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Cite this article: Fan R, Reader SM, Sakata JT. 2022 Alarm cues and alarmed conspecifics: neural activity during social learning from different cues in Trinidadian guppies. *Proc. R. Soc. B* **289**: 20220829. https://doi.org/10.1098/rspb.2022.0829

Received: 29 April 2022
Accepted: 15 July 2022

Subject Category:
Neuroscience and cognition

Subject Areas:
behaviour, cognition, neuroscience

Keywords:
social learning, social information, fear conditioning, conditioned threat learning, anti-predator behaviour, pS6

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6124062.

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Alarm cues and alarmed conspecifics: neural activity during social learning from different cues in Trinidadian guppies

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Learning to respond appropriately to novel dangers is often essential to survival and success, but carries risks. Learning about novel threats from others (social learning) can reduce these risks. Many species, including the Trinidadian guppy (*Poecilia reticulata*), respond defensively to both conspecific chemical alarm cues and conspecific anti-predator behaviours, and in other fish such social information can lead to a learned aversion to novel threats. However, relatively little is known about the neural substrates underlying social learning and the degree to which different forms of learning share similar neural mechanisms. Here, we explored the neural substrates mediating social learning of novel threats from two different conspecific cues (i.e. social cue-based threat learning). We first demonstrated that guppies rapidly learn about threats paired with either alarm cues or with conspecific threat responses (demonstration). Then, focusing on acquisition rather than recall, we discovered that phospho-S6 expression, a marker of neural activity, was elevated in guppies during learning from alarm cues in the putative homologue of the mammalian lateral septum and the preoptic area. Surprisingly, these changes in neural activity were not observed in fish learning from conspecific demonstration. Together, these results implicate forebrain areas in social learning about threat but raise the possibility that circuits contribute to such learning in a stimulus-specific manner.

1. Introduction

Recognizing and responding appropriately to danger is essential for survival and success in risky environments, with both excessive caution and excessive risk-taking having maladaptive consequences [1]. In variable environments with new risks, animals can directly learn about novel threats like predators when they co-occur with aversive events, such as physical injury or pursuit [2]. Given the risks of direct learning, it is often advantageous to learn to respond to novel threats using social information, where the cues that evoke defensive responses come from conspecifics [3–8]. Animals can form associations between a novel stimulus and conspecific cues that indicate threat such as chemical cues released during predation or defensive responses by experienced conspecifics ('demonstrators' [9–11]). This learning can rapidly lead to defensive responses to the novel stimulus alone [9,12,13]. Furthermore, such processes can result in the spread of novel threat responses through populations.

Considerable attention has been devoted to the adaptive significance of conspecific threat cues [2,5,10]. However, in contrast to the extensive work on the function and neural mechanisms of non-social conditioned threat learning (or 'fear conditioning') in a variety of vertebrates [14,15], much less is known about the neural substrates underlying conspecific-mediated (social) threat learning, particularly in non-mammalian vertebrates (but see [14,16–19]). Revealing the neural circuits underlying ethologically important forms of social learning across species can provide important insights into the mechanisms underlying evolutionary and experience-dependent variation in cognition

and plasticity. Research in rodents and humans has identified various brain areas that are important for threat learning through observation, including the amygdala, but relatively little is known about whether these neural systems represent general substrates for learning about risk from conspecifics and whether these processes are conserved across vertebrate species [14,16].

Here, we work towards addressing this knowledge gap by characterizing neural activity when learning about novel dangers in the Trinidadian guppy (*Poecilia reticulata*), a model species for ecology, evolution and behaviour [20–22]. We investigated the degree to which two types of social cues—chemical alarm cues and the behaviour of experienced conspecifics—can be used for threat learning and the extent to which common patterns of neural activity are observed during the acquisition of threat in response to these different social cues. Alarm cues (also referred to as alarm substance or Schreckstoff) are chemicals passively released into the water by epidermal rupture during predation [23]. Just as in other fish species, anti-predator responses to both alarm cue and alarmed conspecifics such as increased freezing, increased grouping, and reduced activity have been documented in guppies [24,25], but whether guppies can acquire responses to novel and arbitrary cues through association with these types of social information has not been directly determined.

