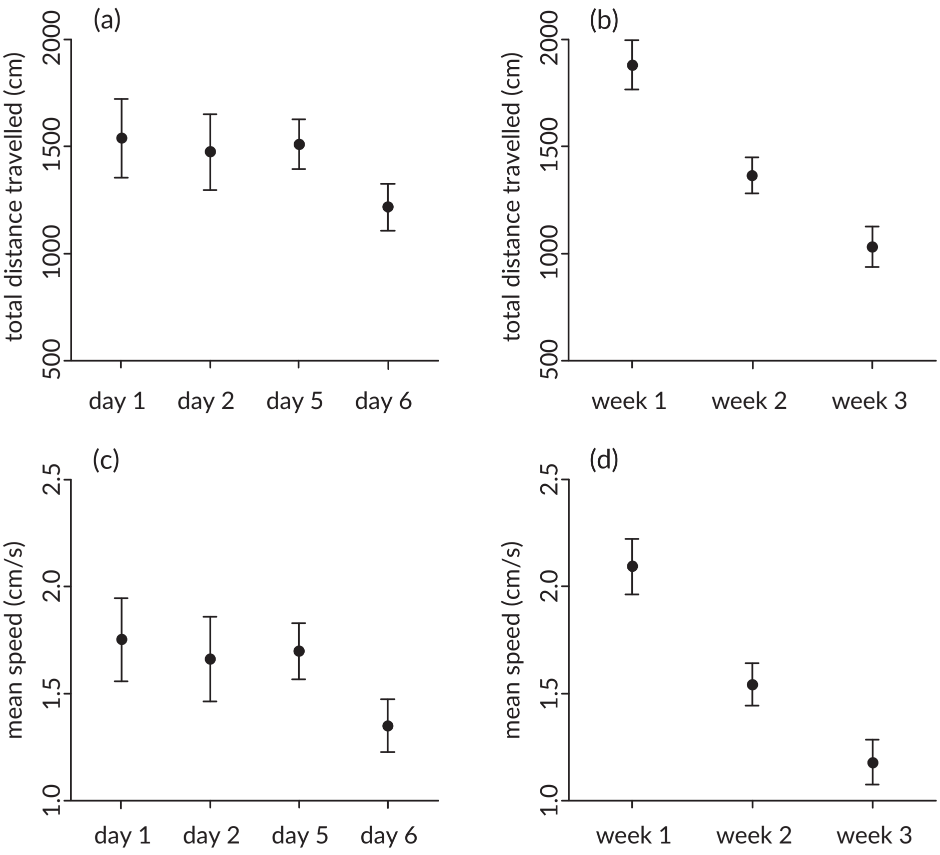

FIGURE 1 Plots of mean values (±s.d.) for the total distance travelled by shoal members of juvenile *Pelvicachromis taeniatus* preceding the shock stimulus (data set "before") in the course of (a) days that shoals were together and (b) weeks that individuals were involved in the experiment and for the (c, d) respective mean speed

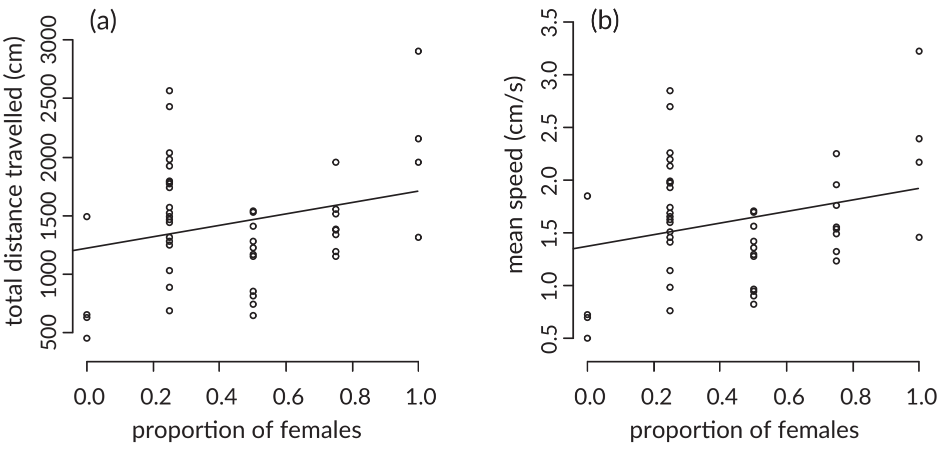

FIGURE 2 The relationships between the proportion of females (sex ratio) in shoals of juvenile *Pelvicachromis taeniatus* and (a) the total distance travelled and (b) the mean speed swam by the fish preceding the stimulus (data set "before"). Each data point represents the mean value for one trial of one shoal. Least square regressions are represented by black solid lines

significantly correlate with the *sex ratio* nor *SL variance* (Spearman's rank correlation test, $N = 12$, all $P \geq 0.263$). Group comparisons did not reveal significant differences between *days* 1 and 4 for the changes in S_v and *freezing* (Friedman's test, $N = 12$, $df = 3$, all $P \geq 0.301$).

