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Do male sticklebacks use visual and/or olfactory cues to assess a potential mate's history with predation risk?

Marion Dellinger [a], Weiran Zhang [b], Alison M. Bell [b, c, d, e], Jennifer K. Hellmann [b, *]

[a] COMUE Université Bretagne Loire, Oniris, Nantes-Atlantic College of Veterinary Medicine and Food Sciences, Nantes, France
[b] Department of Animal Biology, School of Integrative Biology, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.
[c] Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.
[d] Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.
[e] Program in Ecology, Evolution and Conservation, University of Illinois at Urbana-Champaign, Urbana, IL, U.S.A.

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Differential allocation occurs when individuals alter their reproductive investment based on their mate's traits. A previous study showed that male threespine sticklebacks, *Gasterosteus aculeatus*, reduced courtship towards females that had previously been exposed to predation risk compared to unexposed females. This suggests that males can detect a female's previous history with predation risk, but the mechanisms by which males assess a female's history are unknown. To determine whether males use chemical and/or visual cues to detect a female's previous history with predation risk, we compared rates of courtship behaviour in the presence of visual and/or olfactory cues of predator-exposed females versus unexposed females in a 2 × 2 factorial design. We found that males differentiate between unexposed and predator-exposed females using visual cues: regardless of the olfactory cues present, males performed fewer zigzags (a conspicuous courtship behaviour) when they were exposed to visual cues from predator-exposed females compared to unexposed females. However, males' response to olfactory cues changed over the course of the experiment: initially, males performed fewer courtship displays when they received olfactory cues of predator-exposed females compared to unexposed females, but they did not discriminate between cues from predator-exposed and unexposed females later in the experiment. A follow-up experiment found that levels of cortisol released by both predator-exposed and unexposed females decreased over the course of the experiment. If cortisol is linked to or correlated with olfactory cues of predation risk that are released by females, then this suggests that the olfactory cues became less potent over the course of the experiment. Altogether, these results suggest that males use both visual and olfactory cues to differentiate between unexposed and predator-exposed females, which may help ensure reliable communication in a noisy environment.

Because courtship and parenting are costly and risky (Chellappa, Huntingford, Strang, & Thomson, 1989; Magnhagen, 1991; Reguera & Gomendio, 1999; Woods, Hendrickson, Mason, & Lewis, 2007), individuals often adjust their reproductive efforts according to the characteristics of their mates (differential allocation: Burley, 1986, 1988; Sheldon, 2000). Visual traits (e.g. bright and large sexually selected ornaments: Andersson, 1994), acoustic cues (e.g. high-quality songs or vocalizations: Christie, Mennill, & Ratcliffe, 2004; Holzer, Jacot, & Brinkhof, 2003; Wyman et al., 2012) and olfactory cues (e.g. pheromones: Rantala, Kortet, Kotiaho, Vainikka, & Suhonen, 2003) can all differentiate high-quality mates from low-quality mates. Because high-quality mates produce offspring with increased survival (Hasselquist, Bensch, & von Schantz, 1996; Møller & Alatalo, 1999; Petrie, 1994), better growth and condition (Petrie, 1994; Welch, Semlitsch, & Gerhardt, 1998) and increased sexual ornamentation (Griffith, Owens, & Burke, 1999; Norris, 1993), individuals often increase their courtship effort and parental investment when they receive visual, acoustic or olfactory cues indicating a high-quality potential mate (Gil et al., 1999, 2004; Sheldon, 2000; Soma & Okanoya, 2013). Individuals may rely primarily on one type of cue or may use different types of cues simultaneously to assess mate quality, which may increase the reliability of signals in a variable environment (Bro-Jørgensen, 2010).

* Correspondence: J. K. Hellmann, Department of Animal Biology, School of Integrative Biology, University of Illinois at Urbana-Champaign, 505 S. Goodwin Avenue, Urbana, IL 61801, U.S.A.
E-mail address: hellmann@illinois.edu (J. K. Hellmann).

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In addition to indicating quality, cues can covary with current environmental conditions such as food availability (Holzer et al., 2003; Kotiaho, Simmons, & Tomkins, 2001; Rantala et al., 2003; Velando, Beamonte-Barrientos, & Torres, 2006), predation risk (Godin, 1995; Sih, 1994) and risk of parasitism (Zuk, Simmons, & Cupp, 1993), which may induce plasticity in courtship and reproductive investment. In some cases, individuals adjust their mating preference for sexually selected traits that fit current environmental pressures; for example, although female guppies, *Poecilia reticulata*, generally prefer brighter males, they prefer duller males under high perceived predation risk (Gong & Gibson, 1996). In other cases, individuals might reduce their reproductive investment in response to cues from potential mates that indicate a low-quality or risky environment (Ghalambor, Peluc, & Martin, 2013). For example, female sand gobies, *Pomatoschistus minutus*, avoid laying eggs in males' nests when they detect a water mould odour (Lehtonen & Kvarnemo, 2015). Similarly, experimentally depriving males of food alters both chemical (Rantala et al., 2003) and visual cues (Velando et al., 2006) of male condition and causes females to reduce preference for these males. Although acquiring cues from mates may be useful for estimating future offspring quality, they also may help individuals evaluate the state of the environment and make decisions regarding trade-offs between conspicuous, energetically costly reproductive behaviours and antipredator behaviour or foraging.