We examined five brain areas previously implicated in threat learning or social behaviour and broadly predicted that pS6 expression (a cellular marker of neural activity) would be elevated in these regions in guppies undergoing experiences that lead to threat learning, compared to guppies experiencing control conditions that do not lead to learning. We examined the medial zone of the dorsal telencephalon (Dm; putative homologue of the mammalian basolateral amygdala) because it has been implicated in cued threat learning in zebrafish *Danio rerio* [17,26–28], suggesting potential conservation of function. In addition, because of the fundamental role of social stimuli in learning from others, we examined brain areas in the social behaviour network, a network of brain areas that mediate a suite of social behaviours—namely the preoptic area (POA), the supracommissural zone of the ventral telencephalon (Vs; putative homologue of the mammalian medial amygdala/bed nucleus of the stria terminalis) and the ventral zone of the ventral telencephalon (Vv; putative lateral septum (LS) homologue) [15,18,29–32]. The mammalian LS has been found to regulate fear conditioning [33,34], and the POA is a conserved locus for the integration of social context information and relays amygdalar input to the periaqueductal grey to modulate motor responses to fear [15,35]; consequently, these areas could regulate threat learning based on conspecific cues. The hippocampus plays a role in various forms of learning, including some types of threat learning, and we thus examined the lateral zone of the dorsal telencephalon (Dl), the putative homologue of the mammalian hippocampus [16,36–39]. However, while associative learning about threat could increase activity in the Dl, it remains possible that the Dl is not recruited for the cue-based threat learning examined here [16,27].

The effect of learning from two different social cues on pS6 expression in the brain remains unclear, as analyses in mammals have found regions that are either shared or unique for different forms of learning (reviewed in [16]). Nevertheless, by analysing learning of two distinct and ethologically relevant social cues, we gain new perspective on the neural systems that may mediate social learning in nature and raise questions about the specificity and origins of such mechanisms.

2. Material and methods

Subjects were 145 adult female guppies from a laboratory-bred population of mixed wild Trinidadian origin that had been bred in captivity for at least four generations. Male and female guppies differ in anti-predator behaviour, learning performance and response to conspecifics [22,40], and we focused on females because of their greater responsiveness to predators [22,41].

(a) Behaviour testing and scoring

Nine litre experimental tanks (30 (l) × 15 (w) × 20 (h) cm; water depth 12 cm) were marked with lines to divide them into three vertical sections of 0–4 cm, 4–8 cm and 8–12 cm from the bottom of the tank, respectively. Using BORIS coding software [42], an experimenter positioned behind a visual barrier scored the amount of time that guppies spent in the bottom third of the water column, foraging at the substrate and freezing. Foraging was defined as active pecking at gravel and ended when the subject was no longer oriented towards the gravel and had not pecked for 2 s [43]. When presented with alarm cues, many fishes reliably engage in defensive behaviours, including avoiding areas where alarm cue is released, decreasing activity, and lowering their position in the water column [23,44]. This latter response, time spent near the substrate, has been repeatedly used to quantify defensive responses towards alarm cues and novel stimuli [28,45–48]. We predicted that fish would increase substrate use upon alarm cue presentation both as a typical anti-predator response and to move away from the alarm cue, which was delivered at the surface of the water during Experiment 1 training trials. We predicted that fish simultaneously exposed to a novel light cue and either alarm cues (Experiment 1) or alarmed conspecifics (Experiment 2) would respond defensively when tested with the light cue alone. We calculated our main variable of interest, the proportion of an observation period spent in the bottom third of the tank without foraging (henceforth 'substrate use') by subtracting the time (in seconds) spent foraging from the time spent in the bottom third of the water column and dividing this value by the total observation time.

Freezing (defined as the subject resting immobile for 1 s or longer) has been found to increase in response to threatening stimuli [48]. However, freezing (and erratic movements or 'dashing') was infrequently displayed in our study during key experimental trials and, consequently, not analysed.

(b) Experiment 1a: learned aversion through alarm cue exposure

Subjects were isolated and habituated for 24 h in experimental tanks, then randomly and evenly assigned to one of three training stimulus combinations (detailed below). An LED light array that flashed red, green and blue in a consistent order at 500 ms intervals was used as a conditioning stimulus and was fixed to a ring-stand positioned 3 cm above the centre of the experimental tank. A flashing rather than a static light was used to increase the salience of the light cue and to avoid a single-coloured light. Subjects in all groups underwent two training trials (detailed below) separated by six hours, followed by one testing trial to determine learning 24 h after the first training trial. Training and test trials each consisted of a 2 min pre-stimulus period for baseline behavioural observations, followed by a 2 min post-stimulus period (figure 1*a*).