4 | DISCUSSION

This study aimed at investigating the effects of the sex ratio within a shoal, increasing levels of familiarity among shoal members and long-term habituation to an experimental setup on shoal performance regarding baseline shoaling as well as reaction to a shock stimulus. We showed that activity (*mean speed*, *total distance travelled*) increased when shoals were composed of higher proportions of females in a baseline shoaling context. It declined in the course of days that shoal members were kept together as well as across weeks that experimental fish were involved in the experiment. In addition, we found declining shock reactions with higher proportions of females as well as over time (across *days* being kept together as well as *weeks* involved in the experiment).

Preceding the shock stimulus, *total distance travelled* and *mean speed* increased with the number of females. In addition, less time was spent *freezing* at higher proportions of females in the shoals. The

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TABLE 3 Results of linear mixed-effects models examining the impact of shoal members' size variance (SL variance), familiarity to shoal members (day), sex ratio of shoals (sex ratio) and habituation to experimental conditions (week) on relative changes of shoal performance after a shock stimulus (total distance travelled, average speed, *etc.*) of juvenile *Pelvicachromis taeniatus*

Dependent variable	Explanatory variable	χ^2	P
Change in total distance travelled	Sex ratio	<0.001	0.985
	SL variance	0.448	0.503
	Day	4.079	**0.043**
	Week	15.580	**<0.001**
Change in mean speed	Day	0.246	0.620
	SL variance	0.347	0.556
	Sex ratio	5.610	**0.018**
	Week	8.222	**0.004**
Change in interindividual distance	Week	0.362	0.547
	Sex ratio	0.700	0.403
	SL variance	1.828	0.176
	Day	2.266	0.132

Notes: Shoal identity was included as a random factor in each model (all variance of shoal identity ≤0.004, all s.d. of shoal identity ≤0.079). The degrees of freedom always differed by 1 during stepwise model reduction. *P*-values refer to ANOVA tests comparing the model including the respective explanatory variable to the model excluding the variable of interest. Significant results are in bold ($P < 0.05$). SL: standard length.

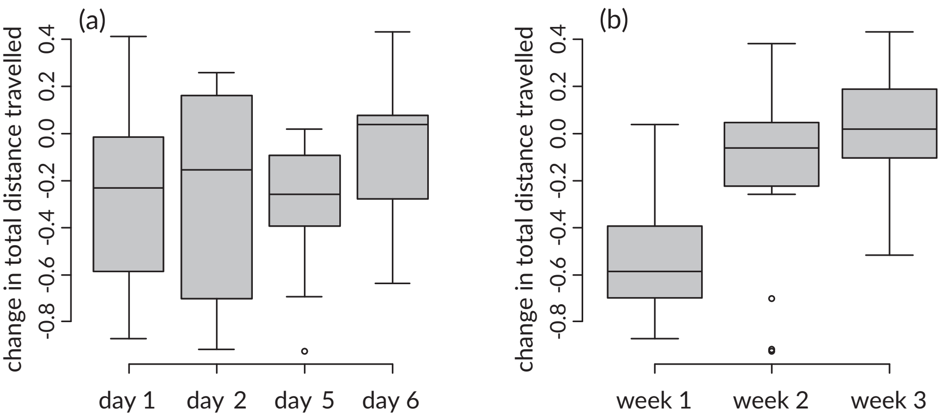

FIGURE 3 The relative changes in total distance covered by shoal members of juvenile *Pelvicachromis taeniatus* following the shock stimulus (data set "change") in the course of (a) days that shoals were together and (b) weeks of the experimental phase. Shown are medians and first and third quartiles. Whiskers are defined as last data points within the 1.5 inter-quartile range (IQR). Circles describe data points outside of the 1.5 IQR

changing activity levels at different sex ratios suggest that female *P. taeniatus* are generally more active than male conspecifics in shoaling situations. Higher activity probably allows for greater foraging effort by covering larger areas; thus, it should lead to higher food intake, as, *e.g*., shown in the Chinese sturgeon, *Acipenser sinensis* (Qian *et al*., 2002). Increased investment in foraging might be necessary for females, because of higher energetic costs associated with female compared to male gonadal tissue generation (Blumer, 1986). Accordingly, higher feeding rates in females than in males are common in many taxa (reviewed in Shine, 1989).

