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The location of damage-released alarm cues in a cichlid fish

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ABSTRACT

Background: Damage-released alarm cues are reliable indicators of predation risk for prey fishes. Based on observations early in the twentieth century, alarm cues were for a long time assumed to be produced and located exclusively within skin-based club or sacciform cells. However, recent studies have suggested that these cells may be unrelated to the alarm function and that fish respond similarly to blood cues, mucus or to muscle tissue extracts as they do to skin extracts. Continuing to assume that alarm cues are primarily located within the skin and using skin extracts in contemporary alarm cue research may thus be problematic.

Hypothesis: In fish, damage-released alarm cues are not located exclusively within the skin but are also present elsewhere in the body.

Organism: *Pelvicachromis taeniatus*, a Western African river cichlid.

Site of experiments: Nine-litre ($30 \times 20 \times 20$ cm) experimental tanks in the wet lab of the Institute for Evolutionary Biology and Ecology at the University of Bonn, Germany.

Methods: Since reduced activity is a common adaptation to reduce predation risk in prey animals, we measured the change in activity of individual fish in response to extracts obtained from skin, muscle tissue, blood, mucus, liver tissue, intestine tissue, and spleen tissue, as well as to a distilled water control. First, we measured individual responses when extracts were diluted proportional to their contribution to a whole-body extract concentration that is known to induce a change in activity. Then, in a second experiment, we exposed fish to the same extracts but now diluted to have the same concentration for each tissue. Furthermore, we histologically analysed the *P. taeniatus* epidermis.

Results and conclusion: Only muscle tissue extracts induced a significant reduction in activity relative to the water control in both experiments. Although *P. taeniatus* possesses sacciform cells in its epidermis, the effects of skin extracts and other tissue extracts on activity remain ambiguous in this species. Consequently, our research provides support for other studies indicating that alarm cues are not exclusively located in skin tissue and suggests a more careful approach to future alarm cue research such as applying whole-body extracts instead of skin extracts until the identity of alarm cues has been elucidated.

Keywords: alarm cues, club cells, *Pelvicachromis taeniatus*, predation risk, predator, skin extract.

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INTRODUCTION

As predation is an important but often variable selective force (Lima and Dill, 1990; Lima, 1998; Sih *et al.*, 2000), prey animals often assess the level of current predation risk through recognition of cues emitted by conspecifics (Smith, 1986). In aquatic ecosystems, prey-borne damage-released chemical alarm cues (hereafter referred to as 'alarm cues'), which are innately recognized by conspecifics, are used as an indicator of the presence of a predator (Chivers and Smith, 1994b, 1998; Chivers *et al.*, 2012; Hettyey *et al.*, 2015). This is because chemical cues readily dissolve and disperse in water (Wisenden, 2000), which allows reliable predator detection over long distances, and are less risky than visual signals (Dodson *et al.*, 1994; Brown, 2003; Ferrari *et al.*, 2010). Alarm cues have been identified across a wide range of aquatic taxa, including insect larvae (Sih, 1986; Wisenden *et al.*, 1997), crustaceans (Laforsch *et al.*, 2006), asteroids (Parker and Shulman, 1986; Lawrence, 1991), gastropods (Kempendorff, 1942; Sleeper *et al.*, 1980; Dalesman *et al.*, 2007), amphibians (Hews and Blaustein, 1985; Kats *et al.*, 1988; Schoeppner and Relyea, 2005, 2009), and fishes (Wisenden, 2000).

The evolution and existence of alarm cues in fishes has puzzled evolutionary ecologists for decades (Chivers *et al.*, 2007). First, their evolution remains unresolved. While cue perception is beneficial for the receiver, as the receiver can exhibit anti-predator strategies upon detection, alarm cues are suggested to be costly to produce for the sender (Wisenden and Smith, 1997; Mathis, 2009). This has led to the search for how alarm cues could have evolved. Senders might yield direct fitness benefits. For example, the 'predator-attraction hypothesis' postulates that alarm substances attract additional predators and the interference among predators allows the sender to escape (Mathis *et al.*, 1995). In contrast, the 'kin-selection hypothesis' predicts indirect fitness benefits for the sender by warning kin (Smith, 1992; Meuthen *et al.*, 2014). And the 'by-product hypothesis' assumes the main function of alarm cues is immune-related, which makes the alarm function a by-product (Chivers *et al.*, 2007). Second, the chemical composition of alarm cues remains vague. Potential substances include pteridines such as ichthyopterin (Hüttel, 1941; Hüttel and Sprengling, 1943; Tschesche and Korte, 1951; Kauffmann, 1959; Pfeiffer and Lemke, 1973; Pfeiffer, 1978; Win, 2000), purines (Hüttel, 1941; Brown *et al.*, 2000), hypoxanthine-3-*N*-oxide (Pfeiffer *et al.*, 1985; Brown *et al.*, 2001b, 2003), and chondroitin (Mathuru *et al.*, 2012). Therefore, alarm cues are now considered to be a species-specific mixture containing a range of components, which include the previously suggested substances (Døving *et al.*, 2005; Mathuru *et al.*, 2012; Faulkner *et al.*, 2017).