Threespine stickleback, *Gasterosteus aculeatus*, fish are suitable subjects for investigating differential allocation because male sticklebacks invest heavily in paternal care. During the breeding season, male sticklebacks build nests and attract females to lay their eggs in the nest using a courtship ritual, which includes a conspicuous nuptial dance that involves swimming swiftly from left to right, alternating which side of the body is exposed (zigzags). After a female lays her eggs in a male's nest, the male provides care to the developing embryos and fry, both oxygenating the embryos and protecting them from conspecific and heterospecific predators. Recently, we found that male threespine sticklebacks altered their courtship and parenting behaviour in response to a mate's previous exposure to predation risk: male sticklebacks performed fewer zigzags towards predator-exposed females compared to unexposed females, and they also provided less care for the offspring of predator-exposed females (McGhee, Feng, Leasure, & Bell, 2015). Similar patterns have been found in other species: male guppies performed fewer courtship displays and more coercive mating behaviours when they encountered a predator-exposed female compared to an unexposed female (Evans, Kelley, Ramnarine, & Pilastro, 2002), while male jumping spiders displayed less to predator-exposed females compared to unexposed females (Su & Li, 2006). It is possible that male sticklebacks reduce courtship to predator-exposed females because females provide cues about imminent danger. Indeed, in a wide array of species, males reduce conspicuous courtship behaviour under high perceived predation risk (Acharya & McNeil, 1998; Candolin, 1997; Fuller & Berglund, 1996; Godin, 1995; Koga, Backwell, Jennions, & Christy, 1998; Sih, 1994). This may be mediated by olfactory cues, such as the release of alarm pheromones (Brown, 2003; Wisenden, 2015), or by visual cues, such as changes in female courtship behaviour (Su & Li, 2006). Alternatively or in addition, males may reduce courtship to predator-exposed females because of the stress associated with predation exposure: individuals prefer mates with low glucocorticoid levels, as high levels of cortisol often suppress sex hormones (Husak & Moore, 2008). This stress may be detectable via visual changes in secondary sexual characteristics (Husak & Moore, 2008) or via detectable olfactory changes in female pheromone or hormone levels (Stacey, 2015).

To our knowledge, none of the studies examining male detection of female predation history have differentiated among the types of cues that males may use to detect female predation status. Understanding how males use visual and olfactory cues to assess predation history is important not just for understanding the mechanism underlying differential allocation with respect to predation risk, but also for generally understanding the transmission of social information about predation risk in the environment. Here we test whether male sticklebacks use visual and/or olfactory cues to assess their mates' previous exposure to predation risk by systematically controlling for the presence of visual and/or olfactory cues from predator-exposed and unexposed females. Males were presented with a predator-exposed or unexposed female confined to a transparent compartment, while simultaneously receiving water from predator-exposed and unexposed females' tanks. If males use visual cues to assess a female's history, we predicted that males would perform fewer courtship behaviours when they visually encountered a predator-exposed female. If males use olfactory cues to assess a female's history, we predicted that males would perform fewer courtship behaviours when they received water from the tanks of predator-exposed females. To confirm that our simulation of predation risk (chasing by a model sculpin) elicited an antipredator response in females, we performed a follow-up experiment that compared excreted cortisol and antipredator behaviour of predator-exposed and unexposed females over time. By exploring the influence of olfactory and visual cues in isolation and in tandem, we can better understand when and to what extent males use different types of cues to gain information regarding females' past experiences and determine whether these cues are redundant (i.e. whether or not males need both cues present to alter courtship behaviour).

METHODS

Housing Conditions

Threespine sticklebacks were captured in 2015 and 2016 as juveniles in Putah Creek, CA, U.S.A., transported to the University of Illinois and reared in the laboratory. During the experiment, they were maintained at 20 °C on a summer photoperiod (16:8 h light:dark cycle). Tanks were on a recirculating system where water was continuously filtered through particulate, biological and ultraviolet (UV) filters; a constant water flow replaced around 10% of the tank's volume per hour. There was no detectable level of UV light in the housing room. Males were fed once a day with a mixture of frozen bloodworms, brine shrimps, mysis shrimps and cyclopeez. Females were also fed with this mixture as well as live bloodworms twice a day to encourage them to become gravid.

Experiment 1: Male Courtship Behaviour

Sixty females were housed in groups of 10 fish each in six 37.9-litre tanks (53 × 33 cm and 24 cm high, 'group tank'). Each group tank contained gravel and two plastic plants and was surrounded by opaque plastic partitions on the lateral sides and the back of the tank. Three group tanks were randomly assigned to a predator-exposed treatment and the other three to the unexposed control treatment. To simulate predation risk, a clay model (21 cm head-to-tail length, 6 cm head width) of a sculpin (a common stickleback predator in Putah Creek) was used to chase the predator-exposed females for 90 s each day at a random time to make exposure to predation risk unpredictable (Giesing, Suski, Warner, & Bell, 2011; McGhee, Pintor, Suhr, & Bell, 2012). Control group tanks were left undisturbed. Females remained in the group tanks until they become gravid, at which point they were used as stimulus fish.

Some females became gravid faster than others; time spent in the experimental treatment varied among females from 5 to 46 days. When a female was removed from her group tank, she was replaced by a new female to maintain density in the group tanks. Each new female added was marked with a unique spine clip combination to keep track of the date at which she was added to the group tank. Because individuals in the same tank co-regulate their cortisol levels (Fürtbauer & Heistermann, 2016), it is likely that new females that were put in the tanks later in the experiment had cortisol responses that resembled females that had already been in the tank for longer periods. After a female was used as a stimulus, she was either placed in a nonexperimental tank for other purposes or marked with a ventral fin clip, returned to her group tank and never used again as a stimulus. Female length did not vary significantly between treatments (Wilcoxon two-sample test: $W = 423.5$, $P = 0.32$).

Thirty males were kept singly in 10-litre tanks (32 × 21 cm and 19 cm high) within sight of one another, with a plastic plant and a sandbox and algae to encourage nest building. Males were left undisturbed. Once a male had completed his nest, as judged by the characteristic entrance hole, his tank was visually isolated from the other males' tanks with opaque partitions and he began the behavioural test.