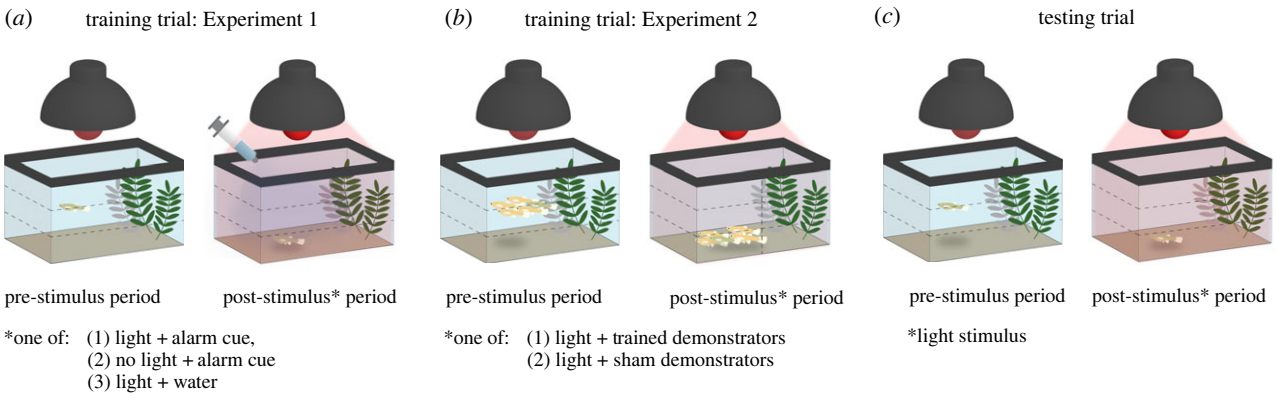

Figure 1. Overview of behavioural paradigms. (*a*) Training trial for Experiment 1. Fish were trained with one of three stimulus combinations: light + alarm cue (pictured), no light + alarm cue or light + water. Following a 2 min pre-stimulus period, fish (excluding the no light + alarm cue group) were presented with the light stimulus. Alarm cue (or water control) was presented 45 s after the onset of the light stimulus, which stayed on for a total of 2 min. (*b*) Training trial for Experiment 2. Fish were exposed to one of two demonstrator shoal types: 'trained' demonstrators or 'sham' demonstrators (4 demonstrators per tank). Following a two-min pre-stimulus period, fish were presented with the light stimulus for a total of two min. (*c*) Testing trial for both experiments. Following a two-min pre-stimulus period, isolated fish were presented with the light stimulus for two min. The proportion of time spent in the bottom third of the tank (substrate use) was recorded as a measure of defensive behaviour. (Online version in colour.)

There were three experimental treatments: (1) in the light + alarm cue group ($n = 17$), following the 2 min pre-stimulus period, fish were exposed to the novel light stimulus for two min. Forty-five seconds after the light was turned on, 6 ml of freshly prepared alarm cue [49] (preparation methods detailed in supplementary material) was administered to the water surface with a syringe. (2) In the no light + alarm cue group ($n = 18$), following the 2 min pre-stimulus period, fish were presented with 6 ml of alarm cue 45 sec into the 2 min post-stimulus period. This treatment controls for broad effects of recent alarm cue exposure, which could for example sensitize fish to any stimulus. (3) In the light + water group ($n = 16$), following the 2 min pre-stimulus period, fish were presented with the novel light stimulus for 2 min. Forty-five seconds after the light was turned on, 6 ml of tank water was administered to the water surface with a syringe. This treatment controls for the broad effects of light exposure and was used to confirm the neutral valence of the light cue. Fish in each group underwent this procedure two times, each separated by 6 h. In addition to a sponge filter running throughout, two-thirds of water in all tanks was changed approximately 30 min after each trial to facilitate the dilution of any remaining alarm cues and to encourage fish to return to pre-stimulus (baseline) behaviours. Twenty-four hours after their first training trial, all subjects underwent the same protocol for testing (test trial) in which they were exposed to the light stimulus alone for two min following a 2 min pre-stimulus period (figure 1*c*).

(c) Experiment 1b: neural correlates of learning from alarm cue

To reveal the neural correlates of the acquisition of threat learning from alarm cues, we repeated the training procedure of Experiment 1a with new subjects and collected brain tissue 30 min after the start of the second training trial (significant learning was observed after two trials in Experiment 1a). We observed similar patterns of behaviour to Experiment 1a during the last training trial of Experiment 1b (electronic supplementary material). In addition to the stimulus treatments matching Experiment 1a (light + alarm cue: $n = 10$; no light + alarm cue: $n = 10$; light + water: $n = 10$), we added an additional no cue control group ($n = 8$) in which fish were subject to experimental tank conditions but not exposed to any additional stimuli.

(d) Experiment 2a: learned aversion through exposure to alarmed demonstrators

Adult female guppy 'demonstrators' were either trained to respond defensively to the flashing light stimulus ('trained', 16 fish) or went through a sham training procedure for a control condition ('sham', 16 fish). 'Trained demonstrators' acquired anti-predatory responses to a flashing light using an alarm-cue training protocol similar to the light + alarm cue group from Experiment 1a, and 'sham demonstrators' were trained in a manner similar to the light + water group in Experiment 1a. Following training, trained and sham demonstrators were housed in separate 19 l tanks and re-trained periodically to prevent extinction or social buffering from repeated exposure to naive subjects.