The above studies on fish alarm cues, however, have a common premise: that the putative alarm cue is assumed to be exclusively located within the skin. This is because during the initial discovery of alarm cues, they were suggested to be skin-based in subjective, observational studies (von Frisch, 1938, 1941). Fish appeared to become frightened only after exposure to conspecific skin extracts but not in response to extracts from liver, spleen, gonads or the heart, while muscle and intestine tissue extracts caused intermediate responses (von Frisch, 1938). Subsequently, the avoidance response to conspecific skin extracts was correlated with the presence of so-called 'club cells' within histological sections of the skin (Pfeiffer, 1963, 1977). This relationship between club cells and alarm cues was then further confirmed by circumstantial evidence. First, the fluorescence emission spectra of the putative alarm cue [at this time a pterin-like ichthyopterin (see Pfeiffer and Lemke, 1973)] and of club cells appeared to be similar (Reutter and Pfeiffer, 1973). Second, in the fathead minnow *Pimephales promelas*, a testosterone-induced seasonal loss of club cells was correlated with the loss of the fright-inducing properties of skin extracts (Smith, 1976). In non-Ostariophysan fishes, skin-based sacciform cells were suggested to be analogous to club cells and to contain

the putative alarm cue (Whitear, 1986; Bryer *et al.*, 2001; Mathis, 2009). A single quantitative study further suggested that fish decrease their activity in response to skin extracts but not to muscle tissue extracts (Smith, 1979), thereby confirming the exclusive presence of alarm cues within the skin. Based on these findings, contemporary studies have employed club and sacciform cell counts as a means of quantifying cues in studies of the evolution of alarm cues (e.g. Chivers *et al.*, 2007). Moreover, attempts to elucidate the composition of alarm cues have often focused on analysing the contents of skin extracts (Hüttel, 1941; Hüttel and Sprengling, 1943; Pfeiffer and Lemke, 1973; Døving *et al.*, 2005) and frequently comparing the behavioural response of animals to other chemicals with those that they exhibit in response to conspecific skin extracts (Pfeiffer *et al.*, 1985; Brown *et al.*, 2000, 2003; Mathuru *et al.*, 2012; Faulkner *et al.*, 2017). For the same reason, recent studies on the information value of alarm cues (Wisenden *et al.*, 2009; Manek *et al.*, 2012; Brown *et al.*, 2015; Crane and Ferrari, 2015; Elvidge and Brown, 2015) and the simulation of predator presence so as to induce anti-predator phenotypic plasticity (Stabell and Lwin, 1997; Pollock *et al.*, 2005; Abate *et al.*, 2010) have predominantly used conspecific skin extracts. Still, further studies suggest that the behavioural responses of fish to alarm cues are unrelated to the presence (Hugie and Smith, 1987; Carreau-Green *et al.*, 2008; Wisenden *et al.*, 2008; de Souza-Bastos *et al.*, 2014; Lienart *et al.*, 2016; Mathuru, 2016) or to the quantity of club or sacciform cells (Barreto *et al.*, 2012; Pollock *et al.*, 2012; Manek *et al.*, 2013). At the same time, other taxa such as crustaceans (Wisenden *et al.*, 2009), molluscs (Trussell and Nicklin, 2002), and amphibians (Relyea and Werner 2000; Hettyey *et al.*, 2015) lack club cells but whole-body extracts still elicit behavioural anti-predator responses. This questions the relationship between these cells and the alarm function. Moreover, other studies have suggested that tissues other than skin also contain an alarm cue that triggers anti-predator responses. For example, in the sea lamprey *Petromyzon marinus*, not only extracts from skin tissue but also those from muscle tissue successfully induced an avoidance response (Bals and Wagner, 2012). In the zebrafish *Danio rerio*, mucus was sufficient to elicit the full extent of the alarm response (Mathuru *et al.*, 2012). In the Nile tilapia *Oreochromis mossambicus*, blood cues extracted without damaging sacciform cells were suggested to elicit an anti-predator response similar to that of skin extracts (Barreto *et al.*, 2013). Furthermore, in the lake sturgeon *Acipenser fulvescens*, skin extracts were shown to elicit an anti-predator response but no associative learning of simultaneously presented predator odours, which is a major property of alarm cues (Chivers and Smith, 1994a, 1994b; Magurran, 1989; Brown *et al.*, 2001a; Manassa *et al.*, 2013; Mitchell *et al.*, 2011, 2013); this association was observed only when fish were subjected to whole-body extracts from conspecifics (Sloychuk *et al.*, 2016).

Under these circumstances, assuming that alarm cues are exclusively located within the skin – or within skin-based club or sacciform cells – may complicate studies on the evolution of alarm cues and also makes it more difficult to determine the chemical composition of alarm cues. Moreover, when skin extracts alone are used to simulate predator presence, the evolutionary consequences of predator presence as well as those of anti-predator phenotypic plasticity in nature may be severely underestimated. Such ambiguity thus requires a more comprehensive study that quantitatively compares the response of fish to extracts derived from different tissues of the same donor fish.

The aim of the present study was to identify the putative location of alarm cues by conducting a histological analysis of the skin and by comparing the behavioural response of fishes to extracts derived from different tissues of conspecifics. We used the Western African river cichlid *Pelvicachromis taeniatus* as a model system. Cichlids in general (Brown *et al.*, 2006; Abate *et al.*, 2010; Barreto *et al.*, 2010) and *P. taeniatus* in particular (Meuthen *et al.*, 2014, 2016a, 2016b, 2018) are well-established as model systems for alarm cue research. First, we conducted

a histological analysis of the *P. taeniatus* epidermis to determine whether it contains sacciform cells. Then, we tested the behavioural response of individual *P. taeniatus* to extracts obtained from skin, muscle tissue, blood, mucus, liver tissue, intestine tissue, and spleen tissue, as well as to a distilled water control. Reduced activity under predation risk is a common behaviour in prey fishes to make them less conspicuous to predators (Lima and Dill, 1990; Kats and Dill, 1998; Lima, 1998; Bourdeau and Johansson, 2012). Therefore, as often done previously (Gelowitz *et al.*, 1993; Mirza *et al.*, 2001; Meuthen *et al.*, 2014), we focused on measuring changes in fish activity as a proxy for the successful perception of alarm cues. As the response of *P. taeniatus* to whole-body extracts has been established previously (Meuthen *et al.*, 2014, 2016a, 2016b, 2018), in our first behavioural experiment we measured the response of fish to different extracts from individual tissues that were diluted proportional to their contribution to the same whole-body extract concentration. Then, to reveal whether the effects of different tissues are concentration-specific, we conducted a second behavioural experiment in which fish were instead exposed to extracts that were diluted to the same concentration as the whole-body extract that has been shown to induce a behavioural response.

