Neotropical Ichthyology, 12(1): 125-132, 2014

DOI: 10.1590/S1679-62252014000100013

Skin extract from *Rhamdia quelen* (Siluriformes: Heptapteridae) does not promote stress in conspecifics

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Chemical communication is widely used in aquatic environments, where visual or auditory signals may not be always effective. Fish of the superorder Ostariophysi are known to display epidermal cells (club cells) that produce and store alarm substances, which are released to the water when the skin is damaged. Responses to alarm substances range widely, between active searches for refuge to a complete stop in any locomotor activity. In this study a large number of binucleated club cells (average density of 11 cells /μm^2) were histologically observed in the skin of the catfish *Rhamdia quelen* (known as jundiá). Skin extract (2, 5, and 10% w/v) applied for 15 minutes to conspecifics elicited increase in swimming activity and in the area visited by the fish inside the tank. However, exposure to the epithelial alarm cue did not evoke any stress response: plasma osmolality, ions (sodium, chloride, magnesium, and potassium), glucose and cortisol remained unchanged. In conclusion, the conspecific alarm cue of the jundiá induces behavioral responses but not an acute stress response upon short-term exposure, compatible with its role in fostering physical integrity without representing major stress activation. Considering that in the natural environment such stimuli must quickly disappear due to dilution and that rapid protection responses may be necessary upon the possibility of an approaching predator, a faster mechanism to assure survival may come into play, such as sympathetic nervous system activation.

Comunicação química é amplamente utilizada por animais que vivem em ambiente aquático, onde sinais visuais e auditivos nem sempre são facilmente identificados. Os Ostariophysi são conhecidos por apresentarem células club na epiderme, as quais produzem e estocam substância de alarme que são liberadas para o ambiente quando a pele é lesionada. As respostas dos peixes a substância de alarme variam entre exploração ativa por refúgios até a parada completa de atividade locomotora. Neste estudo, grande número de células club binucleadas (densidade média de 11 células/μm2) foram histologicamente observadas na epiderme do jundiá, *Rhamdia quelen*. Peixes expostos a extrato de pele de conspecíficos (2, 5, e 10% peso/vol) por 15 minutos apresentaram aumento da atividade locomotora e da área de dispersão. No entanto, essa exposição não promoveu nenhuma resposta de estresse - osmolalidade plasmática, íons (sódio, cloreto, magnésio e potássio), glicose e cortisol não sofreram alteração. Concluímos que a exposição aguda a extrato de pele de conspecíficos promovem respostas comportamentais de fuga, que essa espécie apresenta grande concentração de células club, as quais devem estar envolvidas nessas respostas e que a exposição aguda ao estímulo não promoveu respostas bioquímicas indicativas de estresse. Considerando que no ambiente natural tais estímulos devem desaparecer rapidamente dados a diluição do meio e que respostas rápidas de proteção devem ser desencadeadas frente à possibilidade de presença de predador, vias rápidas de suporte a essas respostas, como sistema nervoso simpático, por exemplo, devem estar envolvidos.

Key words: Anti-predator behavior, Chemical communication, Club cell, Fish stress, Jundiá.

Introduction

In the natural environment, fast and generalized transmission of information is frequently crucial for the survival of any species. In the aquatic environment specifically, high turbidity and density do not favor the effective transmission and adequate perception of visual and auditory signals, what renders chemical signals very useful, since they are quickly spread in the water column, easily and specifically detected, and remain for a

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relatively long period in the water. In fish, chemical substances are involved in the mediation of several physiological and behavioral responses related to the identification of conspecifics, localization of food sources, reproduction, and identification of predation risks (Smith, 1992; Chivers & Smith, 1994; Carreau-Green *et al*., 2008; van de Nieuwegiessen *et al.*, 2009; Wisenden *et al.*, 2010; Daghfous *et al*., 2012).

