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HIGHLIGHTED STUDENT RESEARCH

Predator-induced neophobia in juvenile cichlids

Denis Meuthen[1] · Sebastian A. Baldauf[1] · Theo C. M. Bakker[1] · Timo Thünken[1]

Abstract Predation is an important but often fluctuating selection factor for prey animals. Accordingly, individuals plastically adopt antipredator strategies in response to current predation risk. Recently, it was proposed that predation risk also plastically induces neophobia (an antipredator response towards novel cues). Previous studies, however, do not allow a differentiation between general neophobia and sensory channel-specific neophobic responses. Therefore, we tested the neophobia hypothesis focusing on adjustment in shoaling behavior in response to a novel cue addressing a different sensory channel than the one from which predation risk was initially perceived. From hatching onwards, juveniles of the cichlid *Pelvicachromis taeniatus* were exposed to different chemical cues in a split-clutch design: conspecific alarm cues which signal predation risk and heterospecific alarm cues or distilled water as controls. At 2 months of age, their shoaling behavior was examined prior and subsequent to a tactical disturbance cue. We found that fish previously exposed to predation risk formed more compact shoals relative to the control groups in response to the novel disturbance cue. Moreover, the relationship between shoal density and shoal homogeneity was also affected by experienced predation risk. Our findings indicate predator-induced, increased cross-sensory sensitivity towards novel cues making neophobia an effective antipredator mechanism.

Keywords *Pelvicachromis taeniatus* · *Pelvicachromis kribensis* · Alarm cues · Shoaling · Predation risk

The neophobia hypothesis suggests predation-induced sensitivity of prey animals to novel cues. However, definitive evidence was lacking because in all previous studies cues signaling predation risk and novel cues were based on the same sensory modality. Here we show that predator-induced neophobia is not sensory-specific, but generalized across sensory channels. Thus we report a new antipredator mechanism of general interest for ecologists.

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Introduction

Predation is a major factor in evolution (Lima and Dill 1990; Lima 1998). To evade predators, animals commonly adopt antipredator strategies, e.g., they escape (Lima and Dill 1990; Dix and Hamilton 1993; Smith 2000), hide (Lima and Dill 1990; Martel and Dill 1993; Mikolajewski et al. 2006; Bourdeau and Johansson 2012; Briffa 2013) or form groups (Krause and Ruxton 2002). Such antipredator strategies are in conflict with investment into other fitness-related behaviors such as foraging, territorial defense and mating (Helfman 1989; Sih 1980; Houston et al. 1993), and are consequently costly (Adler and Harvell 1990; DeWitt and Langerhans 2004). Furthermore, predation is temporally and spatially highly fluctuating (Sih 1992; Sih et al. 2000). Thus, antipredator phenotypic plasticity, which allows individuals to alter their phenotype in accordance to current predation risk (Ghalambor et al. 2007), is widespread in nature (Adler and Harvell 1990; Clark and Harvell 1992; Hoverman et al. 2005).

The investment into antipredator strategies should critically depend on the amount of perceived predation risk as postulated by the threat-sensitivity hypothesis (Helfman 1989; Helfman and Winkelman 1997). As an extension of this hypothesis, the recently developed neophobia hypothesis further predicts that prey animals display an antipredator response towards novel cues dependent on their perceived predation risk (Brown et al. 2013). However, empirical evidence for the neophobia hypothesis is still scarce. One of the few available instances suggests that fish originating from high-predation populations changed their shoaling behavior and activity in response to novel olfactory cues, whereas fish from low-predation populations did not (Brown et al. 2013). In other studies, a response similar to that of fish from high-predation populations was observed when predator-naïve fish were previously exposed to predator-related olfactory cues (conspecific alarm cues; see Brown et al. 2014; Chivers et al. 2014). Woodfrog tadpoles responded in a similar way (Ferrari 2014). Those neophobia tests stimulated a single sensory channel—in particular, in these studies, only chemical cues which exclusively stimulated the olfactory system were applied in order to induce neophobia. Hence, the observed neophobic responses could have been caused by an increased sensitivity of a specific sensory channel (sensory channel-specific neophobia). For example, neophobia could be modulated via a plastic increase in quality or quantity of sensory cells caused by previous stimulation; specific sensory stimulation has been suggested to alter the visual sensory system by plastically modifying receptor shape, receptor abundance, opsin expression and synaptic connections (Kröger et al. 1999; Wagner and Kröger 2000; Shand et al. 2008). Likewise, changes in the olfactory system may arise through stimulation with specific odors (e.g., Dudley and Moss 1999). However, while general presence of a predator in a habitat might be inferred by occasional occurrence of predator-related chemical cues, e.g., by alarm cues (Chivers and Smith 1998; Chivers et al. 2012), an immediate predator attack might only be detectable via non-chemical cues. Hence, rather than sensory-channel specific neophobia, generalized cross-sensory neophobia, which integrates multiple sensory modalities (hereafter referred to as 'generalized neophobia'), should be adaptive. A recent study reporting that coral reef fish alter their behavioral lateralization in response to alarm cue exposition (Ferrari et al. 2015b) hints at the possible existence of such generalized neophobia, but direct evidence for this hypothesis is still lacking.

Grouping is an effective antipredator behavior: independent of specific animal taxa, group members generally benefit from improved predator detection, predator avoidance and predator confusion (reviewed in Krause and Ruxton 2002). Additionally, grouping animals may benefit from social foraging, e.g., by accelerated discovery of novel resources or by enhanced feeding rates, such as by coordinated hunting (Clark and Mangel 1986; Valone 1989; Vickery et al. 1991; Krause and Ruxton 2002). On the other hand, grouping also invokes competition costs for the group members, which makes grouping decisions a trade-off between costs and benefits (Krause and Ruxton 2002). Fish shoals are a well-studied example of animal groups. The antipredator benefits of shoaling are analogous to those from other animal groups (Magurran 1990; Pitcher and Parrish 1993). Hence, fish form denser shoals in both natural habitats with predators (Magurran et al. 1992, 1995) and laboratory experiments during predator presence (Rüppell and Gösswein 1972; Andörfer 1980; Pitcher and Parrish 1993). This also becomes apparent through the stronger tendency of fish to join a shoal when they are exposed to predation risk for a prolonged time period (Chapman et al. 2008; Foam et al. 2005).