METHODS

Experimental fish

Pelvicachromis taeniatus is a small, sexually dimorphic and socially monogamous cichlid species that inhabits small streams and rivers in Western African countries including Benin, Nigeria, and Cameroon. Like other cichlids (Escobar-Camacho and Carleton, 2015), *P. taeniatus* has well-developed olfactory capabilities (Thünken *et al.*, 2009; Meuthen *et al.*, 2011; Hesse *et al.*, 2012) and is sensitive to alarm cues. Similar to other species (Bourdeau and Johansson, 2012), it displays responses that are adaptive in a predatory context when it detects damaged conspecifics (as communicated through whole-body extracts). These responses include reduced activity in females (Meuthen *et al.*, 2014), reduced aggression in males (Meuthen *et al.*, 2016a) and, in the long term, increased neophobia in juveniles (Meuthen *et al.*, 2016b), an altered morphology in males (Meuthen *et al.*, 2018), and an altered mate choice in both sexes (D. Meuthen, S.A. Baldauf, T.C.M. Bakker, and T. Thünken, unpublished data).

The *P. taeniatus* used for the experiments were F1, F2, and F3 progeny of wild-caught fish collected in June 2007 from the Moliwe river (04°04′N, 09°16′E) near Limbe, Cameroon. Prior to the experiments, fish were kept in indoor holding tanks (50 cm long × 30 cm wide × 30 cm high) as mixed-sex sibling groups of 5–15 individuals [12 hour light/12 hour dark cycle (light from 9 am to 9 pm); room temperature maintained between 26 and 27°C]. Each day, fish were fed *ad libitum* with a mixture of defrosted mosquito larvae of the genera *Chironomus*, *Culex*, and *Chaoborus* as well as *Artemia* sp. in a ratio of 2 : 1 : 0.25 : 1. We used both males and females (equal sex ratio) as donor fish, but only females as test fish because female within-individual activity is more consistent over time than that of males (Meuthen *et al.*, 2011). For this reason, previous experiments of the effect of alarm cues on fish activity in the same species also used exclusively females as test fish (Meuthen *et al.*, 2014). Histological analyses were conducted in October 2011, and the behavioural experiments between November 2013 and May 2016 (Experiment 1: November 2013 to January 2014; Experiment 2: April to May 2016).

Skin histology

To ascertain whether the skin of *P. taeniatus* contains sacciform cells, we conducted a histological analysis of its epidermis. For this purpose, we killed two members of our F1 lab-bred progeny (one male, total length 6.5 cm, standard length 4.8 cm, body mass 3.252 g; and one female, total length 5.1 cm, standard length 3.9 cm, body mass 1.653 g) with a blow to the head followed by cervical dislocation in accordance with §4, §8b, and §9(2) of the German animal welfare act (BGB l. I S. 1207, 1313). We then removed 1 × 1 cm sections of the epidermis, including the attached underlying muscle tissue. These sections were fixed in 10% formaldehyde, and then transferred to 100% ethanol via a gradually ascending alcohol series. Beginning at 30% ethanol, we raised the concentration in 10% steps to 90% ethanol and then 5% steps from 90% to 100% ethanol. Thereafter, sections were transferred to methyl benzoate, and then to butanol. They were then mounted in paraffin via an ascending butanol/paraffin series (3 : 1, 1 : 1, 1 : 3) and embedded in Paraplast (Leica Microsystems, Wetzlar, Germany). Subsequently, sections were sliced with a Leica RM Microtome to a thickness of 7 μm and stained with Heidenhain's Azan trichrome stain (aniline blue-orange G for 13 minutes, carmalaun for 20 minutes). The slices were then examined with light microscopy using 400× magnification with a Zeiss Forschungsmikroskop Universal including a Carl Zeiss Plan 40/0.65 160/0.17 objective (Carl Zeiss Microscopy, Jena, Germany) at 400× magnification so as to check for the presence of sacciform cells.

Behavioural responses to different tissue extracts

For the behavioural experiments, we exposed each individual fish to the following extracts in random order: (1) skin extract, (2) muscle tissue extract, (3) blood, (4) mucus, (5), liver tissue extract, (6) intestine tissue extract, and (7) spleen tissue extract, as well as (8) a distilled water control. First, we measured the response to tissue extracts that were diluted proportional to their contribution to the whole-body extract (Experiment 1). Then, we repeated the experiment but exposed fish to extracts that were diluted to the same concentration (Experiment 2).