Behavioural trials

Behavioural trials for experiment 1 were conducted during June–July 2016. During the behavioural trials, we compared male courtship behaviour towards a predator-exposed or an unexposed female that was confined in a transparent water-tight compartment (18 × 8.2 × 15.2 cm) that was fastened to the outside of a male's tank. In this configuration, the male could see the female and the pair could interact visually, but olfactory communication between the focal male and the stimulus female was not possible (Fig. 1). Each male ($N = 30$ unique males) was presented with a confined predator-exposed female twice and a confined unexposed female twice, in random order at a rate of one female per day for four consecutive days. At the same time as a male was presented with a confined female, olfactory cues were manipulated by adding water from tanks from either predator-exposed or predator-unexposed females (Fig. 1). Half the males were randomly assigned to receive water from predator-exposed females and the other half were assigned to receive predator-unexposed water, such that each male only received one type of water. Males did not receive both types of water to avoid bias caused by potential olfactory cues that might remain in the tank between trials. Consequently, we compared male behaviour across four different conditions: (1) visual and olfactory cues from predator-unexposed females; (2) visual cues from predator-exposed females and olfactory cues from unexposed females; (3) visual cues from unexposed females and olfactory cues from exposed females; (4) visual and olfactory cues from predator-exposed females.

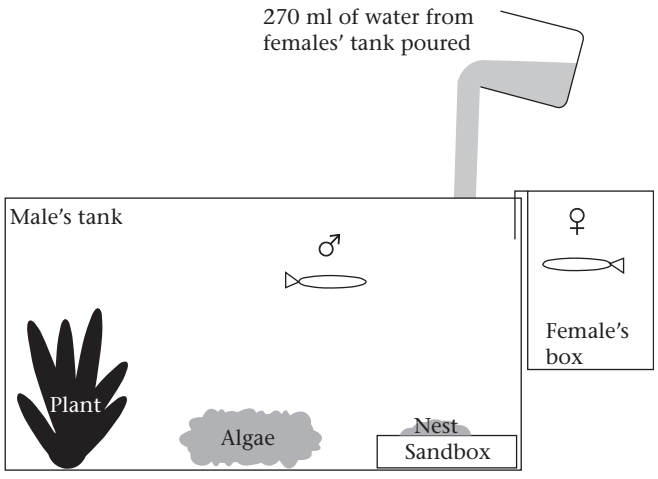

Figure 1. Experimental set-up. Each male was presented with a female that either had or had not been exposed to predation risk in the past. The female was confined to a transparent box, such that only visual cues of predation exposure were available to the male. Each male simultaneously received water from the tanks of predator-exposed or unexposed control females. This procedure was repeated for a full factorial combination of olfactory and visual cues across subjects.

Before a trial, we turned off the water flow in the females' group tanks. We immediately chased females in the predator-exposed treatment for 90 s with the clay model predator the same way as described above (unexposed females were not chased). One hour afterwards, we gently stirred the tank to distribute any potential olfactory cues and collected 90 ml of water from each tank. Water samples from each of the three replicate tanks per treatment were combined for a total water sample of 270 ml per treatment. We then turned on the water flow in the females' group tanks, gently netted a gravid female from her group tank and placed her in a transparent water-tight compartment, which was fastened to the outside of the front of a male's tank. At the same time, we gently poured a water sample from either predator-exposed or unexposed females into the male's tank to introduce olfactory cues (see Fig. 1 for experimental set-up). During behavioural observations, the water flow in the males' tanks was turned off to prevent olfactory cues in the water sample from being flushed out of the tank. During all other times, a steady water flow was maintained in the males' tanks in order to flush out any olfactory cues.

Each female was used as a visual stimulus twice in a random order: once for a male that received predator-exposed water and once for a male that received predator-unexposed water. After the trial with the first male, the compartment containing the female was removed from the male's tank and transferred to the second male's tank. After those two trials, the female was weighed and measured. Because there might be differences in how a female behaves on her first encounter with a male compared to her second encounter, each male saw two females on her first trial and two females on her second trial; the order in which he saw these females was randomized.

Once the focal male oriented towards the female, the behaviour of the male was observed for 5 min. We recorded the focal male's behaviour, which consisted of aggression (bites and chases) and display (zigzags, pokes, fanning) behaviours, as well as the number of approaches to the female, using JWatcher software (http://www.jwatcher.ucla.edu/). We also observed female courtship behaviour (head up, in which females elevate their head in a posture that indicates sexual receptivity), but these were relatively rare (occurred in only four trials), as were male pokes and fanning. Therefore, none of those behaviours were included in further analyses. All trials were scored by an observer (M.D.) who was blind to the treatment of the stimulus female (predator exposed versus unexposed control) and the type of water that was added to the male's tank. We weighed and measured males after completing the four behavioural trials.

Experiment 2: Female Cortisol and Behaviour

The objective of experiment 2 was to test whether the behaviour and cortisol stress response of females changed in response to the regimen of repeated exposure to predation risk used in experiment 1. In October–November 2016, we assembled six tanks (53 × 33 cm and 24 cm high) of 10 females (a mix of 2015 and 2016 populations) as described above. An equal number of tanks were randomly assigned to either the predator-exposed or unexposed treatment.

Females were weighed and added to a tank where they were allowed to acclimate for 7 days. After the acclimation period, females in the predator-exposed treatment were chased for 90 s per day with the model sculpin predator, as described in experiment 1. We drew a 21-square grid (3 squares high by 7 squares wide) on the front of the tank, which allowed us to measure the total number of squares occupied by all the females, which we used as a proxy for shoaling behaviour, and the number of fish in the bottom third of the tank, which we interpret as a proxy for fish seeking cover. We video-recorded tanks immediately before, immediately after and 1 h after chasing; we later scored the videos to extract data on the position of all 10 females every 10 s for 3 min immediately before, immediately after and 1 h after chasing. One hour after chasing, we gently stirred the tank water and collected 270 ml of tank water from each tank (the equivalent amount of water that was poured into the males' tank) and froze the water for later hormone extraction. For both unexposed and predator-exposed tanks, we recorded the behaviour of the females every 2 days for 42 days ($N = 21$ observation days) and collected water for hormone analysis every 4 days for 42 days ($N = 11$ collections). On the days that we collected water samples, we chased the females and collected all water samples at approximately the same time (chased at 14:00 hours, water collection at 15:00 hours) to control for circadian effects; on days without water collection, we chased females at a different time every day. We immediately replaced any fish that died within the 42-day period ($N = 10$ fish from the predator-exposed tanks, $N = 8$ fish from the predator-unexposed tanks) with predator-unexposed fish to maintain tank densities at $N = 10$ females per tank.