Here, we aimed to test the generalized neophobia hypothesis by investigating whether predation risk perceived via chemical cues also drives neophobia against cues perceived through a different sensory channel. We focused on shoaling as a group-based antipredator behavior in order to test this hypothesis in juveniles of the fish *Pelvicachromis taeniatus*. This socially monogamous river cichlid species from Western Africa, which shows complex mate choice (Baldauf et al. 2009; Thünken et al. 2012), displays distinct shoaling behavior throughout the juvenile phase (Lamboj 2004). Juvenile *P. taeniatus* benefit from shoaling as they grow better in a group (Hesse and Thünken 2014) and develop social competences enhancing cooperation, e.g., during predator inspection (Hesse et al. 2015a). They are able to differentiate between kin and non-kin (Hesse et al. 2012), and prefer to shoal with kin which seems to be adaptive because individuals grow better in kin-shoals than in shoals of mixed relatedness (Thünken et al. 2015). Furthermore, siblings form denser shoals (Hesse and Thünken 2014) and are more cooperative during predator inspection than unrelated fish (Hesse et al. 2015b). In the present experiment, we simulated predation risk by regularly exposing fish from hatching onwards to conspecific alarm cues which reliably signal predator-unspecific predation risk (Chivers and Smith 1998; Chivers et al. 2012). Conspecific alarm cues are known to induce antipredator behavior in fish including tighter shoaling behavior (Heczko and Seghers 1981; Nordell 1998), and have been shown to induce behavioral responses in adult *P. taeniatus* (Meuthen et al. 2014, 2015). Juvenile *P. taeniatus* were raised in a split-clutch design, in which they were exposed to either chemical cues signaling the presence of a predator preying on conspecifics (conspecific alarm cues), chemical cues signaling a predator preying on allopatric heterospecifics (heterospecific alarm cues) or control cues

(distilled water). We investigated shoaling density and the change in density following a novel cue perceived by a different sensory channel: a tactical predator-disturbance cue perceived by mechanoreception. Compact shoals enhance the antipredator benefits of shoaling (Magurran 1990) as, for example, short interindividual distances between shoal members potentially allow fish shoals to apply cooperative escape tactics (Pitcher and Parrish 1993). Following the generalized neophobia hypothesis, we first expected that shoals exposed to conspecific alarm cues would become more sensitive towards the novel predator disturbance and in response decrease the distance between individual fish. Second, we expected that fish exposed to predation risk would form denser shoals in general in order to enhance the antipredator benefits of shoaling independent of current predator presence. Lastly, we investigated the relationship between shoaling density and shoal homogeneity concerning body size. The degree of homogeneity of a shoal affects both costs and benefits of individual group members (members of heterogenous shoals have disadvantages in predator avoidance; see Conradt and Roper 2000; and may face higher inter-shoal competition; see Lindström and Ranta 1993; Ranta et al. 1994). Thus, it is assumed that previous exposure to predation risk also may differently affect shoaling behavior in homogenous and heterogenous shoals by shifting the trade-off between the costs and benefits of shoaling.

Materials and methods

Study species

We collected adult *Pelvicachromis taeniatus* from the Moliwe river, Cameroon (04°04′N, 09°16′E) in June 2007 (a recent study suggests *Pelvicachromis kribensis* as a revalidated species name for several *P. taeniatus* populations including the studied one; see Lamboj 2014) and bred them at the Institute for Evolutionary Biology and Ecology, University of Bonn. Subsequent to parental care, we raised the F1 generation in sibling groups of 5–15 individuals in 60 × 45 × 30 cm (length × width × height) tanks to maturity. From May to October 2012, we used adults of this F1 generation (mean ± SD; male size: 7.45 ± 0.50 cm, female size: 5.23 ± 0.21 cm) as parental stock for the current experiments. We formed random pairs of fish from different families (so as to generate outbred clutches) and transferred them to 50 × 30 × 30 cm (l × w × h) tanks containing a breeding cave. We removed eggs immediately upon deposition and transferred them to egg-rearing tanks. If a pair did not deposit eggs within 2 months, we replaced them by a new pair. Clutches had to contain at least 27 eggs from which at least 18 fry had to hatch in order to be used for further experiments. We continued sampling for 6 months until 12 pairs had reproduced with sufficiently large clutches. One breeding pair spawned twice; thus, 13 clutches were available for the experiment.