Chemical cue preparation: Experiment 1

We took the different extracts for Experiment 1 from eight adult F1 and F2 lab-bred donor *P. taeniatus* (four males: standard length 6.20 ± 0.94 cm, body mass 6.84 ± 2.90 g; four females: standard length 4.25 ± 0.19 cm, body mass 2.25 ± 0.39 g; mean ± SD). Prior to tissue extraction, donor fish were isolated in holding tanks (50 cm long × 30 cm wide × 30 cm high) with internal filters for 3 weeks to allow for controlled feeding, thereby preventing confounding condition-related differences in alarm cue quality (e.g. Roh *et al.*, 2004). For this purpose, we fed isolated fish 6 days a week according to their body mass: up to 3 g wet weight (w/w) they received 120 μL, between 3 and 5 g w/w they received 240 μL, between 5 and 7 g w/w they received 360 μL, between 7 and 9 g w/w they received 480 μL, and fish heavier than 9 g received 600 μL food per day. Food consisted of frozen *Artemia* sp., red mosquito larvae (*Chaoborus* sp.), and black mosquito larvae (*Culex* sp.) in a ratio of 1 : 2 : 1. Two days before extract preparation, fish were starved to ensure that residual food did not contaminate the tissue samples. After 3 weeks, donors were removed from their tanks. We then measured the standard length (SL, from the tip of the snout to the end of the caudal peduncle) of every individual to an accuracy of ± 0.5 mm on graph paper. We later weighed

fish in a water-filled bowl to the nearest milligram on an electronic scale (ED-153-CW, Sartorius, Germany) and photographed fish in the lateral view with a size scale using a digital camera (D5000 with AF-S Micro Nikkor 105mm 1:28G macro objective, Nikon, Japan). Subsequently, we euthanized fish with a blow to the head followed by cervical dislocation in accordance with §4, §8b, and §9(2) of the German animal welfare act (BGB l. I S. 1207, 1313).

Fish were dissected and the different tissues collected from each fish. First, blood was collected by puncturing the heart from below the gill covers with a 10 µL syringe (Hamilton Microliter 701 ASN, USA). Then, by gently scraping a microscope slide along both flanks, we were able to collect mucus without damaging skin or scales. For the collection of livers, intestines, and spleens we made a small opening between the pelvic and anal fins with a scalpel. The organs were removed and carefully isolated from each other. Skin tissue was collected by making a vertical incision and then pulling the skin away with tweezers, a procedure known as 'skin filleting' (Brown *et al.*, 2003; Roh *et al.*, 2004; Foam *et al.*, 2005; method of G.E. Brown, personal communication). Muscle tissue was collected by cutting fillets along the flanks and removing any adhering skin. After collection, all tissues were weighed with an analytical scale (Explorer E11140, Ohaus, USA, error of ±0.1 mg) and skin tissue was photographed along with a size standard (D5000 with AF-S Micro Nikkor 105mm 1:28G macro objective, Nikon, Japan) so as to measure skin area. For blood, mucus, liver, spleen, and intestines, we tried to collect all available tissue of each individual fish; for simplicity, we thus assume that we removed 100% of available tissue in each fish. However, for skin and muscle tissue, we had to calculate the proportion of tissue removed. For skin, we measured the area of removed skin relative to the lateral projection area of the whole fish (excluding the fins) using ImageJ software (Rasband, 1997–2014, US National Institutes of Health, Bethesda, MD), which automatically converts digital dimensions to metric units according to size standards. For muscle tissue, we calculated the weight of the tissue that we removed relative to the weight of all other muscle tissue of an individual fish (which we also removed but did not use in our experiments).

Previous studies of *P. taeniatus* suggest that they respond with reduced activity to conspecific alarm cues in a concentration of 3.6 $mg \cdot L^{-1}$ (Meuthen *et al.*, 2014), and similar conspecific alarm cue concentrations also elicit other behavioural and morphological anti-predator responses in *P. taeniatus* (Meuthen *et al.*, 2014, 2016a, 2016b, 2018), in several cichlid species (Roh *et al.*, 2004; Pollock *et al.*, 2005; Abate *et al.*, 2010), and in other fish taxa (Chivers and Smith, 1994a). To ensure that we exposed fish to cues capable of inducing an anti-predator response, we instead used a slightly higher concentration of 4.73 $mg \cdot L^{-1}$ as our reference. However, due to the differences in abundance of certain tissues within the body of a fish, they differ in their contribution to this reference concentration. Using fish whole body weight as well as our knowledge of the removed ratio of tissues, we were able to calculate the dilution factors for all homogenates according to the proportions of the same tissues available in each fish. For example, we know that liver tissue (average weight 57.425 ± 28.824 mg) always constitutes 100% of the same tissue available in each fish. Thus we calculated liver homogenates to be diluted with the same amount of water as we would use to dilute the whole-body extract of the same fish (based on whole-fish wet weight). Consequently, the absolute concentration of tissue used was different for each extract (Table 1). Solid tissues were ground using a pestle and mortar so as to rupture cells and allow alarm cues to be released. Then the homogenates were diluted with the calculated amount of distilled water. Extracts were then passed through filter floss and stored in 1 mL aliquots (Eppendorf Varipette 100–1000 µL,

Eppendorf, Germany) at −20°C until use. At this temperature, alarm cues retain the capacity to elicit a fright response over a long period (Lawrence and Smith, 1989). Likewise, we prepared 1 mL aliquots of distilled water for the control treatment.