Large epidermal club cells presented in the skin of many teleost fish, and particularly in those of superorder Ostariophysi, are supposed to be damaged during predatory attacks, releasing a chemical alarm cue that has been frequently reported to cause anti-predatory responses in conspecifics (Ide *et al*., 2003; Carreau-Green *et al*., 2008; Barbosa Júnior *et al*., 2012; Manek *et al.*, 2013). More recently, studies have shown that at least for some species, the alarm substance produced by club cells is not solely responsible for the prey response. Carreau-Green *et al*., (2008), for example, observed that the skin of juvenile fathead minnows promoted an alarm response in conspecifics even before the club cells appear along their development. Manek *et al*. (2013) suggest that alarm cues may also be mixtures of chemicals from different parts of the epidermis, including the ECC (epidermal club cells).

Independent of the sources of alarm cues, many prey fish exposed to injured congeners or their skin extracts do display an alarm reaction, which are diverse and species-specific, and include fast swimming against the bottom (erratic movement), freezing, increased school cohesion and sheltering, putatively avoiding the area containing the alarm substance. Neurovegetative correlates to alarm responses such as increase in metabolic and ventilatory rates have also been described. Barreto *et al.*, (2010), for example, showed an increase in ventilatory rate in Nile tilapia exposed to its alarm cue for 4 minutes, suggesting an autonomic anticipatory escape response to a predation signal. Other studies report typical stress responses in fishes exposed to alarm cues, such as raised plasma cortisol levels (Rehnberg *et al.*, 1987; Smith, 1992; Toa *et al.*, 2004; Sunardi-Takashi & Manatunge 2007). However, most of these results have been reported after several hours of exposure to alarm substances, which is a rather unlikely situation to occur in the natural environment. In this study we tested the effect of exposure to skin extract on the exploratory behavior and biochemical indicators of stress and homeostasis maintenance, after 15 minutes of exposure, in a native Siluriform (*R. quelen*). We also evaluated the presence and density of club cells in its epidermis.

The catfish *R. quelen* (jundiá) has Neotropical distribution, is endemic to South America and has high commercial value, being widely employed in aquaculture in Brazil (Barcellos *et al.*, 2001, 2004; Borges *et al.*, 2004). The alarm response is a potential problem in aquaculture systems, since epidermal club cells are easily ruptured and the alarm cue may be released during agonistic confrontations and handling, what can lead to changes in the taste of the meat or even cause yield losses (van de Nieuwegiessen *et al.*, 2009).

Material and Methods

Adult fishes (n = 46), provided from the Laboratório de Pesquisa e Piscicultura of the Pontifícia Universidade Católica do Paraná, were acclimated for seven days in a 250-liter tank, under a 12:12 h L:D photoperiod, water temperature of 20±2ºC, constant aeration and biological filtration. They were daily fed with 1% wet weight commercial food (extruded, Supra, 32% protein) until 24h prior to the start of the experiments, in order to standardize metabolism and prevent water from getting dirty, what could compromise the alarm cue properties, fish sensibility and the quality of the video film.

Epidermal club cells identification. Using the methodology described in Ribeiro *et al.* (2012), four juveniles (3 males, 1 female, mean weight 41.5±1.9 g) were anesthetized with benzocaine (80 mg/L, ~ 2 min), sacrificed (spinal section) and had the dorsal skin gently removed from both sides using a scalpel, yielding a total of 8 fragments of ~ 3 cm of length. The tissue fragments were fixed in ALFAC for 16 h, washed twice in 70% ethanol, dehydrated in an ethanolic series and embedded in Paraplast® (Sigma P3558). Blocks were sectioned (5 µm), stained with PAS (Periodic acid-Schiff) and counterstained with hematoxylin; seven sections were observed (Leica DMLS2) and images captured (Leica XXC300FX, Leica application software V3.1.0.). Cells were visualized and measured using the UTHSCSA Image Tool Software for Windows v 3.0.