Rearing protocol

We split each clutch into three equally sized groups (13–32 eggs each, dependent on clutch size) and incubated them in 15.5 × 9 × 11 cm (l × w × h) tanks supplied with 1 l of substrate-treated water (for a description of this method, see Meuthen et al. 2011) and an airstone for oxygen supply. To visually isolate each group from other fishes, we covered the sides of each tank with tar paper. The water temperature in the tanks was 23 ± 0.5 °C, and they were illuminated in a 12:12 light:dark cycle (from 0800 to 2000 hours). Every day, we replaced 80 % of the water volume with fresh substrate-treated water. From hatching onwards, we exposed offspring to the three treatments 5 days a week. This 'split-clutch' design allowed us to control for genetic effects while investigating the environmentally induced effects (i.e. predator-induced neophobia). After passing through the wriggler stage (the first stage after hatching which is ubiquitous in cichlids; see Barlow 2000) and entering the free-swimming stage, we matched the amount of fry of the same clutch (7–28 fry, dependent on egg number) between the three tanks with a maximum number of ten fry per tank. If 19 or more fry entered the free-swimming stage in each of the three groups of one clutch, we split each group and placed them into two separate tanks (this was the case for two clutches). Subsequently, we fed fry with 10 µl of live *Artemia* nauplii per fish 6 days a week. Following a time period of 2 weeks, we transferred them to 20 × 30 × 20 cm (l × w × h) juvenile rearing tanks with 6 l of water, 105 ml of sand, 0.4 g java moss (*Taxiphyllum barbieri*) and a sponge filter. Here, we kept juveniles at the same temperature and light cycle as before, but fed them with 20 µl live *Artemia* nauplii per fish 6 days a week. Once a week, we replaced 800 ml of water with fresh substrate-treated water. After further 4 weeks, at an age of 50–55 days, we photographed juveniles in order to assess their size (see below for more details). Thereafter, we increased the food amount to 40 µl *Artemia* nauplii per fish 6 days a week. Two weeks later, at an age of 64–69 days (at approx. 15 mm body size), we transferred the fish to the shoaling assays; all treatment groups derived from the same clutch were tested concurrently. In total, we reared and tested 45 groups originating from 13 clutches. Throughout the experiments, we always kept group size constant between the three treatment groups derived from a single clutch; however, group size varied from 6 to 10 fish between different clutches.

Predator-exposure treatment

During the predator-exposure treatment, we exposed the three groups of each clutch to different chemical cues: (1) alarm cues derived from conspecifics (CON); (2) alarm cues derived from allopatric heterospecifics (*Xiphophorus helleri*, HET) and (3) distilled water (DW). *P. taeniatus* respond to conspecific alarm cues with decreased activity (Meuthen et al. 2014) and reduced interspecific aggression (Meuthen et al. 2015). We used swordtail alarm cues as heterospecific alarm cues to control for a generalized response to injured fish. Swordtails have developed an alarm cue system within their skin (Mirza et al. 2001), but are taxonomically distant from cichlids. Furthermore, swordtail alarm cues are a common heterospecific alarm cue control in cichlid studies (e.g., Brown et al. 2004; Foam et al. 2005; Pollock et al. 2005). To control for possible effects of frequent water disturbance (as by the introduction of alarm cues), we used distilled water as a second control stimulus.

Alarm cue preparation and delivery

We produced conspecific alarm cues from adult F1 and F2 laboratory-bred donor *P. taeniatus* (32 individuals, mean ± SD standard length 4.80 ± 0.83 cm). Heterospecific alarm cues were obtained from adult *X. helleri* of our laboratory stock, which were descendants of a commercial supplier population (40 individuals, mean ± SD standard length 4.08 ± 0.54 cm). All fish had previously been starved for 2 days to exclude any effects caused by the individual's selective diet. During a single alarm cue preparation event, we always derived alarm cues from four males and four females to control for sex effects. We euthanized donor fish with a blow to the head followed by cervical dislocation in accordance with § 4, § 8b and § 9(2) of the German animal welfare act (BGB l. I S. 1207, 1313). Afterwards, we ground the whole fish in a mortar using a pestle. This procedure ruptured cells and thus allowed alarm cues to be released. By using whole fish, we additionally accounted for the possible existence of alarm cues located outside the skin (e.g., blood cues; see Barreto et al. 2013). We diluted the homogenate with distilled water, passed it through filter floss and froze it in 1-ml aliquots at −20 °C until use. The final concentration of alarm cue we exposed each fish to 5 days a week was 7.2 mg donor fish wet body mass per liter of tank water. This concentration is approximately twice as high as the concentrations which have previously been shown to induce a significant change in the activity (Meuthen et al. 2014) and interspecific aggression (Meuthen et al. 2015) of *P. taeniatus*. Likewise, we prepared 1-ml aliquots of pure distilled water for control experiments. To apply the predator-exposure treatment, we thawed these aliquots and introduced them in the respective rearing tanks. We exposed fish to alarm cues between 1000 and 1500 hours daily; this exposure occurred at least 1 h after fish were otherwise disturbed (by water changes or photographs) and we did not supply food until at least 1 h after the alarm cue exposure period.

Size measurements

For accurate size measurements, we removed juveniles from their rearing tanks and transferred them individually into a distortion-free and orthrochromatic quartz glass cuvette (Hellma 100-5-20 macro cuvette, outer dimensions 1.25 × 0.75 × 4.4 cm, inner dimensions 0.95 × 0.5 × 4.4 cm) together with 1.75 ml of tank water. Subsequently, we photographed fish under standard lighting conditions including a scale with a digital single lens reflex camera (Nikon D5000 with AF-S Micro Nikkor 105 mm 1:28G macro-objective). Afterwards, we returned fish to their respective tanks.

Shoaling assays

To investigate how previous exposure to alarm or control cues affects the shoaling behavior of juvenile *P. taeniatus*, we carried out shoaling trials which were run in three 30 × 20 × 20 cm experimental tanks with visual 2 × 2 cm grid markings at the bottom (Fig. 1). Tanks were covered with translucent plastic film on the inside in order to minimize reflections. Each experimental tank contained 2 l of substrate-treated water (approx. 3 cm water height). Such shallow water levels are a common method in shoaling studies as it is more accurate to evaluate between-fish distances in two dimensions (Kelley et al. 2012). Furthermore, the natural habitat of *P. taeniatus* consists of shallow rivers (Lamboj 2004); hence, we conducted experiments in a naturally realistic context. Water temperature was kept at 24 ± 0.3 °C. We covered the three experimental tanks with white polystyrene from all sides to isolate them visually from the other shoals and the experimenter. Then, we placed them inside a wooden cage-like setup. By using this setup, we could create a novel cue which addresses a different sensory channel from olfaction—a tactical predator disturbance. Next to highly sensitive inner ears, fish have developed superficial neuromasts spread over the body's surface and canal neuromasts which are bundled into a highly sensitive lateral line (Blaxter 1987; Kalmijn 1989). Through these neuromasts, fish perceive water disturbances via mechanoreception which also aids them in detecting and avoiding predators (Fuiman and Magurran 1994; Montgomery et al. 1995). They are particularly sensitive towards low frequencies of water vibrations which are indicative of physical predator movement (Kalmijn 1989). In our setup, we created a tactical predator disturbance of