Chemical cue preparation: Experiment 2

We derived the alarm cues for Experiment 2 from 16 (eight males: standard length 5.88 ± 0.43 cm, body mass 4.58 ± 0.48 g; eight females: standard length 4.00 ± 0.18 cm, body mass 1.79 ± 0.15 g; mean ± SD) adult F3 lab-bred donor *P. taeniatus*. For the same purpose as in Experiment 1, all donor fish were isolated for 3 weeks in 20 × 30 × 20 cm tanks with airstones for aeration. During isolation, donor fish were fed the same quantity of food as in Experiment 1, but this time it consisted of frozen *Artemia* sp., *Chironomus* sp., *Culex* sp., and *Chaoborus* sp. in a ratio of 1 : 2 : 1 : 0.25. As in the first experiment, fish were starved for 2 days prior to alarm cue preparation. After euthanization, we collected the same tissue samples as in Experiment 1 with the exception of spleen tissue. This is because the amount of spleen tissue in each individual is very small and obtaining a sufficient volume of spleen extract with the same concentration would have required the sacrifice of more than 50 donor fish. During calculations of the dilution factor for Experiment 2, rather than using the same reference concentration as in Experiment 1 (4.73 $mg \cdot L^{-1}$), we aimed for a slightly lower concentration of 3.6 $mg \cdot L^{-1}$ for all tissue extracts so as to minimize the number of donor fish required. This concentration is the same as that for whole-body extracts that has previously been shown to induce a significant decrease in activity in *P. taeniatus* (Meuthen *et al.*, 2014). Tissues were weighed as above, pooled across donor fish, and then ground with a pestle and mortar so as to release alarm cues. As before, we diluted homogenates with distilled water, passed them through filter floss and – along with a distilled water control – stored them in 1 mL aliquots at −20°C until use.

Experimental setup

Behavioural trials of Experiments 1 and 2 were run in four experimental tanks (30 cm long × 20 cm wide × 20 cm high). Each tank was supplemented with a grey square plastic tube (0.5 × 0.5 × 15 cm) taped to the middle of one of the short sides of the tank and leading 5 cm below the water surface. This duct allowed for the introduction of any cues while minimizing any disturbance to the fish. Experimental tanks contained 6 litres of aged tap water that was previously supplied with substrate; this method is known to facilitate fish activity in empty tanks (for details, see Meuthen *et al.*, 2011). Water temperature was maintained at 26 ± 1°C throughout the experiments. Tanks were visually isolated from each other by a layer of white polystyrene between them. Moreover, the whole experimental setup was surrounded on all sides except the top with white polystyrene so as to visually isolate fish from the experimenter.

Experimental procedure

One week prior to the behavioural trials, we randomly chose 47 female *P. taeniatus* from 15 different families (Experiment 1: 24 females from seven families, standard length 3.93 ± 0.19 cm, body mass 2.05 ± 0.33 g; Experiment 2: 23 females from nine families, standard length 4.00 ± 0.26 cm, body mass 1.91 ± 0.36 g; mean ± SD). These individuals were then placed in experimental tanks (30 cm long × 20 cm wide × 20 cm high) filled with 6 litres of water, 210 mL sand as substrate, and an airstone for aeration. Keeping *P. taeniatus* in isolation before an experiment instead of removing them from a shoal immediately before

an experiment substantially increases the number of active fish when tested individually and thus minimizes the number of fish required (D. Meuthen, personal observation). When transferring fish, we measured the standard length and body mass of each individual as described before. During isolation, tanks were visually isolated from each other with black tar paper and fish received 120 µL of frozen *Artemia* sp., *Chironomus* sp., *Culex* sp., and *Chaoborus* sp. in a ratio of 1 : 2 : 1 : 0.25 daily. On the morning of an experiment, fish received an additional small water change whereby 1 litre of tank water was replaced with 1 litre of fresh substrate-treated water. Such a water change increases fish activity in subsequent experiments (S.A. Baldauf, personal communication).

Experiments were run by first adding the substrate-treated water to the experimental tanks. Then, we transferred individual fish to their respective experimental tank with a hand net. Fish behaviour was recorded for 60 minutes from above using a video camera at 640×480 pixel resolution (Quick Cam 9000, Logitech, China). This period constituted the pre-stimulus phase. Subsequently, experimental stimuli (tissue extracts or distilled water) were temperature-adjusted to tank temperature, and then introduced into the tanks through the tubes. Thereafter, fish behaviour was recorded for an additional 60 minutes, which constituted the post-stimulus period. At the end of each experiment, fish were returned to their isolation tanks. Each fish was exposed to each of the experimental stimuli once (in random order) but experimental stimuli assigned to individual experimental tanks were alternated between trials. Up to eight fish were tested each day, and each fish had an interval of at least 24 hours before being exposed to another stimulus in a different trial. Between trials, experimental tanks were cleaned with 3% hydrogen peroxide and then rinsed with tap water so as to remove any remaining olfactory traces (McLennan, 2004; Mehlis *et al.*, 2008).

In Experiment 1, each fish was exposed to every stimulus, and the choice of donor fish was randomized between experimental fish. In Experiment 2, each fish was exposed to four randomly selected extracts due to the scarcity of some tissues that could be collected from donor fish. In one instance (during Experiment 1), the fish escaped from its tank before the end of the experiment and in other cases (during Experiment 2) some fish remained completely inactive during the pre-stimulus period, which meant we removed these trials from our analysis. Although we settled on minimum sample sizes in advance (24 fish for Experiment 1 and 15 fish for Experiment 2), the exclusion of trials in Experiment 2 required us to re-run this experiment with eight additional individuals. In total, we analysed 278 trials: 191 trials in Experiment 1 (skin extract $n = 24$, muscle tissue extract $n = 24$, blood $n = 24$, mucus $n = 24$, liver tissue extract $n = 24$, intestine tissue extract $n = 24$, spleen tissue extract $n = 24$, and the distilled water control $n = 23$) and 87 trials in Experiment 2 (skin extract $n = 13$, muscle tissue extract $n = 13$, blood $n = 9$, mucus $n = 11$, liver tissue extract $n = 12$, intestine tissue extract $n = 12$, and the distilled water control $n = 17$).

