

Social cichlid fish change behaviour in response to a visual predator stimulus, but not the odour of damaged conspecifics



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ABSTRACT

Predation is one of the primary drivers of fitness for prey species. Therefore, there should be strong selection for accurate assessment of predation risk, and whenever possible, individuals should use all available information to fine-tune their response to the current threat of predation. Here, we used a controlled laboratory experiment to assess the responses of individual *Neolamprologus pulcher*, a social cichlid fish, to a live predator stimulus, to the odour of damaged conspecifics, or to both indicators of predation risk combined. We found that fish in the presence of the visual predator stimulus showed typical antipredator behaviour. Namely, these fish decreased activity and exploration, spent more time seeking shelter, and more time near conspecifics. Surprisingly, there was no effect of the chemical cue alone, and fish showed a reduced response to the combination of the visual predator stimulus and the odour of damaged conspecifics relative to the visual predator stimulus alone. These results demonstrate that *N. pulcher* adjust their anti-predator behaviour to the information available about current predation risk, and we suggest a possible role for the use of social information in the assessment of predation risk in a cooperatively breeding fish.

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1. Introduction

Predation has direct costs in terms of loss of life or injuries sustained during unsuccessful predation attempts (Beauchamp et al., 2007; Nonacs and Blumstein, 2010). Predation can also carry substantial indirect costs in terms of the time and energy required for anti-predator behaviours, which can detract from other important activities such as foraging and reproduction (Lima and Dill, 1990). Because anti-predator behaviour is in itself costly, there should be strong selective pressure for individuals to closely match their anti-predator responses to the current risk of predation in the environment (Helfman, 1989). Information regarding the presence and threat level of a predator can come from visual, olfactory, auditory, and tactile cues emitted directly by the predator, or indirectly

through information about predation produced by conspecifics or heterospecifics (Thorson et al., 1998).

In the aquatic environment, chemical cues are often used to assess predation risk (see reviews by Smith, 1997; Wisenden, 2000, 2015; Ferrari et al., 2010). These may include disturbance cues (i.e. chemical cues released by nearby individuals that have detected a predator; Hazlett 1990; Chivers and Smith, 1998; Ferrari et al., 2010; Wisenden, 2000), kairomones (i.e. chemical cues that are directly released by a predator, Kats and Dill, 1998; Ferrari et al., 2010) and damage-released cues (Chivers and Smith, 1998; Dalesman et al., 2007; Ferrari et al., 2010). Damage-released cues appear to be a particularly salient chemical cue of predation risk, as these cues are released during the attack and capture stages of the predation sequence, and indicate that a predator is actively foraging nearby (Brown, 2003). Thus, damage-released cues are a good predictor of a clear and imminent risk of predation, and concordantly, elicit intense anti-predator responses (Ferrari et al., 2010; Wisenden, 2015). Hundreds of studies have investigated the importance of these cues in mediating predator–prey interactions (see reviews by Chivers and Smith, 1998; Wisenden, 2000, 2015; Ferrari et al., 2010; Chivers et al., 2013), and a wide variety of aquatic taxa, including fishes, respond to damage-released cues (reviewed by

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Chivers and Smith, 1998), suggesting a widespread benefit to the use of damage-released cues by prey animals (Ferrari et al., 2010; Wisenden, 2015).

By making use of multiple sensory inputs, and relying on both direct and indirect sources of information about predation risk, individuals can increase their accuracy of risk assessment, and optimise their anti-predator behaviour (Dalesman and Inchley, 2008). In aquatic environments, turbidity or habitat complexity may reduce the utility of visual cues in assessing predation risk (Wisenden, 2015), while variable water currents may confound chemical information about the direction or proximity of a predator. Therefore, the reliability of individual cues in predator assessment may be reduced (Chivers and Smith, 1998). As a result, aquatic organisms in particular are expected to use multiple sources of information in order to accurately assess predation risk and respond appropriately.

Here, we assessed the response of the highly social cichlid fish, *Neolamprologus pulcher* to multiple indicators of predation risk. *N. pulcher* is a cooperatively breeding cichlid endemic to Lake Tanganyika, East Africa (Taborsky, 1984; Wong and Balshine, 2011). These small-bodied cichlids live in colonies containing up to 200 distinct, clustered social groups (Heg et al., 2005; Stiver et al., 2006). Each social group includes a dominant male and female that gain the majority of the reproduction within the group, and up to 20 subordinates (Balshine et al., 2001; Heg et al., 2005; Hellmann et al., 2015) that assist in communal defence and maintenance of permanent territories in the rocky littoral zone (Taborsky 1984; Wong and Balshine, 2011). *N. pulcher* is highly amenable to experimental study both in the field and in the lab, and as a result *N. pulcher* has recently emerged as a powerful model system for the integrative study of social behaviour and cooperation in vertebrates (Wong and Balshine, 2011). In the wild, *N. pulcher* live under the constant threat of predation (Balshine-Earn et al., 1998; Balshine et al., 2001). Predators of both adult and juvenile *N. pulcher* occur at high densities within and around colonies (Heg et al., 2004), and smaller subordinates have little chance of survival outside of a territory that is defended by larger group members (Taborsky and Limberger, 1981; Taborsky, 1984; Heg et al., 2004). The evolutionary drivers of social living in *N. pulcher* is an area of active research (Wong and Balshine, 2011), but there is strong evidence that high levels of predation pose a constraint to dispersal in juvenile *N. pulcher* (Heg et al., 2004), and predation therefore likely contributed to the evolution of social living and cooperative breeding in this species. Recent work supports the contention that predation pressure is likely a driver of sociality both at the group and colony level in *N. pulcher* (Jungwirth et al., 2015). Furthermore, individual *N. pulcher* show finely tuned responses to visual predator stimuli, responding differentially depending on the threat level of the presented predator stimulus (Zöttl et al., 2013; Fisher et al., 2014). To date, it has not been established whether *N. pulcher* use olfactory cues to gauge predation risk. Here, we aim to determine whether *N. pulcher* exhibits antipredator behaviour in response to odours emitted by damaged conspecifics. We seek to further our understanding of antipredator behaviour in this highly social fish by documenting the responses of individual *N. pulcher* to an indirect cue of predation risk, the odour of damaged conspecifics, to a direct visual predator stimulus and to the combination of these cues. For the direct visual predator stimulus, we used a live *Lepidolamprologus lemairii*, a natural predator of *N. pulcher* in Lake Tanganyika (Heg et al., 2004; Heg and Taborsky, 2010; Witsenburg et al., 2010), presented behind a sealed transparent barrier.