Skin extract preparation. Fresh epithelial alarm cue was obtained using adult jundiá specimens (length: 30-35 cm, weight: 85-91.2 g), as previously described in Ide *et al.* (2003) and Honda *et al.* (2008). Briefly, animals were sacrificed through spinal section, skin patches from both dorsal sides of the body were gently removed using a scalpel (20g total skin weight, ~30 cm^2), were homogenized in 200 mL of deionized water and filtered in common filter paper yielding a skin homogenate considered as the 10% (w/v) stock solution. For the homogenate test solutions of 5% and 2%, 20 ml of the 10% stock were diluted in either 20 or 80 ml of deionized water, respectively. Aliquots of 2 mL were pipetted in the experimental 20 ml glass aquarium, immediately after being diluted. From now on we will refer to groups receiving different concentrations of alarm cue (AC) extracts as AC0, AC2, AC5, AC10.

Behavioral data acquisition. In order to access behavioral data, 40 fishes were individually placed in glass tanks (49 cm x 35 cm x 29 cm, filled with 20 L of water) and after

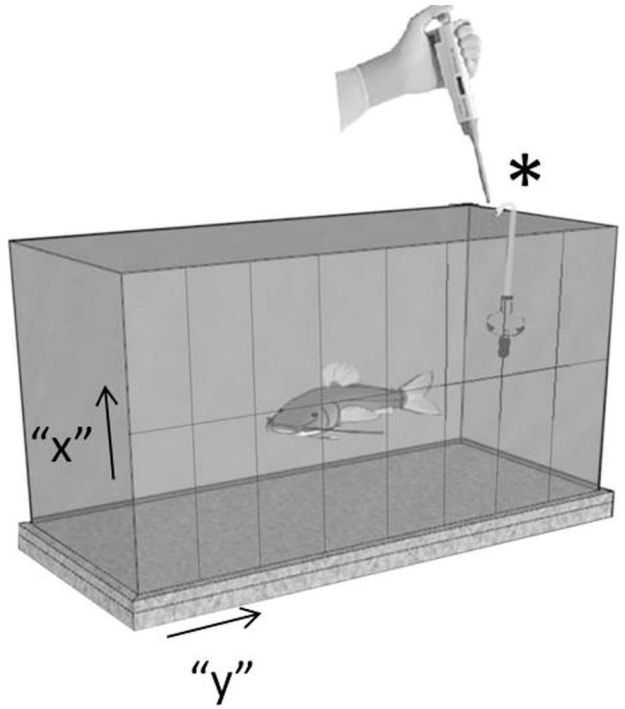

Fig. 1. Schematic drawing of the tank used for behavioral analysis of *R. quelen*, indicating the axes of evaluation of locomotory activity (arrows, "x" and "y") and the location of the aeration stone and placement of alarm substance (*).

a 24 h acclimation, they were exposed to either water (2 mL, AC0) or conspecific skin extract (2 mL, AC2, AC5, or AC10, n=10 for each group), pipetted next to the aeration stone, which was always positioned on the right side of the glass tank (Fig. 1). As this fish species spends most of the time still in aquaria corners and they are very sensitive to disturbances, we chose to delineate an independent group test to identify the individual response to a vehicle introduction (control group - water) and to three alarm cue concentrations, instead of the classic before-after exposure. Each fish was videotaped for 15 minutes.

The behavioral response of the jundiás upon exposure to the alarm cue was followed through the **a)** percentage of fishes that displayed locomotor activity (% of animals that dispersed), **b)** the degree of dispersion and **c)** the time spent in locomotion. As in all groups some animals remained motionless along the whole 15 min of observation, those individuals were not considered for the quantification of locomotor activity. The degree of dispersion, which indicates the spatial occupation of the aquarium by fish, was calculated as described in Jordão & Volpato (2000). Briefly, a grid contained 14 quadrants (7x2, with 7 cm squares) was drawn on the frontal side of the aquaria. At each 15 sec the position of the head of the animal was recorded as *x*/*y* coordinates (in cm) in the quadrants drawn on the aquarium wall. The average of the *x*/*y* values at each measurement, [(*x*+*y*)/2], constitute a baricentric coordinate, from which dispersion (cm) was calculated. The time spent in locomotion was recorded as the total swimming time for each individual fish and was shown as a percentage of the total recording time, *i.e.*, of 15 minutes.