Data analysis

Fish activity was evaluated by tracking individual movement during both the pre-stimulus period as well as the subsequent post-stimulus phase with animal tracking software (Biobserve Viewer2 v.3.0.0.119, St. Augustin, Germany). We later calculated a relative activity index for each trial that represents the relative change in activity induced by the introduction of the experimental stimulus. Positive values represent an increase in activity whereas negative values represent a decrease in activity:

$$\text{Activity index} = \frac{\text{post-stimulus track length (cm)} - \text{pre-stimulus track length (cm)}}{\text{post-stimulus track length (cm)} + \text{pre-stimulus track length (cm)}}$$

Statistical analysis

For statistical analysis, we used R v.3.2.5 (R Development Core Team, 2016). As the activity indices met assumptions of normality according to Shapiro-Wilk tests, we applied parametric tests throughout.

We constructed linear mixed-effect models (function 'lme' in the R package 'nlme') with maximum likelihood parameter estimation throughout. We always entered 'fish identity' and 'fish family' (the former nested in the latter) as random variables so as to account for both the repeated use of individual fish and genetic effects. All results are based on likelihood ratio tests (LRT), and *P*-values represent whether the removal of the explanatory variable led to a significant decrease in model fit.

First, to analyse the results from Experiment 1, we constructed a global model by entering 'pre-stimulus track length' (distance covered during the pre-stimulus period) as the dependent variable and 'stimulus' (skin extract, muscle tissue extract, blood, mucus, liver tissue extract, intestine tissue extract or distilled water control) as the explanatory variable. Thereafter, in a second global model aiming to reveal general treatment effects on fish activity, we entered 'activity index' (where a negative value represents a decrease in activity induced by the stimulus) as the dependent variable and 'stimulus' as the explanatory variable. In the case of a significant effect in the second global model, we conducted further *post-hoc* tests entailing pair-wise comparisons between all stimuli. Then, to elucidate whether a change in activity is correlated with the absolute amount of tissue that was used to produce each extract, we constructed another model based on the dataset from Experiment 1 where the data from the control treatment was removed. Here, 'activity index' was likewise entered as the dependent variable and 'concentration' (mean values from Table 1) as the explanatory variable.

Table 1. The respective amounts of tissue used to derive the extracts in the behavioural experiments

Tissue	Extracts in Experiment 1	Extracts in Experiment 2
Mucus	0.027 ± 0.025 mg·L^{-1} (4.73 mg·L^{-1})	3.60 mg·L^{-1}
Blood	0.031 ± 0.019 mg·L^{-1} (4.73 mg·L^{-1})	3.60 mg·L^{-1}
Spleen	0.032 ± 0.034 mg·L^{-1} (4.73 mg·L^{-1})	N/A
Liver	0.070 ± 0.022 mg·L^{-1} (4.73 mg·L^{-1})	3.60 mg·L^{-1}
Intestine	0.117 ± 0.040 mg·L^{-1} (4.73 mg·L^{-1})	3.60 mg·L^{-1}
Skin	0.513 ± 0.192 mg·L^{-1} (4.73 mg·L^{-1})	3.60 mg·L^{-1}
Muscle	4.786 ± 0.020 mg·L^{-1} (4.73 mg·L^{-1})	3.60 mg·L^{-1}

Note: Values in the left-hand column are means ± standard deviation with the (theoretical) concentration of the corresponding whole-body extract in parentheses. N/A: not available.

As we established treatment effects in Experiment 1 and repeated the same treatments with different concentrations in Experiment 2, we only conducted *post-hoc* tests for analysis. Thus, we constructed the same *post-hoc* models as for Experiment 1 and compared each stimulus with each of the other stimuli.

RESULTS

Skin histology

In both sexes, the *P. taeniatus* epidermis was found to contain sacciform cells (Fig. 1).

Experiment 1

Fish activity pre-stimulus did not differ across treatments (LRT, $\chi^2 = 4.127$, $df = 7$, $P = 0.765$) but the change in fish activity did (LRT, $\chi^2 = 14.216$, $df = 7$, $P = 0.048$; Fig. 2). The reduction of activity following exposure to muscle tissue extract was significantly higher than in the distilled water control (LRT, $\chi^2 = 4.683$, $df = 1$, $P = 0.031$). In contrast, exposure to blood cues (LRT, $\chi^2 = 0.845$, $df = 1$, $P = 0.358$), mucus (LRT, $\chi^2 = 1.565$, $df = 1$, $P = 0.211$), liver tissue extract (LRT, $\chi^2 = 0.092$, $df = 1$, $P = 0.762$), spleen tissue extract (LRT, $\chi^2 = 0.201$, $df = 1$, $P = 0.654$), intestine tissue extract (LRT, $\chi^2 = 1.157$, $df = 1$, $P = 0.282$), and skin extract (LRT, $\chi^2 = 0.003$, $df = 1$, $P = 0.960$) did not induce a significantly greater reduction in activity compared with the distilled water control. Moreover, the change in activity in response to exposure to muscle tissue differed significantly from all other extracts apart from liver and intestine tissue (Fig. 2, Table 2).

Excluding the distilled water control, the reduction in activity was positively correlated with the amount of tissue that was used to produce each extract (LRT, $\chi^2 = 14.403$, $df = 1$, $P < 0.001$). Fish exposed to extracts derived from higher concentrations of tissues reduced their activity significantly more.