In response to various indicators of predation risk, aquatic prey adopt a number of different anti-predator behaviours that typically involve any or all of the following: reduction in activity, movement out of the water column, seeking shelter, area avoidance, and increased grouping behaviour (Wisenden, 2000). Therefore,

we examined activity, area use, shelter-seeking, and the propensity to seek out conspecifics in *N. pulcher* in response to the direct predator stimulus, the indirect chemical cue, and both indicators of predation risk combined. Based on previous research of other cichlids' responses to indicators of predation risk (e.g. Wisenden and Sargent, 1997; Pollock et al., 2005; Ferrari et al., 2010), we predicted that both the visual predator stimulus and the chemical cue would cause a reduction in activity and exploration, increase shelter seeking, and increase the time spent shoaling. We predicted that the combination of the visual predator stimulus and the chemical cue would elicit a stronger response than either indicator alone (Wisenden, 2000, 2015; Manassa et al., 2013).

2. Methods

2.1. Study animals

All of the *N. pulcher* used in the current study were laboratory-reared descendants of wild-caught fish from Lake Tanganyika, and part of a laboratory population maintained at McMaster University in Hamilton, ON, Canada. We housed focal fish in a 527 L (183 × 48 × 60 cm) communal aquarium containing 3 cm of coral sand substrate, 4 water filters, 2 heaters, and a thermometer. Stimulus fish were housed in a separate but identical aquarium. We obtained the *L. lemairii* used as the visual predator stimulus from a commercial aquarist (Dave's Rare Aquarium Fish, San Antonio, TX), and housed them in a 189 L (92 × 40 × 50 cm) aquarium containing 3 cm of coral sand substrate, 2 water filters, a heater, and a thermometer, as well as PVC tubing and artificial aquarium plants to use as shelters. The water temperatures of all aquaria were held at $26 \pm 2^\circ\text{C}$ and fish were kept on a 13:11 light:dark cycle. We fed all fish dried prepared cichlid food ad libitum six times per week.

2.2. Visual predator stimulus

Prior to the start of the experimental trials, we captured the *L. lemairii* stimulus predators ($n=3$), weighed them on an electronic balance to obtain total wet mass, and measured them for standard length (SL, the distance from the tip of the snout to the end of the caudal peduncle) using callipers. We then uniquely fin-clipped the fish for identification, allowing the individual stimulus predators to be rotated between trials while avoiding unnecessary handling and prey habituation. Fin clipping does not adversely affect the behaviour of other Tanganyikan cichlid fishes (e.g., *N. pulcher*; Stiver et al., 2004) and the removed fin tissue grows back within a few weeks. In order to maintain clear marks, all *L. lemairii* had their fin-clips redone periodically throughout the experiment. With $SL=91.3 \pm 2.8$ mm, and $mass=22.3 \pm 1.4$ g (values here and throughout the manuscript presented as mean \pm standard error of the mean [SEM] unless otherwise noted), all *L. lemairii* were similar in size, and large enough to consume focal *N. pulcher* (Desjardins et al., 2008; Heg and Taborsky, 2010).

2.3. Odour of damaged conspecifics

We obtained the odour of damaged conspecifics from the skin tissue of adult *N. pulcher* housed in the communal aquaria described above, following the methods described by Pollock et al. (2005). We stunned the fish ($n=20$; $SL=75.2 \pm 2.0$ mm; $mass=13.1 \pm 4.1$ g) by submersion in an ice bath, and then swiftly euthanized them by spinal cord severance. We used a mechanical method to euthanize the fish in order to prevent any interference with the chemical cue (Hoare et al., 2004). We prepared the chemical cue in two batches, one in October 2013, and one in June 2014. For each batch, we homogenized the skin tissue harvested from both flanks of 10 *N. pulcher* (approximately 80 cm² of total skin) with 245 mL of

chilled, double-distilled water, passed the solution through a fine stainless steel filter, and diluted to 345 mL with chilled, double-distilled water. We divided the solution into 15 mL aliquots in 20 mL glass vials, while simultaneously preparing 15 mL aliquots of pure double-distilled water, without any chemical cues, in identical 20 mL glass vials. We coded all vials in order to keep the experimenter blind to the treatment during the experiment. All vials were frozen for no more than 10 weeks at -20°C prior to use. This dose for the odour cue was selected based on pilot studies indicating that *N. pulcher* increase activity when exposed to this concentration of damaged conspecific odour, but show no response to similar concentration of cue harvested from the distantly related Trinidadian guppy (*Poecilia reticulata*; A.R. Reddon, unpublished data).