Measuring cortisol

We thawed water samples at room temperature and filtered them through qualitative filter papers (Double Rings 102, medium speed) to remove any solid impurities before concentrating through tC18 cartridges (Waters Sep-Pak, 900 mg sorbent capacity, 37–55 μm particle size) using a vacuum manifold. We then used 5 ml of ethyl acetate (Fisher Scientific, Lenexa, KS, U.S.A., HPLC grade) to elute the free form of cortisol from the cartridge into a glass vial (Fisherbrand, Fisher Scientific, borosilicate glass 13 × 100 mm tubes) for each sample. The solvent ethyl acetate was dried via a SpeedVac concentrator (Savant DNA 110 SpeedVac, Thermo Fisher Scientific, Waltham, MA, U.S.A.) and the cortisol-containing residue was resuspended in 500 μl assay buffer provided in the cortisol ELISA kit (Enzo Life Sciences, Farmingdale, NY, U.S.A.). The free form of the cortisol was assayed as it directly correlates with the cortisol level in fish plasma and is an accurate measurement reflecting the active physiological state, particularly the stress level (Scott & Ellis, 2007; Sebire, Katsiadaki, & Scott, 2007).

To validate the methodology for cortisol measurements above, several tests to evaluate assay performance are necessary (Andreasson et al., 2015; Ligocki, Earley, Hellmann, & Hamilton, 2015). First, in order to test whether there was matrix interference with the ELISA assay, we assessed the parallelism (Plikaytis et al., 1994) between the standard curve and the serial dilution curve derived from pooled water samples. Specifically, the pool was made by combining 66 assay buffer-resuspended samples (10 μl each) and serial dilutions from 1:1 to 1:8 were assayed. Slopes of the standard curves and serial dilution curve were parallel (ANCOVA: $F_{1,2} = 0.06$, $P = 0.83$), indicating that there was negligible matrix interference contributing to systematic measurement error.

Secondly, to test quantitative recovery through the tC18 extraction cartridge, 10 μl of pooled sample was spiked with a known concentration of cortisol ($N = 5$ spikes ranging from 10 000 pg/ml to 500 pg/ml). The sample was then diluted using double distilled water into 270 ml, concentrated and eluted through tC18 cartridges, dried, reconstituted and assayed in ELISA in the same fashion as previously described for the water samples. Our results showed reasonable recovery rates (defined as average = 94.2%, range 84.6–101%). In addition, for the six ELISA plates used in the measurement, the intra-assay coefficients of variation were all within acceptable range (3.6%, 10.5%, 14.4%, 7.7%, 11.6% and 2.8%), the interassay coefficient of variation was 18.1%, and all measurements were run in duplicate. Samples with a coefficient of variation greater than 20% ($N = 3$) were removed from the data set.

Statistical Analysis

Experiment 1

We used generalized linear mixed models (GLMM) with a negative binomial distribution (to account for overdispersed count data; package glmmADMB) to assess variation in male zigzag behaviour, male aggression (bites and chases) and approaches towards the female. All models included fixed effects of visual cues (binomial: control or exposed), olfactory cues (binomial: control or exposed), male standard length, trial number (e.g. the first, second, third or fourth female that the male encountered) and trial day to account for any variation over time or seasonal effects. To examine whether the number of days that a particular female was chased influenced male behaviour, we also reran the same model above for only predator-exposed females and replaced the fixed effect of visual treatment with a fixed effect of the number of days of predation exposure for each female. This is distinct from trial date, as predator-naïve females replaced females that were used for courtship trials throughout the course of the experiment. For all models, we tested for interactions, particularly interactions between visual and olfactory treatments, and only retained interactions that were significant and improved Akaike's information criterion (AIC) values of the model (see Appendix Table A1 for AIC values of models testing interactions between visual and olfactory treatments). To account for repeated measures, we included random effects of male and female identity in all models.

Experiment 2

We used linear mixed effects models to examine variation in (1) female shoaling behaviour and (2) female clustering on the bottom of the tank. For the two models testing predictors of female behaviour, we included fixed effects of treatment (binomial: control or exposed), trial day (continuous: days 1–41) and time period (before, immediately after chasing and 1 h after chasing). For models testing shoaling behaviour (number of squares occupied), data were cubed to achieve normality of the residuals. We also used a linear mixed effects model, with fixed effects of treatment and trial day, to examine variation in female cortisol levels (pg/g of fish mass). We used two additional linear mixed effect models to examine how fixed effects of shoaling behaviour and clustering on the bottom of the tank were linked to cortisol levels. All models had a random effect of tank identity to control for repeated measures.

Behavioural data from two predator-exposed tanks on day 29 and day 31 as well as one predator-exposed tank on day 33 were removed from analysis because fish jumped between tanks ($N = 5$ sets of observations, $N = 15$ observation periods total). The corresponding water samples were also excluded ($N = 3$ cortisol samples). Furthermore, two outliers were removed from the cortisol data set (day 5: one predator-exposed tank; day 41: one control tank), as those measurements were greater than three times higher than any other cortisol measurement taken on the same day from other tanks. Model residuals were examined for normality. We tested for interactions and only retained interactions that were

significant and improved AIC values of the model (see Appendix Table A2 for AIC values of models testing interactions).