There were two experimental treatments: subjects were randomly assigned to experimental tanks with a group of (1) four trained demonstrators ($n = 12$) or (2) four sham demonstrators ($n = 14$). During the training trials, fish were exposed to the flashing light stimulus two minutes after a pre-stimulus period. A preliminary experiment indicated at least three pairings of light stimulus and demonstrator behaviour were necessary to observe noticeable increases in substrate use at test (electronic supplementary material). Consequently, subjects received three training trials with the light stimulus while freely interacting with demonstrators, each separated by a 3 h interval. After the three training trials, demonstrators were removed, and 24 h after the first training trial subjects were tested with the light stimulus alone to determine learning (figure 1*b*,*c*). Subjects were not exposed to alarm cue during training. All other methodology was identical to Experiment 1a.

(e) Experiment 2b: neural correlates of learning from exposure to alarmed demonstrators

To analyse neural activity during learning from demonstrators, we repeated the training procedure of Experiment 2a with new subjects and collected brain tissue 30 min after the start of the third training trial (significant learning was observed after three trials in Experiment 2a). We observed similar patterns of behaviour to Experiment 2a during the last training trial of Experiment 2b (electronic supplementary material). In addition to the stimulus treatments matching Experiment 2a (trained demonstrators: $n = 11$; sham demonstrators: $n = 10$), we added an additional group where subjects were paired with four

demonstrator fish in experimental conditions but were not shown the light stimulus ('uncued shoal': $n = 9$).

(f) Brain collection and imaging

Brains of fish from Experiments 1b and 2b were sectioned at 20 µm using a cryostat and stored at −80°C. Sections were then stained with rabbit polyclonal anti-pS6 antibody (1 : 500; Cell Signalling Technologies #5364, Danvers, MA, USA) and counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) to aid with the identification of brain areas. This specific antibody has been used in previous studies in zebrafish [50] and birds [51,52]. We examined five forebrain areas implicated in social behaviour and aversion learning (see above): the lateral zone of the dorsal telencephalon (Dl), the medial zone of the dorsal telencephalon (Dm), the supracommissural zone of the ventral telencephalon (Vs), the ventral zone of the ventral telencephalon (Vv) and the POA. Single channel pS6 images with ROI overlays were manually counted using the count tool in Adobe Photoshop 2017 (Adobe, Peachpit, CA, USA). Of 38 brains processed for Experiment 1b, we quantified pS6 expression (number of pS6-expressing neurons per 1000 µm^2) in the Dl, Dm, Vs, Vv and POA, respectively, in 37, 31, 28, 30 and 30 individuals, with 5.54 ± 0.58, 2.71 ± 0.06, 2.10 ± 0.09, 2.60 ± 0.07 and 2.63 ± 0.07 (mean ± s.e.m.) sections quantified per area for each individual (omissions were due to sporadic tissue damage). Of 30 brains processed for Experiment 2b, we quantified pS6 expression in the Dl, Dm, Vs, Vv and POA, respectively, in 28, 25, 20, 24 and 25 individuals, with 5.59 ± 0.61, 2.52 ± 0.08, 2.10 ± 0.09, 2.33 ± 0.09 and 2.80 ± 0.07 (mean ± s.e.m.) sections quantified per area for each individual. Further details of methods are in the electronic supplementary material.

(g) Statistical analysis

Statistical analyses were conducted in R v. 3.5.2 [53]. Behavioural data from Experiments 1a and 2a were separately fitted to two-way factorial models to investigate how substrate use varied as a factor of treatment (experimental group) and period (pre- and post-stimulus periods). Fish ID was included as a random variable. We ran planned contrasts across treatment for each period and between pre-and post-stimulus data within each treatment group with false discovery rate (FDR) adjustments. pS6 density data from Experiments 1b and 2b were square-root-transformed, then fitted to two-way factorial linear mixed effects models (using 'lme4' [54]) with the fixed effects of treatment and region (Dl, Dm, Vs, Vv and POA) and random effects of fish ID and batch. Within each region, we compared densities across treatment groups with FDR adjustments. Type II Wald χ^2-tests using the 'car' library [55] were conducted to assess significance within each model, and *post hoc* contrasts were run using 'emmeans' [56]. Effect sizes were calculated using Hedges' g and adjusted for the small bias observed with relatively small sample sizes ($n < 50$) by multiplying g by a correction factor $(1 - (3/(4 * (n_1 + n_2) - 9))$ where n_1 and n_2 refer to sample sizes in each of the contrasted groups) [57]. Group least-squared means from 'emmeans' and variance estimates from 'lme4' (i.e. with random effects accounted for) were used in the calculation of g.