2.4. Experimental set-up

We tested the behavioural responses of $n=80$ focal *N. pulcher* to a visual predator stimulus and to the odour of damaged conspecifics in a 162 L aquarium ($90 \times 45 \times 40$ cm), divided into 4 compartments (Fig. 1). These compartments were separated by both watertight clear acrylic barriers, and by removable black acrylic barriers. The removable barriers were attached to a string-pulley system so they could be raised remotely without disturbing the fish, and the sides and the back of the aquarium were covered to reduce any disturbances to the fish. The central compartment (focal compartment; $60 \times 26 \times 40$ cm) housed the focal *N. pulcher*, while compartments on either side (stimulus compartments; each $15 \times 26 \times 40$ cm) housed a single stimulus *N. pulcher* on one side, and a group of three stimulus *N. pulcher* on the other (Fig. 1), with the side housing the single fish determined by coin toss. This set-up has been used previously for group size discrimination tasks in *N. pulcher* (Reddon et al., 2011), as well as in other fish species (Agrillo et al., 2007; Dadda et al., 2009). Here, we hypothesized that the focal fish would prefer to associate with a group of conspecifics, rather than a single conspecific, when faced with indicators of increased predation risk. A compartment at the rear (predator compartment; $60 \times 19 \times 40$ cm) housed a *L. lemairii* stimulus predator during trials with a visual predator stimulus, or was empty during the visual control trials. All compartments of the experimental aquarium contained 3 cm of coral sand substrate. Between trials, all compartments also contained a heater, aerator, and thermometer, which were removed during the trials.

The central compartment also contained two chemical cue injection tubes, one for the odour of damaged conspecifics, and the other for the blank water control. Each cue injection tube was also connected to an aerator, to promote rapid distribution of the injected solution throughout the aquarium (McCormick and Manassa, 2008; Reddon and Hurd, 2009). Pilot trials using food colouring indicated that the solutions were distributed throughout the central focal section within 30 s. To ensure that any residual conspecific odours were completely eliminated between trials, we ran only a single trial per day, and the aquarium was drained and refilled after each trial. Additionally, the test aquarium was refilled above the top of the watertight dividers between trials, so that all water in all sections could be filtered together using two AquaClear 20 (Hagen Inc., Baie-d'Urfé, QC) power filters filled with activated carbon (filtering at a combined rate of 756 L per h, running for at least 16 h between trials).

2.5. Experimental protocol

Before each trial, the treatment was determined by random draw. If the trial included a visual predator stimulus, a single *L. lemairii* was captured and placed in the predator compartment (Fig. 1). Four stimulus fish were caught and placed as a single fish or as a trio in either the left or the right stimulus compartment. Stim-

ulus fish were always all larger than the focal fish, to reduce any variation social rank between the focal and stimulus fish (Reddon et al., 2011). A coin toss was used to determine whether the single stimulus fish was placed on the right or the left side chamber. All fish were given 10 min to habituate to the experimental set-up with all opaque barriers in place. After the 10 min habituation period, the opaque barriers on the sides of the arena were raised, to reveal the stimulus conspecifics. After an additional 10 min of habituation, the opaque barrier at the back of the arena was raised, to reveal either the *L. lemairii* predator stimulus, or an empty chamber. Either the chemical cue or the blank water control was simultaneously infused into the focal compartment through the cue injection tube, which was then flushed with 100 mL of holding water to ensure that the entire volume entered the experimental arena. Fish were exposed to the treatment for 10 min. The full 30 min duration of all trials recorded with a Canon Vixia HF S100 video camera. One trial was run per day between 9:30 and 18:30. At the end of the trial, the *L. lemairii* was returned to its home aquarium. Both the focal and stimulus *N. pulcher* were weighed using an electronic balance, measured for SL, and then all fish were returned to communal aquaria. Focal individuals were returned to a different, identical, aquarium, to ensure that they were never reused. Stimulus *N. pulcher* were returned to the same aquarium, and may have been reused in different combinations across the trials.

2.6. Behavioural scoring

Only the final 10 min treatment period of each trial, and not the initial 20 min habituation period, was used for analyses. For scoring purposes, the front of the experimental aquarium was marked with a grid of 10×10 cm squares. At the start of the treatment period, the initial position of the focal fish within this grid was recorded. Whenever the fish moved into a different square within the grid, the change in location and the time of the change in location was recorded. For the purposes of scoring, the fish were marked as changing locations when the front 50% of the body (head to the middle of the dorsal fin) crossed one of the lines denoting the squares. From this scored location data, the following metrics were calculated: (1) activity, which was calculated as the total number of lines crossed during the duration of the 10 min treatment period; (2) exploration (i.e. a measure of area use), which was calculated as the proportion of unique squares entered during the 10 min treatment period; (3) shelter seeking, which was calculated as the proportion of the 10 min treatment period spent in the bottom half of the water column; (4) shoaling, which was calculated as the proportion of the 10 min treatment period spent within 10 cm of either stimulus conspecific compartment (i.e. near either the group of 3 conspecifics, or the lone conspecific, and not in the central zone of the focal compartment; Fig. 1); and (5) group preference, which was calculated as the proportion of the time spent with the group of 3 stimulus conspecifics, rather than the single stimulus fish. A scan was also done every 30 s, to note whether the predator and the conspecific stimulus fishes were swimming or holding still.