Ethical Note

All methods were approved by Institutional Animal Care and Use Committee of University of Illinois Urbana-Champaign (protocol ID 15077), including the use of a model predator, and adhered to the guidelines set forth by the Animal Behavior Society/Association for the Study of Animal Behaviour. Spine and fin clipping lasted for approximately 30 s per fish: the fish was gently netted from the tank, removed from the net and gently held between two fingers. For spine clips, we lifted the spine and quickly cut at its base with sharp dissecting scissors. For fin clips, we cut off a small portion of the fin with dissecting scissors. The fish was then gently placed back in stock tanks and monitored; we detected no adverse health effects of spine or fin clipping. All fish were returned to stock tanks at the completion of the experiment.

RESULTS

Experiment 1

Males displayed significantly fewer zigzags in response to visual cues from predator-exposed females relative to visual cues from unexposed females (Table 1, Fig. 2), regardless of olfactory treatment. There was a significant interaction between olfactory cues and trial date on zigzags (Table 1). As a post hoc test, we examined olfactory cues from control and predator-exposed females separately; we found that males exposed to water from predator-exposed females showed significantly fewer zigzags early in the experiment compared to later in the experiment (GLMM with negative binomial distribution: $Z_{50} = 2.37$, $P = 0.018$), whereas this pattern was not present for males that were exposed to water from unexposed females ($Z_{52} = -0.45$, $P = 0.65$; Fig. 3). There was no evidence that exposure to olfactory cues or visual cues influenced male aggression (bites and chases) to the female or the number of times that he approached the female (Table 1). Among predator-exposed females, we found no effect of the number of days that a female was chased by the model predator on male behaviour (zigzag: $Z_{52} = 0.41$, $P = 0.68$; aggression: $Z_{53} = -0.29$, $P = 0.77$; approaches: $Z_{53} = -1.31$, $P = 0.19$). We did not find any evidence for an interaction between visual and olfactory treatments for zigzags, aggression or approaches (Appendix Table A1).

Bigger males were less aggressive to females and tended to display fewer zigzags (Table 1). There was no effect of male size on how frequently a male approached the female (Table 1). Males became less aggressive and exhibited lower rates of courtship behaviour (zigzags) with repeated testing (i.e. between trial 1 and trial 4) (Table 1). In contrast, males approached females more

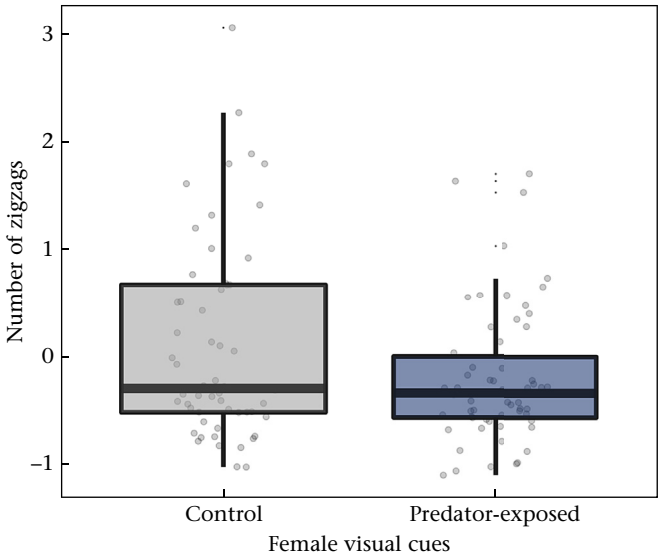

Figure 2. Number of zigzags performed by males in experiment 1 in response to visual cues of predator-exposed and unexposed control females. Data presented are the residuals of the regression model without visual treatment, plotted against visual treatment. The line in the box plot denotes the median value, with the ends of the boxes showing the upper and lower quartile, overlaid on top of individual data points for each male.

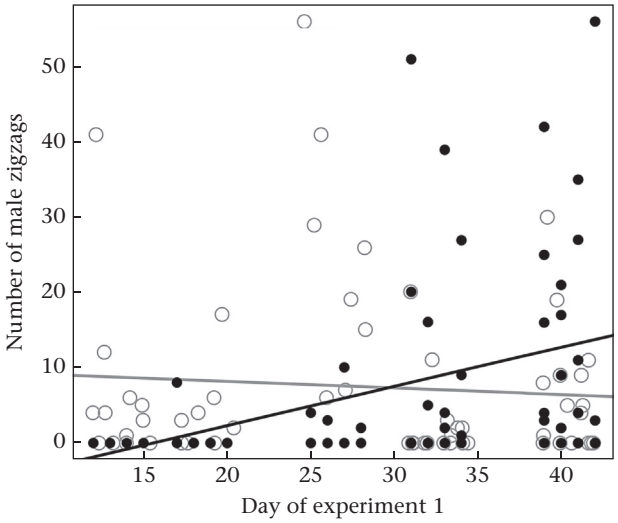

Figure 3. Number of zigzags performed by males in experiment 1 in response to olfactory cues from predator-exposed females (closed circles, black line) and unexposed control females (open circles, grey line).

Table 1
Results of generalized linear mixed models (with negative binomial distribution) testing predictors of male behaviour in experiment 1

	Zigzags		Aggression		Approaches	
	Z_{108}	P	Z_{109}	P	Z_{109}	P
Visual treatment	1.98	**0.047**	1.04	0.30	0.94	0.35
Olfactory treatment	1.90	0.057	0.17	0.86	−0.35	0.73
Trial date	2.02	**0.043**	−0.07	0.94	2.15	**0.032**
Encounter order	−2.63	**0.009**	−3.59	**<0.001**	2.08	**0.038**
Male standard length	−1.93	0.054	−2.38	**0.017**	1.09	0.27
Olfactory∗date	−1.91	**0.048**	–	–	–	–

Fixed effects tested included visual treatment (unexposed control or exposed female), olfactory treatment (water from unexposed control or predator-exposed tanks), trial date, encounter order (first, second, third or fourth trial per male) and male standard length. Significant outcomes are shown in bold.

frequently over the course of the four trials as well as over the course of the entire experiment (Table 1).