a low frequency by using a single pendulum stroke on one of the outer sides of each tank for all three tanks simultaneously; hence, the resulting disturbance was equal across all three tanks. According to our calculations, which applied the simple gravity pendulum formula $F = m \times g \times \sin(\theta)$ as well as the pressure formula $p = F/A$ based on apparatus weight (m), earth gravity (g), traverse angle (θ) and impact area (A), the tactical predator disturbance was comprised of a hit of 0.087 N force (F) with a momentary pressure (p) of 1071.60 Pa in the middle of the short side of each tank. Preliminary tests revealed that during this disturbance, water surface movement was still negligible (no visible waves were formed). However, this tactical cue induced a clear behavioral response in the experimental fish. As the experimental fish did not receive a tactical predator disturbance of this kind prior to the present experiment, it may represent a novel cue. Furthermore, the wooden cage contained three video cameras (QuickCam 9000; Logitech, China) 44.5 cm above tank water level so that fish behavior in each tank could be recorded.

We always tested three shoals derived from the same clutch which received different treatments concurrently in the three experimental tanks; all shoals were used 21–24 h subsequent to the last chemical cue exposure, which should have ensured that alarm cues have degraded (fish alarm cues degrade within 0.5–6 h; see Wisenden et al. 2009; Chivers et al. 2013). Here, we randomly assigned shoals derived from the different predator-exposure treatments to the different experimental tanks every trial. After removing the shoals from their rearing tank (we viewed all the fry in a single tank as a shoal), we placed them into a 15.5 × 9 × 11 cm (l × w × h) transportation tank filled with 200 ml of water, then gently released the shoals into their respective experimental tanks. Following a 20-min acclimation period, we observed shoaling behavior for 10 min (prior to the tactical predator disturbance). Next, we delivered a standard tactical predator disturbance to each experimental tank concurrently. Subsequently, we observed shoaling behavior again for 10 min (following the tactical disturbance) after the fish started to move (i.e. each fish of a shoal moved at least 2 cm). We videotaped the trials in 640 × 480 pixel resolution at 25 frames per second. After a trial, we removed shoals from the experimental set-up and returned them to their home tanks. Between trials, we thoroughly rinsed experimental tanks with hot water in order to remove chemical residues.

Data analysis

Size measurements

For size measurements, we picked a single image from each fish in which it was positioned perpendicular to the camera lens and erected all fins; here, we measured fish size (total length, i.e. from the snout tip to the end of the tail fin) with ImageJ software (Rasband 1997–2014; U.S. National Institutes of Health, Bethesda, MD, USA). Fish size was measured and subsequently converted to metric units according to the known dimensions of the cuvette. As predation risk may also affect the variation of growth between fish in a shoal, we also determined the coefficients of variation (CV, calculated by dividing the standard deviation of fish size by the mean fish size for each shoal) as a proxy for shoal homogeneity in our analyses.

Shoaling assays

We evaluated videotapes of the shoaling assays to estimate shoal densities. For this purpose, we extracted one image every 30 s over 10 min for both time periods (prior and following the tactical predator disturbance) with Free Video to JPG Converter software (DVDVideoSoft; UK). Then, we imported the images into ImageJ (Rasband 1997–2014) and measured distances between fish (as a proxy for shoaling density), which were subsequently converted to metric units according to the known dimensions of the experimental tank. The distances we measured were interindividual distances (IID, the distance from the middle of the head of each fish to the middle of the head of every other fish; Fig. 1) and nearest-neighbor distances (NND, the distance between the middle of the head of each fish and the middle of the head of its nearest neighbor; Fig. 1). For statistical analysis, we subsequently calculated arithmetic means of IIDs and NNDs for each shoal for each of the time periods (prior and following the tactical predator disturbance), respectively. Afterwards, we calculated the average change in IID and NND induced by the tactical predator disturbance for each shoal by subtracting the variables obtained prior to the tactical predator disturbance from the corresponding ones obtained following the tactical predator disturbance. All 45 shoals (15 shoals per treatment) could be evaluated successfully and entered the analyses.

Statistical analysis

We used R 3.0.1 (R Core Team 2014) for statistical analyses. As data did not deviate significantly from normality according to Shapiro–Wilk tests, parametric analyses were applied. Accordingly, we constructed linear mixed-effect models (function 'lme' from the R library 'nlme') in order to include family identity and rearing tank identity as random effects. By using family as random effect, we accounted for the split-clutch rearing design. Variation in shoal size (ranging from 6 to 10 fish) did not affect any shoaling variable (we initially included shoal size as an additional explanatory variable throughout all constructed linear mixed-effect models, all $p > 0.05$).
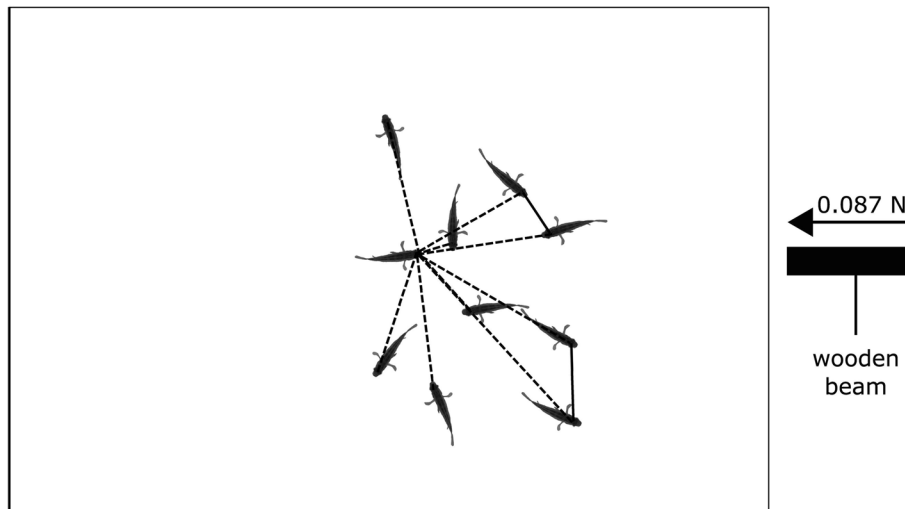
Fig. 1 Shoaling assay. Shoals (6–10 individuals) of 64- to 69-day-old juvenile *Pelvicachromis taeniatus* were transferred into 20 × 30 × 30 cm (l × w × h) tanks. After the pre-disturbance period, they received a tactical predator disturbance of 0.087 N via a wooden beam (*black bar*). Every 30 s throughout the pre-disturbance and post-disturbance period, we measured interindividual distances (exemplified for the *leftmost fish* by *dashed lines*) and nearest-neighbor distances (two examples indicated by *straight lines*) as a proxy for shoaling density from still images