In total, $n=80$ trials were run ($n=20$ control trials; $n=20$ trials with a visual predator stimulus only; $n=20$ trials with the chemical cue only; and $n=20$ trials that included both the visual predator stimulus and the chemical cue). Trials were excluded in 2 cases where the predator never moved throughout the entire 10 min treatment period, as movement is a key factor in determining salience of fish predator stimuli (Wisenden and Harter, 2001), and in another 5 cases where there were mechanical failures in either in the video recording, or in the string-pulley system that raised the barriers. Remaining were a total of 73 usable trials ($n=16$ control trials; $n=20$ trials with a visual predator stimulus only; $n=20$ trials with the chemical cue only; and $n=17$ trials that included both the visual predator stimulus and the odour of damaged conspecifics).

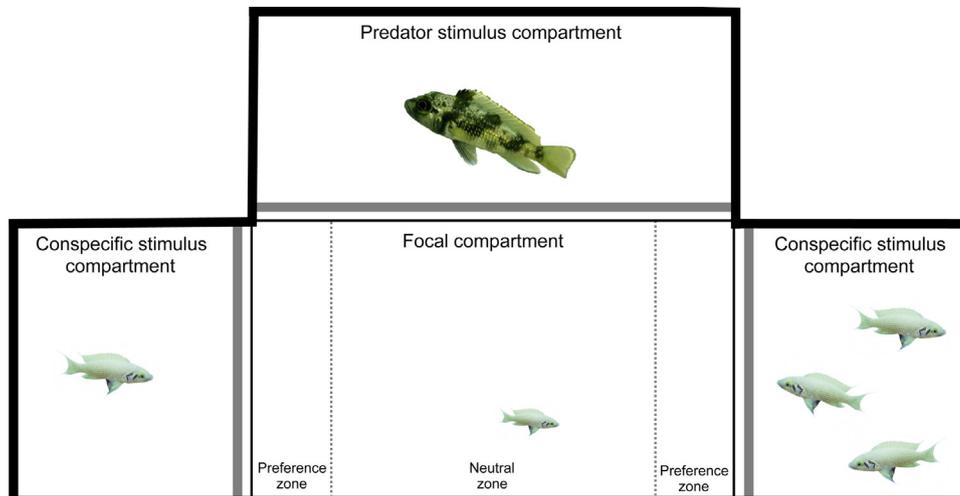


Fig. 1. A top-view schematic of the experimental set-up we used to look at the effect of a visual predator stimulus and the odour of damaged conspecifics on *Neolamprologus pulcher* activity, exploration, and social preferences. We divided an aquarium into 4 compartments, separated by both watertight transparent barriers (thin black lines), and by removable opaque barriers (thick grey lines), which were in place during the initial acclimation period (A), and then removed for the treatment period (B). We used opaque coverings for the sides and the back of the aquarium (thick black lines) to restrict external stimuli. The odour of damaged conspecifics was injected through a syringe and tubing into the focal compartment at the start of the treatment period (not shown). We marked the front of the focal compartment with a grid of $10 \times 10 \text{ cm}^2$ for scoring purposes, and the focal fish was considered to be associating with a conspecific stimulus when it was within 10 cm of either conspecific stimulus compartment (dashed black lines). See Section 2 for full details of the experimental set-up and protocols.

In these trials, the mean SL of the focal fish was $36.7 \pm 0.9 \text{ mm}$ (range: 24.1–56.7 mm), and mean body mass was $1.4 \pm 0.1 \text{ g}$ (range: 0.4–4.8 g). This is within the size range for subordinate *N. pulcher* that would be threatened by our *L. lemairii* predator stimulus fish in the wild (Desjardins et al., 2008; Heg and Taborsky, 2010). The mean stimulus *N. pulcher* SL was $48.9 \pm 0.3 \text{ mm}$, with a mean body mass of $3.0 \pm 0.1 \text{ g}$.

2.7. Statistical analyses

As an overall comprehensive test to examine the effect of indicators of predation risk on all five focal fish behaviours simultaneously, we conducted a multivariate analysis of variance (MANOVA). We scaled and centred the dependent variables, so that they would be equally weighted in the analysis. We used the ‘MVN’ package (Korkmaz et al., 2015) to assess the multivariate distribution of the dependent variables, and given that the multivariate distribution was not normal, we used the ‘adonis’ function within the ‘vegan’ package (Oksanen et al., 2015) to run a permutational MANOVA (McArdle and Anderson, 2001), using 1000 permutations.

Following a significant multivariate test, we then used generalized linear models to look at the effect of indicators of predation risk on each focal behaviour separately: (1) activity (number of lines crossed); (2) exploration (proportion of unique squares entered); (3) shelter seeking (proportion of time near the bottom); (4) shoaling (proportion of time near a conspecific); and (5) group preference (proportion of time spent with the group of stimulus fish). For activity, we used a negative binomial error distribution, appropriate for over-dispersed count data, using the ‘MASS’ package (Ripley et al., 2015). For exploration, shelter seeking, shoaling, and group preference, we used a beta error distribution for proportion data, using the ‘betareg’ package (Zeileis et al., 2014). For all analyses, we included visual predator stimulus and chemical cue as fixed factors. Since smaller fish may perceive the *L. lemairii* predator stimulus as a greater predation risk than would larger fish, we included focal body size fish (i.e. SL) as a covariate in all models. We also included all two-way interaction effects. To make effect sizes comparable among all predictor variables, and to increase the interpretability of interaction effects, focal fish SL was scaled by twice the standard deviation prior to all analyses (Gelman, 2008;

Table 1

Results of permutational multivariate analysis of variance looking at the effect of indicators of predation risk on all five measures of focal fish behaviour: (1) activity (number of lines crossed); (2) exploration (proportion of unique squares entered); (3) shelter seeking (proportion of time near the bottom); (4) shoaling (proportion of time with any stimulus conspecific); and (5) group preference (proportion of shoaling time with the stimulus group rather than the single stimulus fish). The two treatments (i.e. visual predator stimulus and the chemical cue) were included as fixed effects. We included focal fish standard length (SL) as a covariate in all models, and examined all two-way interaction effects. Significant factors ($p < 0.05$) are indicated in bold. Analyses were performed using R version 3.2.1 (R Core Team, 2015) within R Studio (2015).