3. Results

(a) Experiment 1a: learned aversion through alarm cue exposure (behaviour during test trial)

Training stimuli affected substrate use during the test trial (figure 2*a*; LMM, treatment × period interaction, $\chi^2_2 = 16.38$, $p = 0.0003$). While substrate use did not significantly differ across treatments during the pre-stimulus period ($p > 0.1$, $g = 0.06–0.28$ for all comparisons), substrate use during the post-stimulus period (when the light cue was on) was significantly greater for fish trained with light + alarm cue than for fish trained with no light + alarm cue ($t_{81.8} = 2.82$, $p = 0.0273$, $g = 0.42$) or fish trained with light + water ($t_{81.8} = 2.34$, $p = 0.0495$, $g = 0.35$). Only fish previously exposed to the light + alarm cue pairing significantly increased substrate use from the pre-stimulus to the post-stimulus period ($t_{48.0} = 2.39$, $p = 0.0495$, $g = 0.27$). Interestingly, fish trained with the light + water cue significantly decreased substrate use from the pre-stimulus to the post-stimulus period ($t_{48} = 3.06$, $p = 0.0273$, $g = 0.36$).

(b) Experiment 1b: neural correlates of learning from alarm cue

To reveal the neural correlates of the acquisition of threat responses, brains were collected from fish 30 min after the last training trial (see 'Material and methods') and processed for pS6 expression. There was a significant treatment × region interaction (LMM; $\chi^2_{12} = 109.28$, $p < 0.0001$) and a main effect of region ($\chi^2_4 = 1090.90$, $p < 0.0001$) on pS6 expression. Given the significant interaction, we examined group differences within each brain area individually. pS6 expression in the Vv was significantly higher for fish in the light + alarm cue group than for fish in all other groups (figure 2*b*,*c*; versus no cue: $p = 0.0344$, $g = 0.55$; versus light + water: $p = 0.0344$, $g = 0.52$; versus no light + alarm cue: $p = 0.0090$, $g = 0.70$). Similarly, pS6 expression in the POA was significantly higher for fish in the light + alarm cue group than for fish in all control groups (versus no cue: $p = 0.0484$, $g = 0.49$; versus light + water: $p = 0.0043$, $g = 0.71$; versus no light + alarm cue: $p = 0.0001$, $g = 1.02$). pS6 expression in the POA was also significantly higher in no cue control fish than in the no light + alarm cue controls ($p = 0.0377$, $g = 0.53$). No significant differences in pS6 expression between treatments were observed in the Dl, Dm or Vs. The behaviour of fish during training in Experiment 1a and Experiment 1b was indistinguishable (electronic supplementary material, figure S3*a*,*b*).

(c) Experiment 2a: learned aversion through exposure to alarmed demonstrators (behaviour during test trial)

Training stimuli affected substrate use during the test trial (LMM, treatment × period interaction: $\chi^2(1) = 7.17$, $p = 0.0074$). While substrate use did not significantly differ across treatments during the pre-stimulus period (figure 3*a*; $t_{32.1} = 0.41$, $p = 0.8399$, $g = 0.06$), fish that were previously exposed to trained demonstrators spent a greater proportion of the post-stimulus period (when the light was on) near the substrate than fish that were previously exposed to sham demonstrators ($t_{32.1} = 2.47$, $p = 0.0381$, $g = 0.39$). Relatedly, only fish previously exposed to trained demonstrators significantly increased substrate use from the pre-stimulus period to the post-stimulus period ($t_{24} = 4.16$, $p = 0.0014$, $g = 0.34$). These results following three training trials (see 'Material and methods') contrast with a preliminary experiment with only two training trials, which did not provide evidence for a learned aversion at test (see electronic supplementary material). A comparison of behaviour across