First, we tested the effect of the predator-exposure treatment on shoaling density by entering shoal density variables (IID or NND prior to the tactical predator disturbance, change in IID or NND) as dependent variable and treatment (conspecific alarm cues, heterospecific alarm cues, distilled water) as explanatory variable. Subsequently, we analyzed each treatment separately with linear-mixed effect models (LME); here, we tested whether the change in shoal density deviated significantly from the null hypothesis of 'no change'.

Second, we similarly analyzed the effect of the predator-exposure treatment on growth (i.e. average fish size) and shoal homogeneity (CV of fish size within each shoal).

Third, we investigated the relationship between shoal homogeneity (CV of fish size within each shoal) and shoaling density ('shoal homogeneity' × 'treatment' interactions) dependent on the treatment. When significant interactions were present, we conducted additional correlation analyses for every treatment separately. For this purpose, we selected the corresponding shoal density variable as dependent variable and shoal homogeneity as explanatory variable.

When not otherwise stated, all tests of statistical significance were based on likelihood ratio tests (LRT), which assessed whether the removal of a variable caused a significant decrease in model fit; hence, degrees of freedom always differed by two when all three treatments were included in a single model and by one in all other analyses. *p* values refer to the increase in deviance when the respective variable was removed. Test probabilities are two-tailed throughout.

Results

Shoal density

Predator-exposure treatments did not affect shoal densities prior to the tactical predator disturbance. Neither interindividual distances (LRT, $\chi^2 = 2.127$, $p = 0.345$) nor nearest-neighbor distances (LRT, $\chi^2 = 1.720$, $p = 0.423$) differed significantly among the three treatments. However, the change in density in response to the tactical predator disturbance differed between the treatment groups (change in IID: LRT, $\chi^2 = 5.890$, $p = 0.053$; Fig. 2a; change in NND: LRT, $\chi^2 = 6.054$, $p = 0.049$; Fig. 2b). Shoals from the conspecific alarm cue treatment showed a reduced interindividual distance in response to the tactical predator disturbance (LME, $t = -3.317$, $p = 0.006$), but those from the heterospecific alarm cue and distilled water treatments did not (HET: LME, $t = -0.952$, $p = 0.360$; DW: LME, $t = -1.219$, $p = 0.246$). Likewise, the tactical predator disturbance induced a significantly reduced nearest-neighbor distance in the conspecific alarm cue treatment (LME, $t = -3.791$, $p = 0.003$), which it did not in shoals from the other two treatments (HET: LME, $t = 0.262$, $p = 0.798$; DW: LME, $t = -1.056$, $p = 0.312$).

Relationship between shoal homogeneity and shoal density

Treatments neither significantly affected average fish size (LRT, $\chi^2 = 4.158$, $p = 0.125$, mean ± SD: CON 15.857 ± 0.338 mm, HET 15.642 ± 0.355 mm, DW
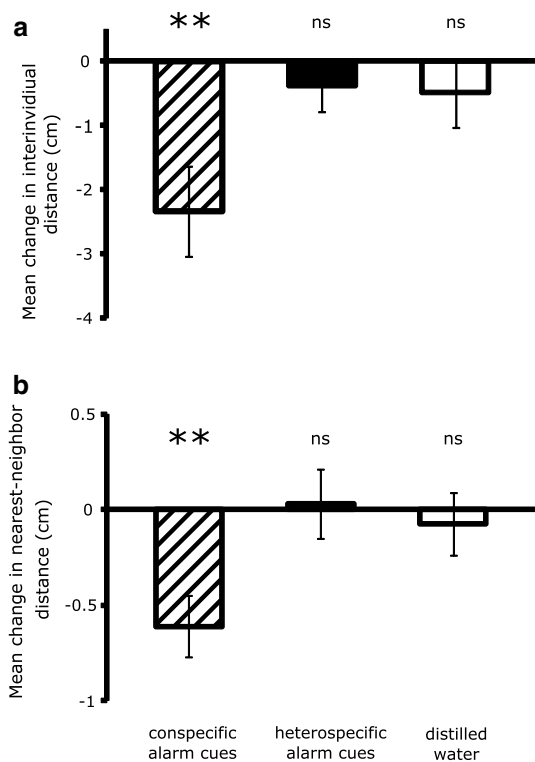
Fig. 2 Mean change in shoaling densities induced by the tactical predator disturbance, showing the change (mean ± SE) in interindividual (**a**) and nearest-neighbor distances (**b**). *Negative* values denote a reduction of within-fish distances and accordingly, an increase in shoaling densities. *Asterisks* indicate the significance of the deviation from the null hypothesis (no change). **$p < 0.01$, ns $p > 0.1$