Sex-biased movement seems to be related to mating systems in the guppy (Croft *et al*., 2003a) and the round goby (Žák *et al*., 2018). Considering that females are the mate-searching sex, this might also be the case in *P. taeniatus*. Under natural conditions, sexually mature males of this species occupy and defend territories (Thünken *et al*., 2011), whereas females search for mating partners with suitable breeding sites (Thünken *et al*., 2007), for which they compete among one another (Baldauf *et al*., 2011). Therefore, female *P. taeniatus* are expected to be more explorative and show higher dispersing rates than males (Vitt *et al*., 2020). In accordance, female-biased dispersal was found in other monogamous, territorial cichlids (Taylor *et al*., 2003; van Dongen *et al*., 2014). Increased activity of females might also be an adaptation to intra-sexual competition for mating opportunities because it can facilitate avoidance of competitors and seeking mating partners elsewhere. Although experimental individuals in the present study were juvenile and showed shoaling behaviour, female-biased activity effects might represent the resulting intra-sexual competition. In the cichlid *Tilapia nilotica*, females mature shortly before males (Babiker & Ibrahim, 1979), which may explain that intra-sexual competition results earlier in females. Nonetheless, nothing is known about temporal sexual differences in maturation of *P. taeniatus*. Moreover, competition avoidance may be expected to result in reduced shoal density (reviewed in Wright *et al*., 2006), which was not found here.

High activity might increase not only foraging and mating opportunities but also predation risk. When shoals were composed of higher proportions of females, the relative change in *mean speed* approached zero, meaning the shoals' speed decline as a result of the

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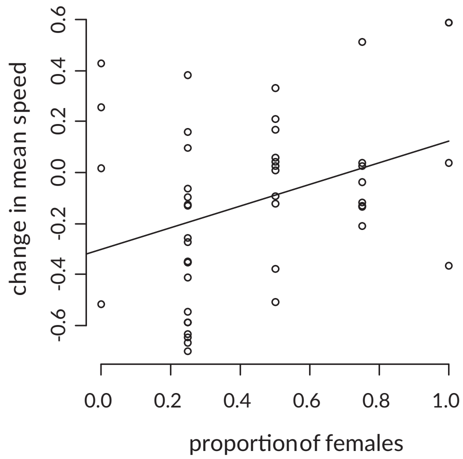

FIGURE 4 The relation between the proportion of females (sex ratio) in shoals of juvenile *Pelvicachromis taeniatus* and the relative change in mean speed following the shock stimulus (data set "change"). Each data point represents the mean value for one trial of one shoal. Least square regression is represented by a black solid line

shock stimulus was less pronounced. This suggests that females might be less cautious after shock stimuli and/or better capable of assessing potential danger. Under natural conditions, this could allow females to spend more time foraging or encountering potential mating partners. Consistently, Oswald *et al.* (2012) found female zebrafish to express bolder behavioural types than males, which was, *e.g.*, reflected in lower feeding latencies. Fright reactions often bear a trade-off between avoiding a potential threat and losing foraging or mating opportunities (Brown & Smith, 1996). For female *P. taeniatus*, the benefits of foraging or mating might outweigh the costs of potential danger earlier than for males, *e.g.*, due to higher energetic needs. Accordingly, female perch were shown to feed at a consistent rate, whereas male conspecifics seem to adjust their hunting attempts to predation risk, which indicates that losses of feeding opportunities have more severe consequences for females (Horppila *et al.*, 2011).