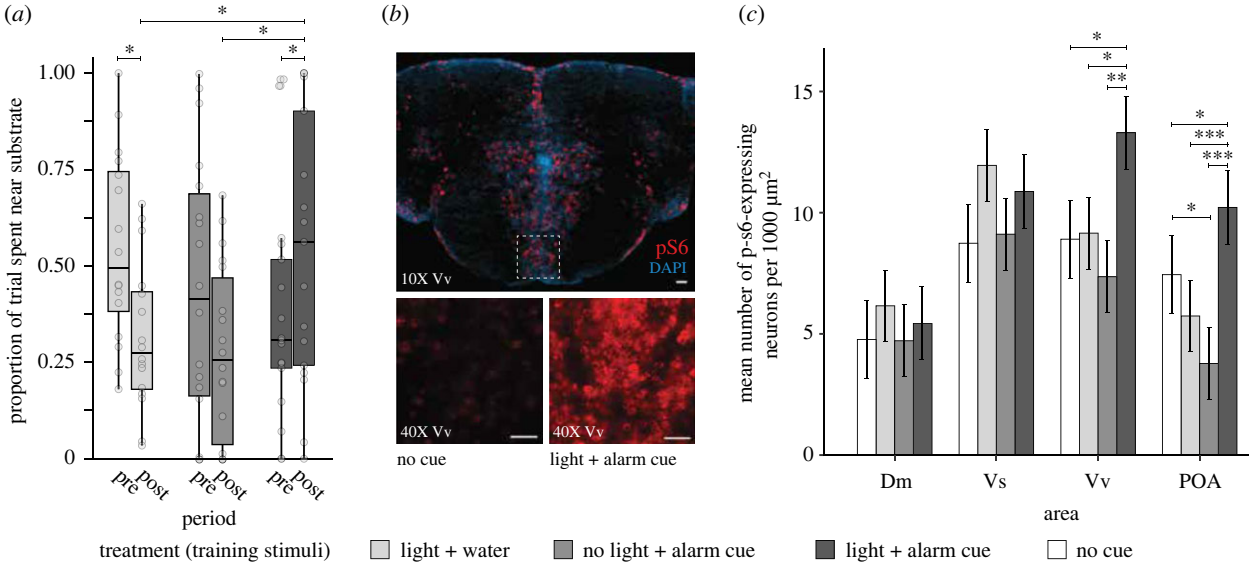

Figure 2. Fish exposed to paired light and alarm cue stimuli show increased defensive behaviour at test compared to controls (*a*), and differential neural activity during learning acquisition (*b*,*c*). (*a*) Experiment 1a: fish trained with a paired light + alarm cue stimulus increased substrate use when presented with the light stimulus alone during testing. Fish trained with a light + water stimulus decreased substrate use under testing conditions, and post-stimulus substrate use was similar between the two control conditions. Box plots show group medians with whiskers indicating upper and lower quartiles, points represent individuals' data. (*b*) Experiment 1b: photomicrograph of DAPI staining (blue; top) and pS6 immunoreactivity (red) in area Vv. Top panel depicts 10× coronal image, with dashed box representing the approximate area examined for pS6 quantification. Bottom panels are representative images of pS6 expression in fish exposed to no cue versus light + alarm cue. Scale bars are 20 µm. (*c*) Experiment 1b: relative to light + water, alarm cue and no-cue controls, fish exposed to light + alarm cue pairings showed a significantly greater density of pS6-expressing neurons in areas Vv and POA. In the POA, no cue control fish showed significantly greater pS6 expression than no light + alarm cue control fish. 'No cue' treatment applies only to Experiment 1b (*c*; see 'Material and methods'). Bar plot values represent square root transformed least square means (±s.e.m.). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. (Online version in colour.)