Table 1 Results of mixed model ANOVAs examining the relationship between shoal density of juvenile *Pelvicachromis taeniatus* (interindividual and nearest-neighbor distance prior to disturbance or the disturbance-induced change in these variables, respectively) and shoal homogeneity (CV of fish size within each shoal)

Treatment	χ^2	p	ρ
Interindividual distance prior to the tactical predator disturbance			
Conspecific alarm cue	0.065	0.799	0.066
Heterospecific alarm cue	**8.638**	**0.003**	**−0.615**
Distilled water	**14.722**	**<0.001**	**0.581**
Nearest-neighbor distance prior to the tactical predator disturbance			
Conspecific alarm cue	0.889	0.766	0.077
Heterospecific alarm cue	**12.039**	**<0.001**	**−0.663**
Distilled water	**15.129**	**<0.001**	**0.774**
Change in interindividual distance			
Conspecific alarm cue	0.962	0.327	0.249
Heterospecific alarm cue	**5.374**	**0.020**	**0.540**
Distilled water	**6.719**	**0.010**	**−0.633**
Change in nearest-neighbor distance			
Conspecific alarm cue	0.698	0.403	0.213
Heterospecific alarm cue	1.083	0.298	0.212
Distilled water	**10.979**	**<0.001**	**−0.633**

The results are presented for each treatment separately. Density estimates based on both interindividual and nearest-neighbor distances are shown. Treatment-specific correlation analyses between response variables and shoal homogeneity include the corresponding Pearson correlation coefficient (ρ). Significant results ($p < 0.05$) are shown in bold

15.790 ± 0.373 mm) nor shoal homogeneity (LRT, $\chi^2 = 5.660$, $p = 0.059$, mean ± SD of the CVs: CON 3.960 ± 1.250 %; HET 5.108 ± 1.448 %; DW 5.724 ± 3.025 %). However, treatments affected the relationship between shoal density and shoal homogeneity prior to the tactical predator disturbance (interaction 'treatment' × 'shoal homogeneity'; IID: LRT, $\chi^2 = 6.460$, $p = 0.040$; Fig. 3a; NND: LRT, $\chi^2 = 11.931$, $p = 0.003$; Fig. 3b). Prior to the tactical predator disturbance, in shoals from the conspecific alarm cue treatment, densities did not correlate significantly with shoal homogeneity (Table 1; Fig. 3a, b). Moreover, shoal density correlated negatively with shoal homogeneity (SD of fish size) in shoals from the heterospecific alarm cue treatment (Table 1; Fig. 3a, b). Instead, in shoals from the distilled water treatment, shoal density correlated positively with shoal homogeneity (Table 1; Fig. 3a, b). Furthermore, the relationship between the degree of change in shoal density and shoal homogeneity was dependent on the treatment (interaction 'treatment' × 'shoal homogeneity'; change in IID: LRT, $\chi^2 = 9.924$, $p = 0.007$; Fig. 3c; change in NND: LRT, $\chi^2 = 6.877$, $p = 0.032$; Fig. 3d). In response to the tactical predator

disturbance, shoal densities of fishes from the conspecific alarm cue treatment again did not correlate with shoal homogeneity (Table 1; Fig. 3c, d). The results of the other two treatments were contrary to the results obtained prior to the tactical predator disturbance. Here, shoal density correlated positively with shoal homogeneity in shoals from the heterospecific alarm cue treatment (however, only concerning interindividual distance; nearest-neighbor distance was not significant; see Table 1; Fig. 3c, d). In shoals from the distilled water treatment, shoal density correlated negatively with shoal homogeneity (Table 1; Fig. 3c, d).