Model term	Estimate	F-value	p-Value
Visual stimulus	0.04	3.68	0.01
Chemical cue	0.01	1.26	0.30
Focal fish size	0.19	17.40	<0.001
Visual stimulus x chemical cue	0.03	2.93	0.04
Visual stimulus x focal fish size	0.01	0.33	0.78
Chemical cue x focal fish size	0.71	0.73	0.53

Schielzeth, 2010). For all models and all model terms, $\alpha = 0.05$. All analyses were performed using R version 3.2.1 (R Core Team, 2015) within R Studio (2015).

3. Results

From the permutational MANOVA, both the visual predator stimulus and focal fish SL significantly influenced focal fish behaviour (Table 1). Although the odour of damaged conspecifics was not significant as a main effect, there was a significant interaction effect between the visual predator stimulus and the chemical cue on focal fish behaviour (Table 1).

When we examined specific behaviours in more detail, we found that activity and shelter-seeking behaviour of subordinate *N. pulcher* were influenced by focal fish size, and by an interaction between the visual predator stimulus and the chemical cue (Table 2). Larger *N. pulcher* were more active, and spent less time near the substrate (Table 2). Taking this variation based on body size into account, focal *N. pulcher* were also less active (Fig. 2A) and spent more time near the substrate (Fig. 2B) when exposed to a visual predator stimulus, but only when the chemical cue was not present.

Table 2

Results of five generalized linear models looking at the effect of a visual predator stimulus and the odour of damaged conspecifics on five measures of focal fish behaviour: (1) activity (number of lines crossed); (2) exploration (proportion of unique squares entered); (3) shelter seeking (proportion of time near the bottom); (4) shoaling (proportion of time with any stimulus conspecific); and (5) group preference (proportion of shoaling time with the stimulus group rather than the single stimulus fish). The two treatments (i.e. visual predator stimulus and the chemical cue) were included as fixed effects. We included focal fish standard length (SL) as a covariate in all models, and examined all two-way interaction effects. For activity, we used a negative binomial error distribution, appropriate for over-dispersed count data. For all other variables, we used a beta error distribution for proportion data. Significant factors ($p < 0.05$) are indicated in bold. For all models and all model terms, $\alpha = 0.05$. All analyses were performed using R version 3.2.1 (R Core Team, 2015) within R Studio (2015).

Dependent variable	Model term	Estimate	t-Value	p-Value
Activity	Visual stimulus	-0.39	-2.90	0.004
	Chemical cue	0.04	0.26	0.79
	Focal fish size	0.40	2.07	0.03
	Visual stimulus x chemical cue	0.37	1.95	0.05
	Visual stimulus x focal fish size	0.07	0.34	0.73
	Chemical cue x focal fish size	0.03	0.17	0.86
Exploration	Visual stimulus	-0.75	-1.93	0.05
	Chemical cue	-0.41	-1.07	0.28
	Focal fish size	-0.02	-0.03	0.97
	Visual stimulus x chemical cue	0.93	1.71	0.08
	Visual stimulus x focal fish size	0.53	0.93	0.35
	Chemical cue x focal fish size	0.36	0.66	0.51
Shelter seeking	Visual stimulus	1.59	5.02	<0.001
	Chemical cue	0.24	0.83	0.41
	Focal fish size	-0.92	-2.14	0.03
	Visual stimulus x chemical cue	-1.29	-2.98	0.002
	Visual stimulus x focal fish size	-0.21	-0.47	0.63
	Chemical cue x focal fish size	-0.71	-1.64	0.10
Shoaling	Visual stimulus	1.42	3.48	<0.001
	Chemical cue	0.59	1.48	0.14
	Focal fish size	-0.91	-1.61	0.11
	Visual stimulus x chemical cue	-1.44	-2.54	0.01
	Visual stimulus x focal fish size	-0.80	-1.36	0.17
	Chemical cue x focal fish size	0.43	0.78	0.43
Group preference	Visual stimulus	-0.22	-0.49	0.62
	Chemical cue	-0.15	-0.32	0.74
	Focal fish size	0.76	1.18	0.24
	Visual stimulus x chemical cue	0.10	0.16	0.24
	Visual stimulus x focal fish size	-0.24	-0.36	0.72
	Chemical cue x focal fish size	-0.89	-1.39	0.16

Although the effect was marginally significant, the focal *N. pulcher* were also less exploratory in the presence of a visual predator stimulus (Table 2; Fig. 3A). Shoaling behaviour (i.e. the proportion of time spent with any of the conspecific stimuli) was influenced by an interaction between the visual predator stimulus and the odour of damaged conspecifics (Table 2; Fig. 3B), such that fish increased shoaling when exposed to a visual predator stimulus, but only if the odour of damaged conspecifics was not also present (Fig. 3B). There was no effect of either the visual predator stimulus or the chemical cue on the preference for the group of conspecifics versus the single conspecific stimulus fish (Table 2).