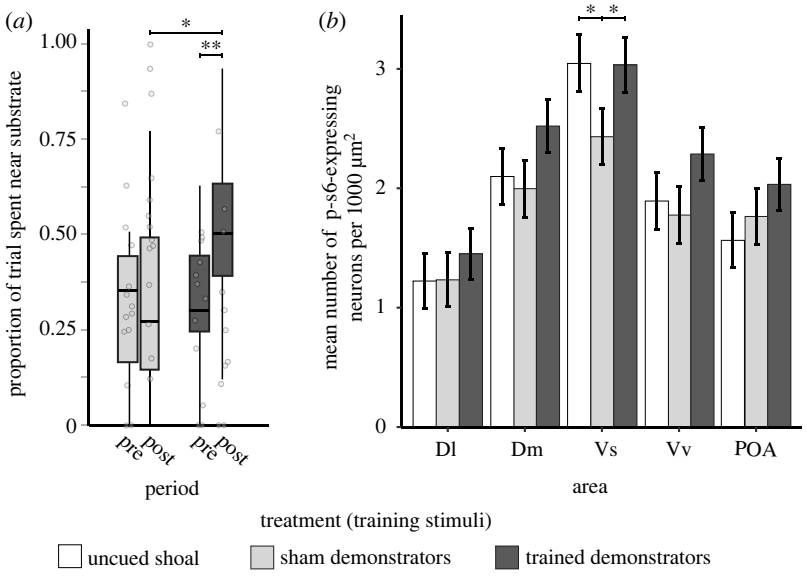

Figure 3. Fish exposed to trained demonstrators show increased defensive behaviour during testing compared to controls (*a*), but no significant differences in pS6 expression compared to both control groups during learning acquisition (*b*). (*a*) Experiment 2a: fish from both demonstration groups showed similar pre-stimulus substrate use during testing. Only fish previously exposed to trained demonstrators were observed to increase substrate use following light cue presentation during testing. Box plots show group medians with whiskers indicating upper and lower quartiles, points represent individuals' data. (*b*) Experiment 2b: relative to fish exposed to sham demonstrators, fish exposed to uncued shoals or trained demonstrators showed significantly greater density of pS6-expressing neurons in area Vs. 'Uncued Shoal' treatment applies only to Experiment 2b (*b*; see 'Material and methods'). Bar plot values represent square-root-transformed least-square means (±s.e.m.). **$p < 0.01$; *$p < 0.05$.

Experiments 1a and 2a showed that fish trained with light + alarm cue and fish trained with trained demonstrators spent a similar amount of time near substrate when tested with the light stimulus (electronic supplementary material, figure S4; $p = 0.904$, $g = 0.09$).

(d) Experiment 2b: neural correlates of learning from exposure to alarmed demonstrators

Similar to Experiment 1b, brains were collected and processed for pS6 expression from fish 30 min after the last training trial (see

'Material and methods') to reveal the neural correlates of threat acquisition. There was a significant region × treatment interaction ($\chi^2_8 = 32.83$, $p < 0.0001$) and a significant effect of region ($\chi^2_4 = 746.10$, $p < 0.0001$) on pS6 expression. Brain areas were analysed individually given the significant interaction (figure 3*b*). In area Vs, the density of pS6 expression was significantly higher in fish exposed to trained demonstrators and uncued shoals compared to fish exposed to sham demonstrators ($p = 0.0260$ for both, $g = 0.59$, 0.58, respectively). pS6 expression was not significantly different across groups for other brain areas. The behaviour of fish during training in Experiment 2a and Experiment 2b was statistically indistinguishable (electronic supplementary material, figure S3*c*,*d*).

4. Discussion

We investigated how guppies learn about a novel threat using two different sources of social information and how activity in focal brain areas was affected during learning from these different social cues (i.e. we examined the neural correlates of threat acquisition). We demonstrated that guppies learn to respond defensively to a novel light stimulus following paired exposures of the light with either conspecific chemical alarm cues or conspecific demonstration of anti-predator behaviour. That is, after paired exposures, they displayed anti-predatory behaviour in response to the light in the absence of either conspecific cue. Thus, guppies use both types of conspecific cues in learning how to respond to novel stimuli. Further, the arbitrary nature of the light stimulus we used could suggest that the social learning we observed is relatively unconstrained, in contrast with work finding enhanced social learning of evolutionary ancient threats over novel ones [6,7]. Notably, we also observed that activation of neural circuitry during learning varied depending on the social cue presented, suggesting that neural circuits contribute to social learning in a stimulus-specific manner.

Our marker of neural activity, pS6 expression, increased in two forebrain areas, the Vv and the POA, in fish learning from alarm cues. In contrast to a study in zebrafish [18], but supporting the conclusion that these areas are involved in threat learning specifically, exposure to alarm cue alone had minimal effects on pS6 expression in the brain areas we examined. The Vv is proposed to be the teleost homologue of the mammalian LS and bed nucleus of the stria terminalis. The activation of the Vv during threat learning in guppies is consistent with existing studies in mammals demonstrating that the LS is active during aversive situations, including during aversion learning (e.g. a foot shock or alarm cue exposue [58]), and is important for selecting stimuli that are predictive of an aversive event [15,35]. The POA has been similarly implicated in the encoding of social stimuli, including predators or aggressive conspecifics [15,35]. As such, our results suggest some functional analogies in the processing and use of social information across disparate taxa.

While we propose that pS6 expression in the Vv and POA reflects a contribution of these areas to learning in response to alarm cue exposure, the increased pS6 expression could represent multisensory integration (and not learning), since guppies in the experimental group are simultaneously exposed to light and alarm cues during training. Indeed, some neuronal populations in the rodent LS and POA are known to integrate multisensory information [59]. While one could eliminate the predictive nature of the light cue by switching the order of stimulus presentation (i.e. light cue after the alarm cue [60]), work in birds has found significant learning about novel threats under these conditions [61]. It is important to highlight, however, that multisensory integration is a central part of associative learning, reflecting a difficulty of dissociating learning from multisensory processing in this context. It is also possible that differences in pS6 expression are consequences of differences in behaviour that emerge as a function of learning (e.g. differences in substrate use). That said, the areas we examined are not motor areas, and an advantage of our focus on acquisition is that we exposed a control group to alarm cue and observed similar substrate use (electronic supplementary material, figure S3) but different pS6 expression in the Vv and POA. Nevertheless, future experiments involving manipulations of neural activity could help reveal the contribution of the Vv (LS) and POA to the social learning of threat.