Discussion

In the present study, we found that exposure to predation risk increases neophobia across sensory channels in juvenile *P. taeniatus* shoals. While shoal density did not differ between predator-exposure treatments when fish were not disturbed, predator-exposed fish increased shoal density in response to a novel predator-disturbance cue whereas control fish did not. *P. taeniatus* living under predation risk thus develop a generally higher sensitivity towards novel
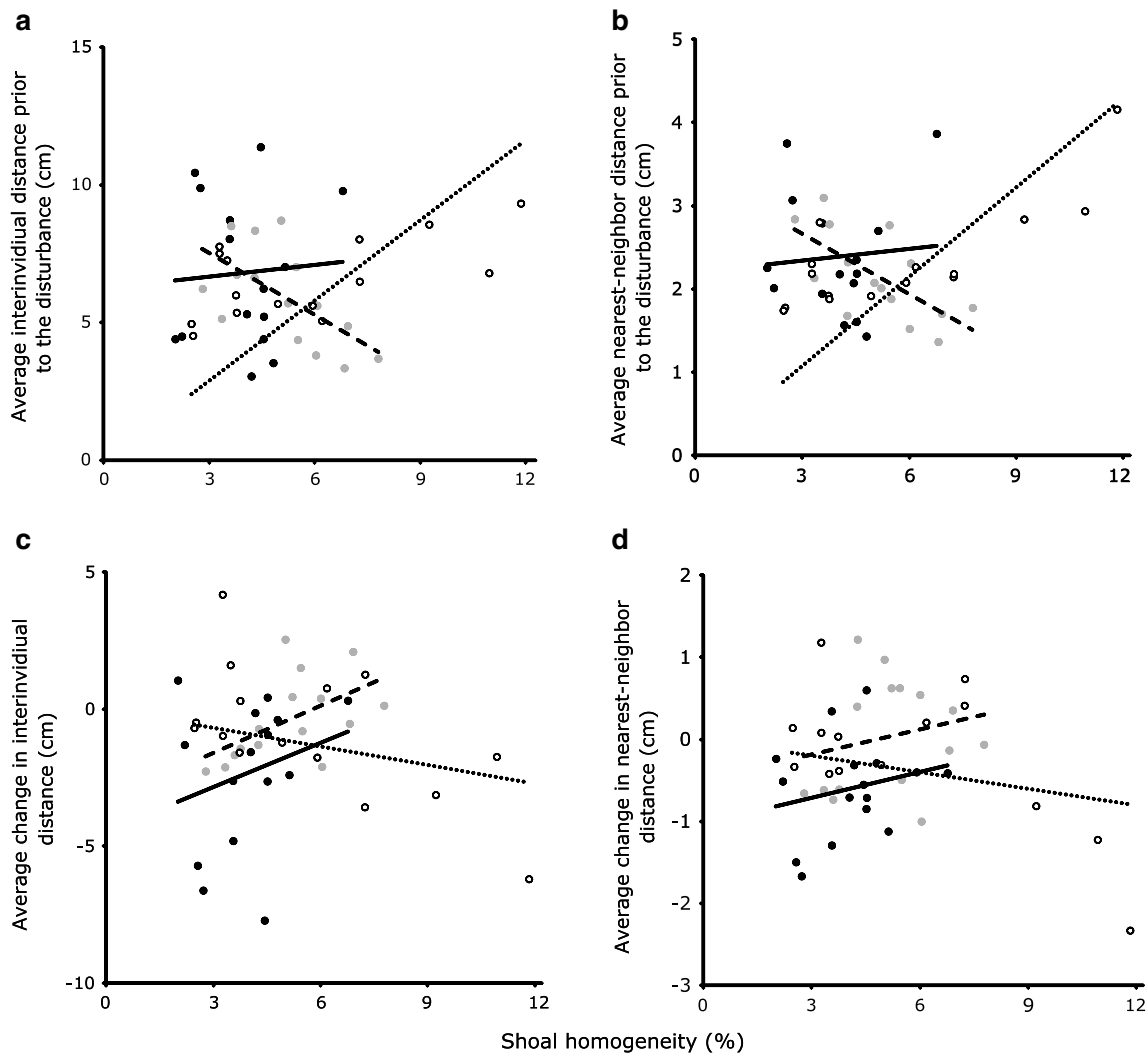
Fig. 3 Correlations between shoaling density and shoal homogeneity (CV of fish size within each shoal) for each of the three predator-exposure treatments: conspecific alarm cues (*n* = 15, *black dots*, *black lines*), heterospecific alarm cues (*n* = 15, *gray dots*, *dashed lines*) and distilled water (*n* = 15, *white dots*, *dotted lines*). Significant correlations between shoal homogeneity and interindividual distances (**a**, **c**) as well as between shoal homogeneity and nearest-neighbor distances (**b**, **d**) dependent on the treatment are shown. All *lines* are least-square regression lines

predator-disturbance cues and form a more compact shoal in response. An increase in shoal density is a well-known group-based antipredator strategy; the presence of a predator (Rüppell and Gösswein 1972; Andörfer 1980; Pitcher and Parrish 1993) or a single pulse of conspecific alarm cues (Heczko and Seghers 1981; Nordell 1998) has been shown to lead to denser shoals. Our result is in accordance with the predictions of the neophobia hypothesis which suggests that prey responses towards novel cues are contingent upon previous predation risk (Brown et al. 2013).

Previous empirical studies testing the neophobia hypothesis have both conditioned and tested animals with chemical cues, which are perceived via olfaction, and thus do not allow a disambiguation between sensory channel-specific neophobia or generalized cross-sensory neophobia (Brown et al. 2013, 2014; Chivers et al. 2014; Ferrari 2014). Our study suggests that predation risk generally induces antipredator responses towards novel predator-related cues independent of the nature of the cues which should theoretically enhance survival (sensu Brown et al. 2013). However, empirical studies suggest that the survival benefits of neophobia induced by olfactory cues still remain unclear: it promoted the survival of coral reef fish against novel predators (Ferrari et al. 2015c) but in woodfrog tadpoles the same treatment increased survival only against novel ambush predators and was detrimental to survival against pursuit predators (Ferrari et al. 2015a). In the present study, fish were exposed to predation risk from birth

onwards, which also contrasts the other neophobia studies that exposed fish to predation risk for not more than 1 week (Brown et al. 2013, 2014; Chivers et al. 2014; Ferrari 2014). Nevertheless, we obtained similar results, suggesting that animals do not habituate to conspecific alarm cues or signals of predation risk in general. This makes sense, as habituation to predator-related signals would be detrimental for fitness under natural conditions. Moreover, our setup which continuously exposed fish from hatching onwards to predation may be more easily extrapolated to natural conditions where predation is continuous (sensu Sih et al. 2000; Sih and McCarthy 2002). Therefore, our results fundamentally add to present knowledge about neophobia in predator–prey contexts.

That shoal density did not differ between predator-exposure treatments prior to the tactical predator disturbance is on the one hand unexpected as the frequent exposure to predation (five times a week during 8 weeks) via conspecific alarm cues should convey that they live in a predator-rich habitat. As predators often appear rapidly, it is expected to be beneficial for fish to constantly form compact shoals which enhances the antipredator benefits of shoaling (Magurran 1990). On the other hand, these results are in accordance to the risk allocation hypothesis. This hypothesis suggests that animals should not display antipredator behavior during safe periods, because individuals must cover their basic energetic requirements and antipredator behavior is predicted to become more costly with prolonged predation (Lima and Bednekoff 1999; Sih and McCarthy 2002). Although shoaling is an effective antipredator behavior and may enhance foraging, it also involves costs such as increased food competition and pathogen transmission (Pitcher and Parrish 1993; Krause and Ruxton 2002). Hence, it might be beneficial for individuals living under enhanced predation risk to form compact shoals only in the close proximity of a predator.