Experiment 2

Female behaviour

Females in the predator-exposed group responded behaviourally to the predator attack (significant time by predator treatment interactions; shoaling: $F_{2,347.00} = 4.20$, $P = 0.02$; clustering on the bottom: $F_{2,352.01} = 7.20$, $P < 0.001$). Specifically, predator-exposed females spent more time on the bottom of the tank after the predator attack compared to before (Tukey's HSD; immediately after: $Z = 4.45$, $P < 0.001$; 1 h after: $Z = 2.34$, $P = 0.05$). Similarly, although predator-exposed females did not shoal more immediately after the simulated predator attack compared to before (linear mixed effects, Tukey's HSD; $Z = 0.59$, $P = 0.83$), they tended to shoal more 1 h after the simulated predator attack relative to before ($Z = -2.26$, $P = 0.06$) or immediately after the simulated predator attack ($Z = -2.85$, $P = 0.01$). There was no difference in the behaviour of unexposed and predator-exposed females prior to the simulated predator attack for either behaviour (bottom: $F_{1,4.03} = 2.25$, $P = 0.21$; shoaling: $F_{1,4.02} = 2.57$, $P = 0.18$).

Females in both treatments spent less time on the bottom of the tank as the experiment progressed ($F_{1,352.06} = 91.50$, $P < 0.001$). We also found a trial day by time period interaction on female shoaling behaviour. Specifically, we found no effect of trial day ($F_{1,112.99} = 1.93$, $P = 0.17$) in the 'before' time period, but for both time periods after the simulated predator attack, shoaling behaviour was higher earlier in the experiment relative to later in the experiment (immediately after simulated predator attack: $F_{1,112.1} = 13.80$, $P < 0.001$; 1 h after simulated predator attack: $F_{1,114.2} = 20.20$, $P < 0.001$).

Cortisol excretion

Cortisol levels were consistently higher in the predator-exposed tanks compared to the unexposed control tanks (linear mixed effects model: $t_{55} = 2.94$, $P = 0.005$; Fig. 4). Cortisol levels decreased over time in both treatments ($t_{55} = -3.92$, $P < 0.001$; Fig. 5). Cortisol levels were positively correlated with the number of fish on the bottom of the tank at the time of water collection ($t_{55.19} = 2.71$, $P = 0.009$), but we found no significant relationship between cortisol and shoaling behaviour ($t_{55.19} = -1.50$, $P = 0.14$).

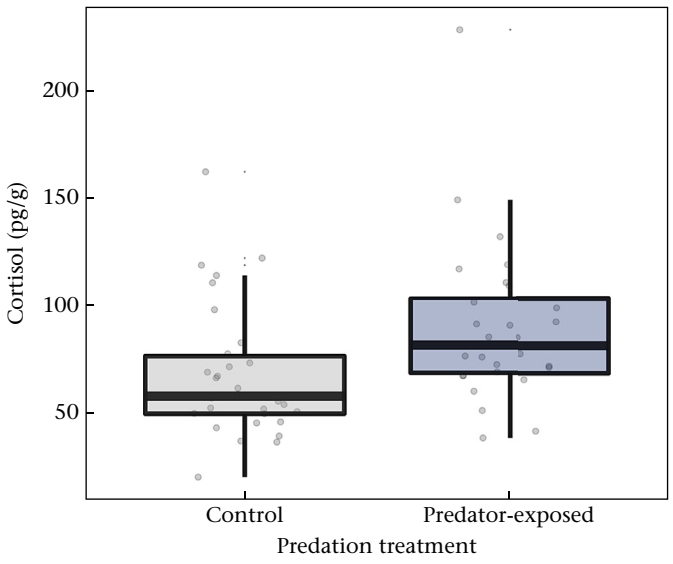

Figure 4. Cortisol levels of predator-exposed and unexposed control females in experiment 2. The line in the box plot denotes the median value, with the ends of the boxes showing the upper and lower quartile, overlaid on individual data points for each tank.

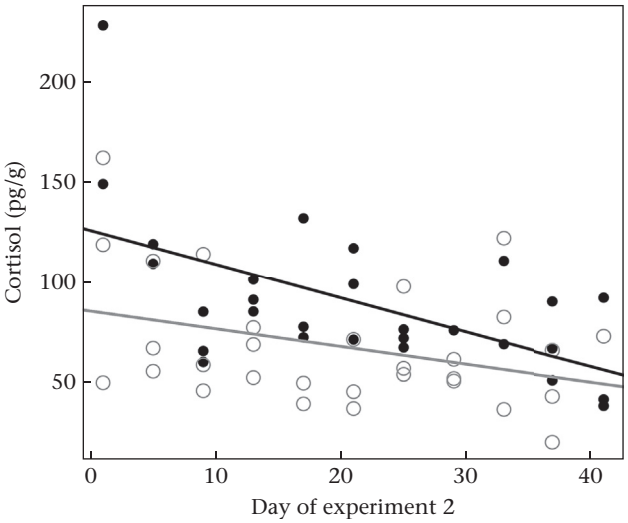

Figure 5. Cortisol levels of predator-exposed females (closed circles, black line) and unexposed control females (open circles, grey line) over the course of experiment 2.