Alarm cue exposure and exposure to demonstrator defensive behaviour led to similar degrees of threat learning (electronic supplementary material, figure S4), but learning-associated changes in neural activity were not consistently observed in fish exposed to experienced demonstrators. This suggests that demonstration-based learning about threats could be mediated by comparatively subtle changes in neural activity in these circuits. Consistent with this interpretation is that more trials were required to observe learning from demonstrators than from alarm cue; this suggests that cue salience and/or learning may be 'weaker' during threat learning from demonstrators, perhaps because demonstrator behaviour is a more ambiguous indicator of risk than alarm cue [19]. This slower learning in response to demonstrators could also stem from the fact that subjects had likely been exposed to some conspecific defensive behaviour but not alarm cue prior to the experiment. Learning from demonstrators was nonetheless rapid, observed after only three stimulus-demonstration pairings. Finally, the two learning paradigms also differed in the sensory modality of the threat cue and, thus, our data suggest that brain areas for demonstrator-based learning could be distinct from alarm cue-based learning.

Neither social learning from alarm cue nor from demonstrators led to significant changes to pS6 expression in the lateral zone of the dorsal telencephalon (Dl; proposed homologue of the mammalian hippocampus). In this respect, our data suggest a minimal role of the Dl in cued fear conditioning in fish, despite playing an important role in the learning and memory of spatial tasks and other forms of learning [36–39,62]. Interestingly, research in mammals suggests that the hippocampus plays a role in contextual fear learning but not cued fear learning [16]; given that both of our paradigms represent cued fear learning, the lack of significant variation in Dl in our experiments is consistent with studies in mammals.

In the experiment investigating the neural correlates of social learning from demonstrators, significant differences in pS6 expression were observed in the Vs: fish that were exposed to trained demonstrators had higher expression than fish exposed to sham demonstrators. However, fish exposed to the uncued shoal also had elevated pS6 expression in the Vs, complicating the interpretation of this finding. The reason for lower pS6 expression in the Vs for fish exposed to sham demonstrators remains unclear, though no behavioural differences were detected between fish exposed to an uncued shoal or sham demonstrators.

Given the importance of understanding the role of experience and evolution in shaping learning from conspecific cues, these findings have the broader implication that learning from different forms of conspecific stimuli could have similar functional consequences but may have distinct mechanistic substrates, as well as distinct developmental and/or evolutionary origins. Overall, we show that guppies can learn about novel threats from both chemical alarm cues and conspecific demonstration, but that there appears to be some distinction between the neural correlates underlying learning from these different social cues. Furthermore, our results emphasize the importance of examinations of neural mechanisms in resolving debates on the origins of social learning.

Ethics. All procedures followed McGill University Animal Care and Use Committee protocols (protocol no. 7133/7708), as well as the guidelines from the Canadian Council on Animal Care, and the Animal Behavior Society/Association for the Study of Animal Behaviour (ABS/ASAB). The experiment employed stressful stimuli, either exposure to alarm cue or to alarmed conspecifics. We employed these ecologically relevant stimuli to promote the rapid learning needed for behavioural and neural analyses.

Data accessibility. Data and code used for this analysis can be accessed on Dryad Digital Repository [63].

Electronic supplementary material is available online [64].

Authors' contributions. R.F.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; S.M.R.: conceptualization, funding acquisition, investigation, methodology, supervision, writing—review and editing; J.T.S.: conceptualization, funding acquisition, investigation, methodology, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors declare no competing interests.

Funding. This work was supported by Natural Sciences and Engineering Research Council (NSERC) of Canada grants to J.T.S. (05016-2016) and S.M.R. (2017-04720), a Fonds de recherche du Québec–Nature et technologies (FRQNT) grant to J.T.S. and S.M.R. (PR-284884), and a Canada Foundation for Innovation grant to S.M.R. (29433). R.F. was supported by an NSERC Master's Canadian Graduate Scholarship (CGS-M), an FRQNT bourse de maîtrise (B1X) and a Biodiversity, Ecology and Ecosystem Services Fellowship (through NSERC-CREATE).

Acknowledgements. We thank Mélanie Guigueno, María Cabrera-Álvarez, Argel Aguilar-Valles, Oliver Hardt, Michael Ryan, William Swaney and Karen Maruska for their contributions to developing protocols and/or comments on experimental design and analyses, Adrian Foster for assistance in administering behavioural experiments, Zhuoling Li for assistance in image analysis, and Karim Nader for access to tissue processing equipment.

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