4. Discussion

In the current study, we found that a visual predator stimulus elicited a classic antipredator response in smaller, subordinate-sized *N. pulcher*. Namely, we found a decrease in activity and exploration, and an increase in shelter seeking and shoaling. However, contrary to our predictions, we found that the odour of damaged conspecifics had no measurable effect on its own, and there was a significant interaction effect between the visual predator stimulus and the chemical cue such that the combination of cues elicited a reduced response relative to the visual predator stimulus alone.

4.1. The response to a visual predator stimulus

We found that the small *N. pulcher* used as focal fish in the current study responded to a visual predator stimulus by reduc-

ing activity and exploration, and increasing shelter-seeking (i.e. spending a higher proportion of the trial time near the substrate) as well as shoaling (i.e. increasing the proportion of time spent near conspecifics), which is consistent with classic anti-predator behaviour across a range of species (Wisenden, 2000), including previous work on *N. pulcher* (Zöttl et al., 2013; Fischer et al., 2014). However, we should note that our experimental design somewhat limits the interpretation of these results. Namely, we found no differences in the propensity to spend time with the group of stimulus conspecifics over the single stimulus conspecific (i.e. no difference in group preference based on treatment), as we initially predicted would occur if *N. pulcher* seek out conspecifics as an anti-predator strategy. Instead, we found only a general tendency to avoid the central portion of the experimental aquarium, which in this case might reflect either a strategy to seek out conspecifics, or may simply reflect general shelter-seeking behaviour. An experimental design that can disentangle these possibilities would help to determine the importance of shoaling as an anti-predator strategy in *N. pulcher*.

4.2. Why did focal fish not respond to the odour of damaged conspecifics?

The *N. pulcher* used in this study did not appear to use the odour of damaged conspecifics as an indicator of predation risk, in that we found no effect of the chemical cue alone on focal fish behaviour. There are several potential explanations as to why the *N. pulcher* used in the current study failed to respond as expected to this chemical cue. First, prey fishes often respond in a graded man-

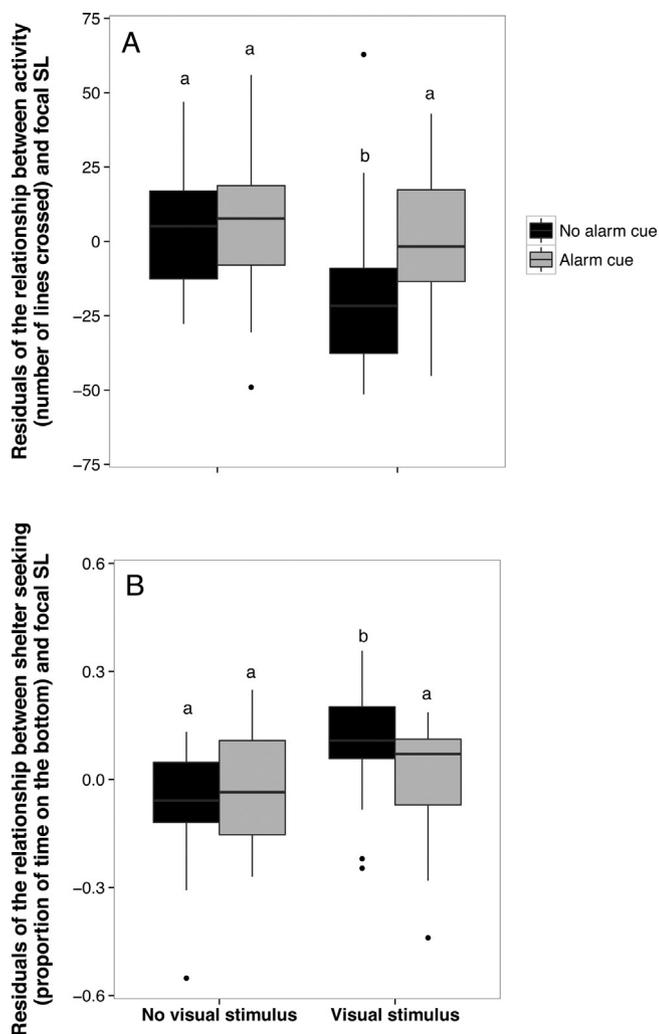


Fig. 2. *Neolamprologus pulcher* are (A) less active, and (B) spend more time seeking shelter when exposed to a visual predator stimulus, but only if the odour of damaged conspecifics is not also present. To determine treatment effects, we used generalized linear mixed models with a negative binomial error distribution for activity, and a beta error distribution for shelter seeking. Different letters indicate differences among treatment groups as determined by post-hoc pairwise comparisons. Since focal fish standard length is has a significant effect on both activity and shelter-seeking, the residuals of the relationship between behaviour and standard length are presented. See Section 2 for full statistical details, and Table 1 for full statistical results.

ner to damage released the scent of damaged conspecifics, with higher concentrations eliciting stronger responses (Ferrari et al., 2010). This is the first study to explore the response of *N. pulcher* to the odour of damaged conspecifics, and the concentration we chose may not have been sufficient to elicit a response. However, the concentration of the cue used in this study was relatively high when compared to doses used for another cichlid fish (Pollock et al., 2005), and we therefore predicted that *N. pulcher* would display a strong response to this chemical cue.