DISCUSSION

In this study we demonstrate that male threespine sticklebacks respond to visual and olfactory cues from females that have been exposed to predation risk. Specifically, males consistently displayed fewer zigzags in response to visual cues from predator-exposed females, suggesting that visual cues alone are sufficient to produce differential allocation with respect to female experience with predation risk. We also found that males reduced their courtship behaviours when they received olfactory cues from predator-exposed females at the beginning of the experiment, but this effect disappeared by the end of the experiment. Corresponding to this, although cortisol from predator-exposed females was always higher than cortisol from unexposed control females in experiment 2, cortisol release decreased over the course of the experiment. If the olfactory cues that males use to detect female predation status are linked to or correlated with cortisol, olfactory cues might have weakened over the course of experiment 1. Interestingly, this suggests that males do not respond in a binary way to the presence versus absence of predator-induced olfactory cues, but instead adjust their behaviour along a continuum in response to weaker versus stronger cues. However, the fact that males did not change their behaviour towards control females during the course of the experiment (despite the fact that control female cortisol also declined) suggests that males only respond along a continuum of olfactory cues when they exceed a certain threshold. In other words, males may not alter their behaviour in response to olfactory cues when they vary within an expected range, but they alter the magnitude of their response based on how far the olfactory cues are from the expected range. This pattern may also occur for the visual cues presented by females; however, given that we do not know the extent to which visual cues varied over the course of the experiment, we cannot assess whether males respond to visual cues in a binary fashion or across a gradient.

Given that we found a decrease in cortisol over time in both predator-exposed and unexposed control treatment groups, it seems unlikely that the drop in cortisol over time is due solely to habituation to repeated chasing. This is consistent with the finding

that the duration of individual female exposure did not significantly influence male behaviour and supports previous studies that have found no effect of the length of female predation exposure on male courtship behaviour (McGhee et al., 2015) or on offspring traits (Giesing et al., 2011; Mommer & Bell, 2013). The general decrease in cortisol over time in both the control and experimental groups might reflect acclimation to a new tank and/or a new social group, as sticklebacks prefer to shoal with familiar conspecifics (Barber & Ruxton, 2000). Indeed, in experiment 2, both predator-exposed and unexposed females showed lower levels of antipredator behaviours (i.e. shoaling, clustering at the bottom) over time. It is unlikely that the decline in response over the course of the experiment reflects seasonal effects because experiments 1 and 2 took place at different times over the summer, but a decline in response (male behaviour in experiment 1, cortisol excreted and behaviour in experiment 2) was observed in both experiments. Furthermore, if males generally became less responsive over the course of the study, then we would expect to see a decline in male response for control females as well as predator-exposed females in experiment 1, which was not observed.

The precise visual cues that males use to discriminate between predator-exposed and unexposed females are unknown. It is unlikely that the visual cue is the female's body shape or gravidity: clutch size does not vary between predator-exposed and unexposed females (McGhee et al., 2015) and female length did not vary between treatment groups. Another possibility is that predator-exposed and unexposed females behaved differently while they were interacting with males. Predator-exposed and unexposed females did not differ in female preference behaviours in a previous study (McGhee et al., 2015), but it is possible that females demonstrated changes in other behaviours that we did not measure. Supporting this, predator-exposed and unexposed females differed in space use and social behaviour 1 h after the simulated predator attack in experiment 2. Given that females in experiment 1 were presented to the males 1 h after the simulated predator attack, males may have been detecting differences in these types of behaviours, which were not recorded in experiment 1. In addition to behavioural differences, predator-exposed and unexposed females may differ in coloration. Signalling in the ultraviolet spectrum is unlikely to play a role in our results because UV light levels were undetectable in the room where the study was carried out. However, predator-exposed and unexposed females might have differed in coloration in other subtle ways; for example, female red–orange spine coloration and body pattern influences courtship in male sticklebacks (Nordeide, 2002; Rowland, Baube, & Horan, 1991), and non-UV female coloration influences courtship in male gobies (Amundsen & Forsgren, 2001).

The precise olfactory cues that differ between predator-exposed and unexposed females are unknown. In experiment 2, we measured free cortisol, which reflects the physiologically active state of the female (Scott & Ellis, 2007). However, males may be more likely to respond to conjugated cortisol, which can act as a pheromone (Scott et al., 2008). Furthermore, males may detect other hormones that are correlated with free cortisol and that indicate female receptivity rather than a female's history with predation risk. For example, cortisol suppresses sex hormones in fish (Wendelaar Bonga, 1997), and previous studies have shown that males prefer to court females with high levels of sex hormones (Crews, 1976). Therefore, stickleback males might have reduced courtship behaviour in response to olfactory cues from predator-exposed females because the water contained lower levels of female sex steroids. A manipulative test, in which male courtship is observed after cortisol or sex steroids are added to the water, would elucidate the extent to which variation in female cortisol underlies differences in male sexual behaviour.

Males that were exposed to olfactory or visual cues from predator-exposed females behaved similarly to males that are directly exposed to predation risk, i.e. reduced zigzags (Candolin, 1997). Therefore, it is possible that males may detect female cues that indicate immediate predation risk. On the other hand, males might have responded to cues that reflect traits that were altered by the predation treatment (e.g. behaviour, coloration), and not because males detected females' experience with predation risk per se. Additionally, it is possible that males might have sensed higher cortisol in females, which elicited a physiological stress response in the males (Fürtbauer & Heistermann, 2016) and caused them to reduce their courtship behaviour. Although moderate levels of cortisol are essential for mobilizing energy required for reproduction, higher levels can often suppress reproduction (Moore & Jessop, 2003; Moore & Miller, 1984; Wingfield et al., 1998). Determining whether males respond to a female's history of predation risk per se or to a female's general physiological state will give insights into the information encoded by visual and olfactory cues.