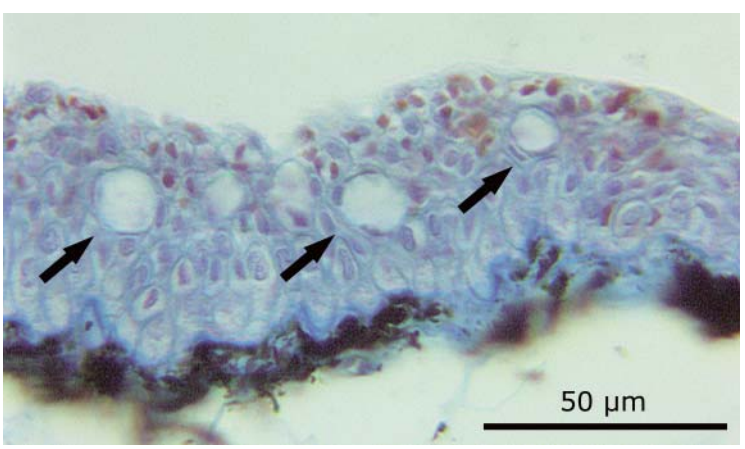

Fig. 1. Microphotograph of *Pelvicachromis taeniatus* epidermis stained with Heidenhain's Azan trichrome stain at 400× magnification. Arrows indicate the epidermal sacciform cells.

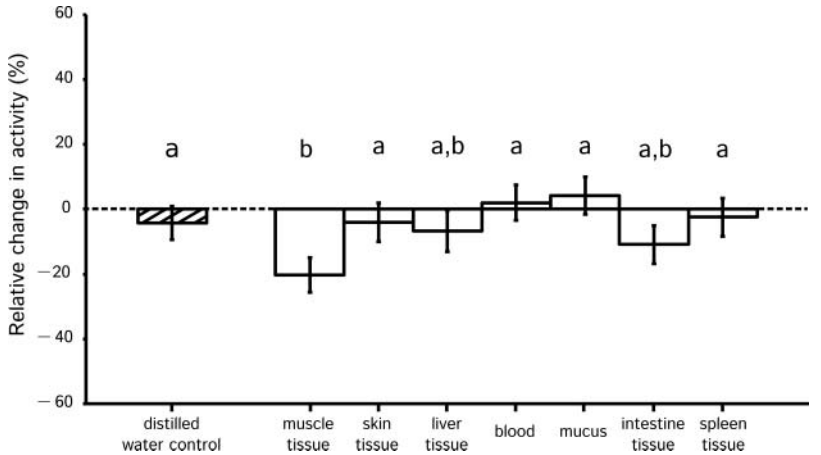

Fig. 2. Relative changes in female *P. taeniatus* activity (mean ± SE) as induced by exposure to either a distilled water control or to different extracts that were diluted proportional to their contribution to the whole-body extract. The dashed line is the zero referent and represents no change in activity. Different letters denote significant differences ($P < 0.05$).

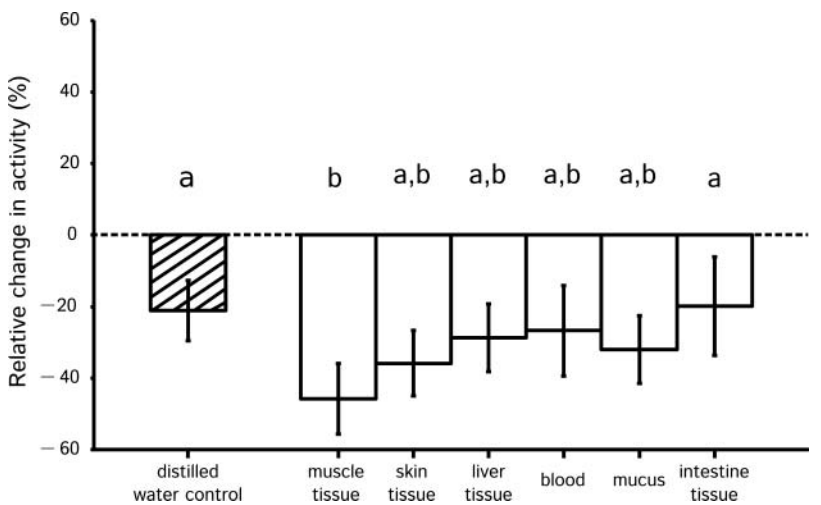

Fig. 3. Relative changes in female *P. taeniatus* activity (mean ± SE) as induced by exposure to either a distilled water control or to different extracts of the same concentration. The dashed line is the zero referent and represents no change in activity. Different letters denote significant differences ($P < 0.05$).

Experiment 2

In Experiment 2, in which fish were exposed to cues of the same concentration, only exposure to muscle tissue extract resulted in a significant reduction of activity compared with the distilled water control (LRT, $\chi^2 = 5.043$, $df = 1$, $P = 0.025$; Fig. 3). Compared with the distilled water control, fish did not change their activity significantly when exposed to

blood cues (LRT, $\chi^2 = 0.158$, $df = 1$, $P = 0.691$), mucus (LRT, $\chi^2 = 0.542$, $df = 1$, $P = 0.462$), liver tissue extract (LRT, $\chi^2 = 0.753$, $df = 1$, $P = 0.386$), intestine tissue extract (LRT, $\chi^2 = 0.056$, $df = 1$, $P = 0.813$) or skin extract (LRT, $\chi^2 = 1.062$, $df = 1$, $P = 0.303$). However, except for intestine tissue, the change in activity in response to the other tissue extracts did not differ from that observed to muscle tissue extract (Fig. 3, Table 2).