Second, it is possible that despite the nearly universal response of aquatic organisms to the scent of damaged conspecifics, *N. pulcher* may be insensitive these cues. Early work on cichlids suggested that this family of fishes might not respond strongly to the odour of damaged conspecifics (Shutz, 1956). However, numerous subsequent studies have contradicted this earlier claim, and confirmed that cichlids do alter their behaviour in response to these cues (Wisenden and Sargent, 1997; Alemadi and Wisenden, 2002; Roh et al., 2004; Brown et al., 2004, 2006; Foam et al., 2005; Reddon and Hurd, 2009; Abate et al., 2010; Barreto et al., 2010; Meuthen et al.,

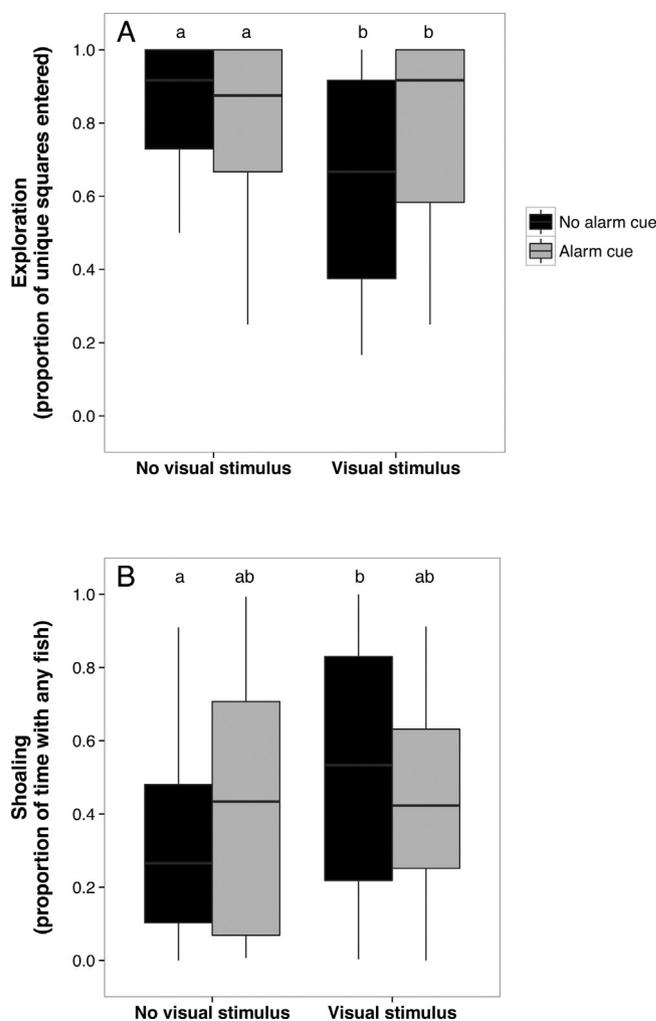


Fig. 3. *Neolamprologus pulcher* (A) reduce exploration when exposed to a visual predator stimulus, and (B) increase shoaling when exposed to a visual predator stimulus, but only if the odour of damaged conspecifics is not also present. To determine treatment effects, we used generalized linear mixed models with a beta error distribution. Different letters indicate differences among treatment groups as determined by post-hoc pairwise comparisons. See Section 2 for full statistical details, and Table 1 for full statistical results.

2012; although it should be noted that the majority of this work has been carried out using only a single species, the neotropical convict cichlid *Amatitlania nigrofasciata*, Keller-Costa et al., 2015).

Third, *N. pulcher* may be sensitive to the source of damage released alarm cues. In the current study, we obtained the damage released alarm cues from very large *N. pulcher* that would certainly be dominant breeding individuals in natural populations (SL = 75.2 ± 2.0 mm; mass = 13.1 ± 4.1 g). Conversely, the focal fish used in the trials were much smaller subordinate-sized individuals (SL = 36.7 ± 0.9 mm; mass = 1.4 ± 0.1 g). Since predators typically have a specific size range that they target (Popova, 1978), damage released alarm cues from individuals of a similar size to the focal individuals are the most relevant to focal fish, and many fish do not respond to damage released alarm cues obtained from much larger or smaller conspecifics (Harvey and Brown, 2004; Mitchell and McCormick, 2013; Elvidge and Brown, 2015). Further research should investigate the responses of *N. pulcher* to damage-released alarm cues obtained from a range of conspecifics, in order to determine how sensitive *N. pulcher* are to the source of these cues, and to better understand the relative salience of different indicators of predation risk.

As a fourth potential explanation for our results, there could have been some issue with the method in which we harvested or stored our cue samples. For instance, damage-released alarm cues can degrade rapidly at room temperature (Chivers et al., 2013). However, we kept our cue frozen for storage following established protocols (B.D. Wisenden, personal communication), and a diversity of cue harvesting and storage methodologies have been applied in the literature, suggesting that damage-released alarm cue responses ought to be a strong phenomenon robust to minor changes in collection or delivery protocols. The fact that the odour of damaged conspecifics did appear to alter the response to the visual predator stimulus suggests that the fish did in fact detect this chemical cue and that the cue was not inert as a result of some technical aspect of the procedure. Furthermore, in an earlier pilot experiment, we found that *N. pulcher* appeared to increase their activity in response to chemical cues collected, stored and delivered using the same methodology (A. R. Reddon, unpublished data). We are uncertain as to the reason for the difference between the results of the pilot study and the current experiment, but we believe further investigation into the possibility that *N. pulcher* treat the odour of damaged conspecifics as an alarm cue is warranted, though the available evidence to date is equivocal.

As a fifth potential explanation for our documented lack of response in *N. pulcher* to the odour of damaged conspecifics, it may be that the response to odour of damaged conspecifics is quite subtle in this species. For example, the response to *N. pulcher* to the odour of damaged conspecifics may involve changes in foraging frequency or posture that enhance vigilance rather than an overt antipredator response (Foam et al., 2005). It is therefore possible that the *N. pulcher* used in this study did show a subtle change in vigilance or preparedness to cope with predation threats in response to the chemical cue, which we did not detect in our observations.