Conclusions

Collectively, these results demonstrate that males respond to both olfactory and visual cues that indicate a potential mate's previous experience with predation risk. It is possible that using multiple types of cues could help ensure reliable communication in a noisy environment (Partan, 2013). Responding to information about predation risk probably has important fitness consequences for both the male himself and his offspring (Stein & Bell, 2014); consequently, there might be strong selection for males to take advantage of any type of information about predation risk, regardless of its source or channel. Furthermore, it is possible that olfactory and visual cues may give information about different female qualities, and males may differ, both within and among populations, in the cues that they use to differentially allocate. For example, Putah Creek is a relatively stable environment in which visual landmarks have been shown to be important (Bensky & Bell, 2018), but olfactory communication may be relatively more important in populations living in habitats that limit visual communication (Heuschele, Mannerla, Gienapp, & Candolin, 2009). Consequently, exploring how differential allocation is impacted by visual and olfactory cues across different populations would be useful for understanding the relationship between differential allocation and cue type.

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Appendix

Table A1
Table of models with all possible combinations of interactions between visual cues (VC), olfactory cues (OC) and experimental date

Model (all models include random effects of male and female ID)	Model AIC	VC∗OC	VC∗Date	OC∗Date	VC∗OC∗Date
Zigzags ~ Visual + olfactory + date + encounter + male SL	611				
Zigzags ~ Visual∗olfactory + date + encounter + male SL	613	0.96			
Zigzags ~ Visual∗date + olfactory + encounter + male SL	612.1		0.34		
Zigzags ~ Visual∗olfactory + visual∗date + encounter + male SL	613.9	0.67	0.3		
Zigzags ~ Visual + olfactory∗date + encounter + male SL	**609**			**0.048**	
Zigzags ~ Visual∗olfactory + olfactory∗date + encounter + male SL	611	0.91		0.048	
Zigzags ~ Visual∗date + olfactory∗date + encounter + male SL	610		0.31	0.044	
Zigzags ~ Visual∗olfactory + visual∗date + olfactory∗date + encounter + male SL	611.8	0.6	0.26	0.042	
Zigzags ~ Visual∗olfactory∗date + encounter + male SL	611.4	0.11	0.07	0.014	0.13
Aggression ~ Visual + olfactory + date + encounter + male SL	**985.6**				
Aggression ~ Visual∗olfactory + date + encounter + male SL	987	0.42			
Aggression ~ Visual∗date + olfactory + encounter + male SL	986.7		0.41		
Aggression ~ Visual∗olfactory + visual∗date + encounter + male SL	988.5	0.5	0.48		
Aggression ~ Visual + olfactory∗date + encounter + male SL	987.6			0.88	
Aggression ~ Visual∗olfactory + olfactory∗date + encounter + male SL	989	0.43		0.89	
Aggression ~ Visual∗date + olfactory∗date + encounter + male SL	988.9		0.41	0.85	
Aggression ~ Visual∗olfactory + visual∗date + olfactory∗date + encounter + male SL	990.5	0.5	0.47	0.86	
Aggression ~ Visual∗olfactory∗date + encounter + male SL	991.3	0.42	0.2	0.87	0.29
Approaches ~ Visual + olfactory + date + encounter + male SL	**507.6**				
Approaches ~ Visual∗olfactory + date + encounter + male SL	509.3	0.65			
Approaches ~ Visual∗date + olfactory + encounter + male SL	509.5		0.91		
Approaches ~ Visual∗olfactory + visual∗date + encounter + male SL	511.3	0.65	0.95		
Approaches ~ Visual + olfactory∗date + encounter + male SL	509.5			0.83	
Approaches ~ Visual∗olfactory + olfactory∗date + encounter + male SL	511.3	0.65		0.83	
Approaches ~ Visual∗date + olfactory∗date + encounter + male SL	511.5		0.92	0.83	
Approaches ~ Visual∗olfactory + visual∗date + olfactory∗date + encounter + male SL	513.3	0.65	0.97	0.84	
Approaches ~ Visual∗olfactory∗date + encounter + male SL	514	0.23	0.44	0.47	0.26

AIC: Akaike's information criterion; SL: standard length. In selecting the final model (shown in bold), we only retained interactions that were significant and improved AIC values of the model.

Table A2
Table of models with all possible combinations of interactions among treatment (Tr), time period (TP) and experimental day

Model (all models include random effects of tank)	Model AIC	Tr∗TP	Tr∗Day	TP∗Day	Tr∗TP∗Day
No. of fish on bottom ~ Treatment + time period + day	1397.27				
No. of fish on bottom ~ Treatment∗time period + day	**1387.37**	**<0.001**			
No. of fish on bottom ~ Treatment∗day + time period	1404.65		0.24		
No. of fish on bottom ~ Treatment∗day + treatment∗time period	1394.7	<0.001	0.24		
No. of fish on bottom ~ Treatment + time period∗day	1406.55			0.02	
No. of fish on bottom ~ Treatment∗time period + day∗time period	1396.22	<0.001		0.02	
No. of fish on bottom ~ Treatment∗day + day∗time period	1413.93		0.24	0.02	
No. of fish on bottom ~ Treatment∗day + day∗time period + treatment∗time period	1403.58	<0.001	0.23	0.02	
No. of fish on bottom ~ Treatment∗day∗time period	1415.91	<0.001	0.23	0.02	0.37
Shoaling ~ Treatment + time period + day	4207				
Shoaling ~ Treatment∗time period + day	4186.62	0.02			
Shoaling ~ Treatment∗day + time period	4207.57		0.72		
Shoaling ~ Treatment∗day + treatment∗time period	4187.32	0.01	0.72		
Shoaling ~ Treatment + time period∗day	4201.39			0.04	
Shoaling ~ Treatment∗time period + day∗time period	**4181.43**	**0.02**		**0.04**	
Shoaling ~ Treatment∗day + day∗time period	4202.08		0.72	0.04	
Shoaling ~ Treatment∗day + day∗time period + treatment∗time period	4182.14	0.02	0.72	0.04	
Shoaling ~ Treatment∗day∗time period	4177.88	0.01	0.72	0.04	0.27

AIC: Akaike's information criterion. In selecting the final model (shown in bold), we only retained interactions that were significant and improved AIC values of the model.