Furthermore, the relationship between shoal density and shoal homogeneity (with respect to body size) was differentially altered by previous predation risk. In the distilled water treatment, we observed that only shoals with high within-shoal homogeneity formed tight shoals while more heterogeneous shoals had a higher average interindividual and nearest-neighbor distance (Fig. 3a, b). This result is in accordance with theoretical predictions that large shoal heterogeneity is disadvantageous for individual fish because of worse hydrodynamic properties (Belyayev and Zuyev 1969; Weihs 1973; Pitcher and Parrish 1993), higher food competition (Lindström and Ranta 1993; Ranta et al. 1994), and higher predation risk (Landeau and Terborgh 1986; Theodorakis 1989; Conradt and Roper 2000). First, it is suggested that shoaling provides hydrodynamic advantages and therefore movement is less costly for individuals within a shoal (Pitcher and Parrish 1993). To maximize

this energy-saving process, fish should swim close to similar-sized individuals (Belyayev and Zuyev 1969; Weihs 1973), which has been empirically supported (Partridge et al. 1983; Pitcher et al. 1985). To maximize hydrodynamic advantages, individuals are required to swim synchronously, which might be more costly in heterogenous shoals as activity synchrony is suggested to be more costly with increasing group heterogeneity (Conradt and Roper 2000). Second, food competition is a well-known cost of shoaling (Pitcher and Parrish 1993; Krause and Ruxton 2002) which intensifies with increasing shoal density (Eggers 1976). Food competition is pronounced in heterogeneous shoals where unequally sized fish compete with each other (Pitcher et al. 1986). As smaller fish are usually poor competitors (Milinski 1984, 1986), they are forced to move around food patches more when large competitors are present within the same shoal (Pitcher et al. 1986). Small fish should therefore not join shoals with large fish and avoid forming heterogeneous shoals (Lindström and Ranta 1993; Ranta et al. 1994). Third, individuals in heterogenous shoals experience higher predation risk as they are worse at confusing predators by coordinated escape: predators require less time to focus on a single target in a heterogenous group (Bernays and Wcislo 1994; Krakauer 1995), odd members are more likely to be fed on by predators (Landeau and Terborgh 1986; Theodorakis 1989), and activity synchrony, which is a pre-requisite for coordinated antipredator responses, is more costly for heterogenous groups (Conradt and Roper 2000).

In contrast, we did not find a relationship between shoal homogeneity and shoal density in fish previously exposed to predation risk independent of the presence of tactical predator disturbances (Fig. 3a–d). The lack of a relationship between homogeneity and density suggests that the fitness benefits from adopting group-based antipredator behavior to evade predation outweigh the aforementioned costs of heterogeneous shoals. This is not surprising because more compact shoals generally enhance antipredator benefits independent of shoal homogeneity (Magurran 1990), including improved predator detection (Magurran et al. 1985; Godin et al. 1988), dilution benefits (Foster and Treherne 1981) and predator confusion benefits (Landeau and Terborgh 1986). The benefits of shoaling are also reflected in the response to the tactical predator disturbance in the distilled water treatment (Fig. 3c, d). Homogeneous shoals, which were already tight, did not respond with a further increase in shoal density to the disturbance, whereas heterogeneous shoals, which were initially less dense, increased their shoal density following the tactical predator disturbance.

The results from the heterospecific alarm cue control group are more elusive to interpret as they neither fully conform with the outcome of the predation risk (conspecific alarm cue) nor the outcome of the water control group

(Fig. 3a–d). Nevertheless, they confirm that the response of *P. taeniatus* towards conspecific alarm cues is a response characteristic to species-specific predation and not a mere generalized response towards injured fish. Little is known about what swordtail alarm cues convey to cichlids. Pollock et al. (2005) have hypothesized that heterospecific alarm cues constitute a food signal to cichlids. According to this hypothesis, regular exposure to heterospecific alarm cues may signal that resources are not limited and therefore the disadvantage of food competition might lose its importance in shoaling decisions. It might therefore appear logical that heterogeneous shoals exposed to heterospecific alarm cues shoaled with a high density prior to tactical disturbance. More generally, food competition may be the primary determinant for the density of heterogeneous shoals in the absence of predation. However, it still remains difficult to explain why, also prior to tactical disturbance, homogeneous shoals exposed to heterospecific alarm cues shoaled with a lower density than did heterogeneous shoals. Moreover, it remains unclear why, following tactical disturbance, heterogeneous shoals exposed to heterospecific alarm cues became less compact whereas homogeneous shoals from the same treatment became tighter. On the other hand, swordtails are not part of the natural habitat of *P. taeniatus*. Consequently, further research testing the response of fish to alarm cues of both sympatric and allopatric heterospecifics may shed light on the interpretation of our results involving heterospecific alarm cues.

Lastly, the results of our study add to other studies on alarm cues in adult *P. taeniatus* (Meuthen et al. 2014, 2015), by showing that not only adult but also juvenile *P. taeniatus* respond behaviorally to conspecific alarm cues. Our results indicate that *P. taeniatus* juveniles up to an age of 64–69 days are capable of conspecific alarm cue recognition. Few other studies on other fish taxa have investigated the age at which juveniles first respond behaviorally to conspecific alarm cues. The common dace *Leuciscus leuciscus* was reported to be incapable of recognizing alarm cues at the age of 38 days, while first responses appeared at the age of 56 days (Schutz 1956). Fathead minnows *Pimephales promelas* did not respond to conspecific alarm cues before an age of 48–57 days (Carreau-Green et al. 2008).

In conclusion, our findings provide the first evidence for predator-induced generalized cross-sensory neophobia. They further suggest that fish do not plastically alter shoaling behavior per se in habitats with frequent predation but rather develop a higher sensitivity towards predator-related cues. However, more studies on generalized neophobia driven by predation risk under controlled conditions and including different sensory channels such as visual or auditory signals are required to deepen our understanding of neophobia and consequently the extent of antipredator plasticity in predator-rich natural ecosystems.

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