Finally, it is important to note that the fish we used were predator naïve, and originated from a laboratory population that has been maintained in the absence of any predators for several generations. The anti-predator responses of these fish may therefore be muted relative to the responses that would be seen in wild fish. However, domestic guppies housed in the absence of predation show an equally strong response to damage released alarm cues as feral domestic-origin guppies that have been reintroduced to strong predation pressure for 20 years (~60 generations; W.T. Swaney, unpublished data). Previous studies in cichlid fishes have also made use of domestic origin populations which have been housed in the absence of predation for many generations (e.g. Wisenden and Sargent, 1997; Alemadi and Wisenden, 2002; Roh et al., 2004; Foam et al., 2005; Reddon and Hurd, 2009; Abate et al., 2010; Barreto et al., 2010), suggesting that the absence of predation does not abolish the response to damage-released alarm cues in related species. Interestingly, our predator naïve *N. pulcher* showed a typical anti-predator response to the visual predator stimulus of the *L. lemairii* predator. In the vast majority of cases, fish appear to learn about specific predators, in part through pairing the appearance or scent of a potential predator with conspecific damage-released alarm cues, rather than showing an innate reaction to predatory species (reviewed in Kelley and Magurran, 2003; Ferrari et al., 2007; Wisenden, 2015). This suggests that *N. pulcher* may show some innate response to certain predatory fish, including their natural predator *L. lemairii*. This is consistent with previous evidence that *N. pulcher* do show innate visual predator recognition. For example, both Zöttl et al. (2013) and Fischer et al. (2014) described innate recognition of predators as well as a fine-tuned ability of *N. pulcher* to differentiate between predators and non-dangerous fish based on visual cues. Future research should explore the degree to which *N. pulcher* show innate anti-predator responses to unfamiliar potential predators. It would be particularly valuable to conduct a study to compare responses between sympatric and

allopatric predators, and explore the importance of chemical cue conditioning (a well established system in other fishes; Ferrari et al., 2007) for *N. pulcher* to learn about novel predators.

4.3. Why did the focal fish have a reduced response to the combination of cues, relative to the response to the visual predator stimulus alone?

Any of the possibilities mentioned above could account the lack of response to the odour of damaged conspecifics. However, none of these explanations addresses the interesting result that the visual predator stimulus alone elicited predicted anti-predator responses in *N. pulcher*, but the addition of the scent of damaged conspecifics negated many of these anti-predator behaviours, suggesting that *N. pulcher* did indeed detect these odours. We suggest two possible explanations for these results. First, because of the group-living and cooperatively breeding social system of *N. pulcher*, even relatively small *N. pulcher* subordinates are involved to some degree in aggressive defence behaviour, and will engage with surprisingly large predators in order to defend the shared territory (Bruitjies and Taborsky, 2008, 2011; Desjardins et al., 2008; Heg and Taborsky, 2010; Witsenburg et al., 2010; Hellmann and Hamilton, 2014). Many factors influence this aggressive defence behaviour in subordinate *N. pulcher*, including the size of the subordinates (Bruitjies and Taborsky, 2008; Desjardins et al., 2008), predation pressure (Heg and Taborsky, 2010), and 'bystander' effects, where subordinate *N. pulcher* with an audience (particularly an unfamiliar audience) will be more likely to engage in aggressive defence behaviour relative to subordinates without an audience (Hellmann and Hamilton, 2014). There is a complex interplay between anti-predator responses that focus on avoiding predators, and anti-predator responses that focus on collectively confronting and deterring predators. We did not explicitly quantify defensive behaviours produced in the current study, and it is not feasible to do so given how the videos were recorded. However, it is possible that behaviours, such as spending more time in the centre of the aquarium or swimming higher in the water column, represented attempts by the focal fish to dissuade the predator from making an attack. *L. lemairii* are ambush predators, and clearly signalling to the predator that the predator has been detected might in itself be an anti-predator strategy (Caro, 1986; Fischer et al., 2014). A study that simultaneously assesses defensive behaviours, as well as the predator avoidance behaviours examined in the current study, would be valuable in disentangling responses of *N. pulcher* to predator threats, and would help determine if an increase in defensive behaviours could explain the pattern of results we found.

Second, it is an intriguing possibility that the focal *N. pulcher* used in the current study were using social information about predation risk during our trials. Previous work has suggested that other fish species can make use of social information when making decisions about predation risk (Brown et al., 2006), and may even trust information gathered from social fellows more than information they have personally collected (Crane and Ferrari, 2015). Because of the design of our experimental arena, the stimulus fish were not exposed to the odour of damaged conspecifics, but at times may have been able to see the visual predator stimulus (Fig. 1). Therefore, the stimulus fish would be predicted to respond more strongly to the visual predator stimulus than to the chemical cue, which could have in turn influenced the behaviour of the focal fish. Studies where only the stimulus fish is exposed to predator cues would be valuable, and provide information about the extent that *N. pulcher* modify their anti-predator responses based on social information from conspecifics.

5. Conclusions

Taken together, the results of this study demonstrate that *N. pulcher* adjust their anti-predator behaviour to both the type and combination of indicators of predation risk. While exposure to the visual predator stimulus alone produced predicted results, the odour of damaged conspecifics alone, or in combination with the visual predator stimulus, unexpectedly dampened anti-predator responses. This result points to future research avenues in better understand the complex interplay between predation risk and social obligation in a cooperatively breeding and group living fish.

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