Table 2. *Post-hoc* tests entailing pair-wise comparisons between stimuli for Experiment 1 (extracts diluted proportional to their contribution to a whole-body extract) and Experiment 2 (extracts diluted to the same concentration)

	Experiment 1		Experiment 2	
Stimulus comparison	χ^2	*P*	χ^2	*P*
Blood–intestine tissue	2.615	0.106	0.598	0.439
Blood–liver tissue	1.594	0.207	0.039	0.844
Blood–mucus	0.088	0.767	0.130	0.719
Blood–muscle tissue	**8.959**	**0.003**	1.553	0.213
Blood–skin tissue	0.594	0.441	0.732	0.392
Blood–spleen tissue	0.372	0.542	N/A	N/A
Blood–distilled water control	0.845	0.358	0.158	0.691
Intestine tissue–liver tissue	0.251	0.616	1.639	0.201
Intestine tissue–mucus	3.707	0.054	0.013	0.909
Intestine tissue–muscle tissue	1.430	0.232	**4.290**	**0.038**
Intestine tissue–skin tissue	0.830	0.362	0.144	0.704
Intestine tissue–spleen tissue	1.071	0.301	N/A	N/A
Intestine tissue–distilled water control	1.157	0.282	0.056	0.813
Liver tissue–mucus	1.657	0.198	0.089	0.765
Liver tissue–muscle tissue	2.681	0.102	1.556	0.212
Liver tissue–skin tissue	0.179	0.672	<0.001	0.998
Liver tissue–spleen tissue	0.319	0.572	N/A	N/A
Liver tissue–distilled water control	0.092	0.762	0.753	0.386
Mucus–muscle tissue	**9.305**	**0.002**	1.270	0.260
Mucus–skin tissue	1.073	0.300	0.145	0.703
Mucus–spleen tissue	0.981	0.322	N/A	N/A
Mucus–distilled water control	1.565	0.211	0.542	0.462
Muscle tissue–skin tissue	**4.376**	**0.036**	1.334	0.248
Muscle tissue–spleen tissue	**5.050**	**0.025**	N/A	N/A
Muscle tissue–distilled water control	**4.683**	**0.031**	**5.043**	**0.025**
Skin tissue–spleen tissue	0.061	0.805	N/A	N/A
Skin tissue–distilled water control	0.003	0.959	1.063	0.303
Spleen tissue–distilled water control	0.201	0.654	N/A	N/A

Note: Statistical significance was calculated with linear mixed-effect models including fish identity nested within family identity as random effects. Degrees of freedom differ by one throughout. Significant differences ($P < 0.05$) are shown in bold font. N/A: not available.

DISCUSSION

Although epidermal sacciform cells were present within the epidermis of *P. taeniatus* (Fig. 1), in both experiments *P. taeniatus* displayed a significant reduction in activity relative to the control treatment only when exposed to muscle tissue extract. However, except for liver and intestine tissue, the response to the other tissue extracts, including skin extract, mucus and blood cues, clearly differed from that to muscle tissue extract only when their concentrations were diluted proportional to their contribution to a whole-body extract concentration that is known to induce a change in activity (Experiment 1, Table 2, Fig. 2) and not when their concentrations were the same (Experiment 2, Table 2, Fig. 3). Thus, alarm cues are not exclusively based within the skin or in any single specific tissue.

The presence of alarm cues within muscle tissue and other tissues is in accordance with a study on the sea lamprey *Petromyzon marinus* (Bals and Wagner, 2012), which suggested responses to both muscle and skin tissue extracts. Furthermore, our results are in line with previous findings that alarm cues may also be present in blood cues (Barreto *et al.*, 2013) and mucus (Mathuru *et al.*, 2012). More generally, our finding that the presence of sacciform cells within the skin does not correlate with an alarm response exclusive to skin extracts supports the theory that club or sacciform cells may not be the putative location of alarm cues (Hugie and Smith, 1987; Carreau-Green *et al.*, 2008; Wisenden *et al.*, 2008; Barreto *et al.*, 2012; Pollock *et al.*, 2012; Manek *et al.*, 2013; de Souza-Bastos *et al.*, 2014; Lienart *et al.*, 2016; Mathuru, 2016).

In Experiment 2 we found responses similar to that elicited by muscle tissue with the other tissue extracts. This suggests that in Experiment 1, non-muscle tissue extracts may not have elicited a behavioural change owing to the alarm cue being below the response threshold. Furthermore, our finding that exposure of *P. taeniatus* to 3.60 $mg \cdot L^{-1}$ blood did not result in a response significantly different from the distilled water control, contrasts with the observation that in another cichlid (*O. mossambicus*), approximately 0.80 $mg \cdot L^{-1}$ blood was sufficient to induce a measurable behavioural response (Barreto *et al.*, 2013). Taken together, our results suggest that while tissues may differ in a species-specific way in the amount of alarm cues they contain, alarm cues are likely present in tissues throughout the body, not just the skin. In terms of alarm cue evolution, if alarm cues are found throughout the body, such cues might not be specific substances with a distinct signalling function. Rather, they are more likely to be injury-related cues that are – similar to other predator cues – recognized by conspecific receivers. This is in accordance with the by-product hypothesis, which postulates that the epidermal club cells primarily have an immune-related function and the alarm function is secondary (Chivers *et al.*, 2007).

In conclusion, our results suggest further research on alarm cues is needed. First, if alarm cues have no specific signal function that is beneficial for the sender, future studies should focus on the evolution of alarm cue recognition in receivers. Second, as tissues other than skin trigger anti-predator responses, we advocate the use of unspecific extracts such as whole-body extracts in studies with the aim of simulating the presence of predators. Third, future studies should address possible species-specific concentrations of alarm cues within separate tissues. Such studies should also consider testing the consequences of exposure to extracts from different tissues not only in the context of short-term behavioural flexibility but also in terms of long-term behavioural and morphological plasticity.

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