The time that shoal members were grouped together affected shoaling activity: both the *total distance travelled* and *mean speed* declined in the course of days that shoals were together. The effects of *days* (shoals kept together) and *weeks* (individuals involved in experiment) are overlapping. Nonetheless, we accounted for these effects separately in the present statistical models, and both appear to have significant impacts on shoaling activity. Therefore, we discuss the effects separately. Within a week that shoals were kept together, shoal mates clearly increased their level of familiarity, which might have caused effects on activity. Familiarity has repeatedly been shown to promote shoal formation and cohesion (Atton *et al.*, 2014; Chivers *et al.*, 1995; Edenbrow & Croft, 2012), but effects on group-level activity are understudied. In addition, although most previous studies investigating familiarity effects on shoal performance compared familiar and completely unfamiliar individuals (Cattelan & Griggio, 2020; Chivers *et al.*, 1995; Davis *et al.*, 2017), all experimental fish had the same background level of familiarity in the present study. Intensified or reinforced familiarity between shoal members may have caused reduced shoaling activity, which may be beneficial because low activity levels can minimise shoals' predation risk by reducing conspicuousness (Krause & Godin, 1995). According to that, shoaling minnows, *Phoxinus phoxinus*, lower their movement rate when facing a predator (Orpwood *et al.*, 2008). Moreover, the experimental fish covered less distance following a shock stimulus, although this reaction declined with increasing familiarity, which might be explained by habituation to the mechanical stimulus. Because individuals repeatedly encountered the shock stimulus, which was not followed by a true threat, over time they might have associated the stimulus with the absence of a threat and reduced fright reactions. Alternatively, the mechanism for the reduced reaction might be found in habituation to disturbance cues of conspecifics, released in response to the stimulus (Bairos-Novak *et al.*, 2019a). In contrast, Ferrari *et al.* (2010) indicate that in convict cichlids, *Amatitlania nigrofasciata*, sensory habituation to visual or chemical cues (damage-released alarm cues as well as disturbance cues) does not explain lowered responses in individuals from high-risk environments. According to the risk allocation hypothesis, reduced fright reactions can minimise energetic costs imposed by antipredator behaviours, such as freeze-hiding (Lima & Bednekoff, 1999). Shoal member familiarity increases the quality or quantity of released cues and might thus improve risk assessment (Bairos-Novak *et al.*, 2019b). In addition, familiarity causes lower aggression rates in shoaling fishes (Edenbrow & Croft, 2012; Johnsson, 1997), which can also explain decreased activity in established shoals.

Familiar shoal members are known to swim more closely and perform better in antipredator manoeuvres (Ward & Hart, 2003). On the contrary, we found no effects of the time that shoals were kept together on cohesion or synchrony. A transformation in these aspects of shoal performance might happen at earlier levels of familiarity than those investigated in the present study. Here, basic familiarity might have overlapped effects on shoal cohesion and synchrony.

In addition to the aforementioned effects of shoal composition, we could show that shoal members got used to the experimental set-up and procedure, because many of the shoaling variables were significantly affected by the *week* (*mean speed*, *total distance travelled* and *freezing* both before and in reaction to the shock stimulus). The findings suggest that environmental familiarisation in fish might take longer than often assumed. Even after several half-hourly encounters with the experimental surrounding, the shoal members' behaviour changed dependent on the amount of time they were involved in the experiment. To study natural shoaling activity, longer acclimatisation times than frequently used may be reasonable, otherwise exploration behaviour may overlay behavioural types that are aimed to be tested. Nonetheless, the appropriate acclimatisation time strongly depends on the study species and context. Little is known about the effects of environmental familiarity and duration of its establishment in fishes. Thus, acclimatisation times seem to be chosen quite subjectively. Consequently, further studies should address this important aspect when designing behavioural experiments.

In summary, this study found significant effects of the sexual composition on swimming activity in a baseline shoaling context as

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well as regarding shock reaction. Sexual differences in activity and other aspects of shoaling behaviour are understudied (but see Richards *et al.*, 2010; Rystrom *et al.*, 2018), although they may ultimately affect individuals' fitness, *e.g.*, in terms of foraging or mating, and may be related to sex-biased dispersal. Further research should address the causes and consequences of sex-biased activity levels in fishes. We examined how groups' behaviour changes with increasing habituation of the group members to the experimental conditions. The ability to identify and trace individuals across trials (*e.g.*, by colour tagging) would have allowed to examine whether and to what extent individuals differ in behavioural responses. This would be an interesting approach for future studies. By examining behavioural changes at group level, this approach allows robust first conclusions about habituation effects to experimental conditions at low stress levels for the experimental fish. Habituation to the experimental conditions strongly affected activity variables as well as responses to the shock stimulus, which should be considered in future behavioural studies on fishes and may be applicable also for other taxa.

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CONFLICT OF INTEREST

We declare that we have no competing interests.

AUTHOR CONTRIBUTIONS

S.V. and T.T. designed the study. R.F.S. conducted the experiment and collected the data. R.F.S., S.V. and T.T. analysed the data. R.F.S. wrote the manuscript supported by S.V. and T.T.

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