Plasma parameters. The biochemical indicators of stress or disturbed homeostasis assayed were a) osmolality - measured in undiluted plasma samples using a vapor pressure micro-osmometer (Wescor® 5520 VAPRO), b) sodium and potassium ions - measured in diluted plasma samples (1:100 in ultrapure water), by flame photometry (CELM FC-180), c) chloride and magnesium ions and d) glucose - measured in undiluted samples, using colorimetric commercial kits (Labtest®, Brazil, ULTROSPEC 2100 pro-Amersham Pharmacia Biotech) and e) cortisol - determined according to the method established by Munro & Stabenfeldt (1984), using the polyclonal antibody R-4866, produced by the University of California at Davis (diluted 1:8,500). Plasma samples were diluted (1:128 or 1:256) in phosphate buffer (pH 7.0) so that the readings would fall within the range of the standard curve (r^2=0.9689), which had concentrations ranging between 3.9 and 1000 pg of cortisol/well. Absorbance was read at 405 nm (Elisa Sunrise Tecan Deutschland Gmbh, Germany).

Statistical treatment of the data. The effect of the skin extract on the time spent in locomotion was reported as medians and percentiles (25^{th} and 75^{th}) and analyzed using the non-parametric Mann-Whitney U test. Total recording time was divided into five periods of three min each (0-3, 3-6, 6-9, 9-12 and 12-15 min), and the area of dispersion within a same treatment was tested using Repeated Measures ANOVA. The dispersion of each experimental group was compared to the control group using the Mann-Whitney U test. The proportion of animals that dispersed in different groups and periods were compared by Fisher's exact test. Plasma parameters were compared using unpaired Student's t-test. The level of significance was set at 0.05.

The experimental protocol agreed to the Ethical Principles in Animal Research - National College of Animal Experimentation (CONCEA) and was approved by the Committee on Ethics and Animal Experimentation of the Federal University of Paraná - certificate #241 of August 9^{th}, 2007.

Results

Identification of club cells in the jundiá epidermis. Histological analysis revealed the presence of club cells localized within the stratified epithelium of the epidermis, displaying 2.79 ± 0.10 µm in their largest diameter and 2.48 ± 0.09 µm in their smallest diameter, with an average density of 11.1 ± 0.58 cells/µm^2 of the epidermis. They were round

in shape and displayed two united and centralized nuclei. No connecting ducts between the cells and the surface of the skin were ever seen (Fig. 2).

Behavioral responses. There was great individual variability in the behavioral data collected from the jundiás. Of the 10 fishes used in each group, 4 fish in the AC0, 3 in the AC2, 2 in the AC5 and 4 in the AC10 group remained completely still during the whole 15 min of recording. Removing these fishes from the statistical treatment, data revealed that some individuals have shown a biphasic response, characterized by a few minutes of stillness followed by intense movement, or *vice versa*, and the AC2 group displayed higher swimming activity (time of locomotion) when compared to the AC0 ($P = 0.037$). The groups that received higher concentrations of the skin homogenate (AC5 and AC10) did not differ from the AC0 (Fig. 3).

In order to more precisely evaluate the fish response to the alarm cue, the video recording was divided into five periods of three min. In the AC0 group, 60% of the fish displayed some locomotory activity at the start of the observation period (0-3 min), while in the groups exposed to the skin homogenate this proportion was lower: 30% (AC2), 40% (AC5), and 30% (AC10, Fig. 4A).

However, during the subsequent 3-min periods, there was a clear inversion in the pattern, with reduction in the proportion of dispersion in the AC0 group (30% in the period 12-15 min), and increase in the groups exposed to the skin homogenate:

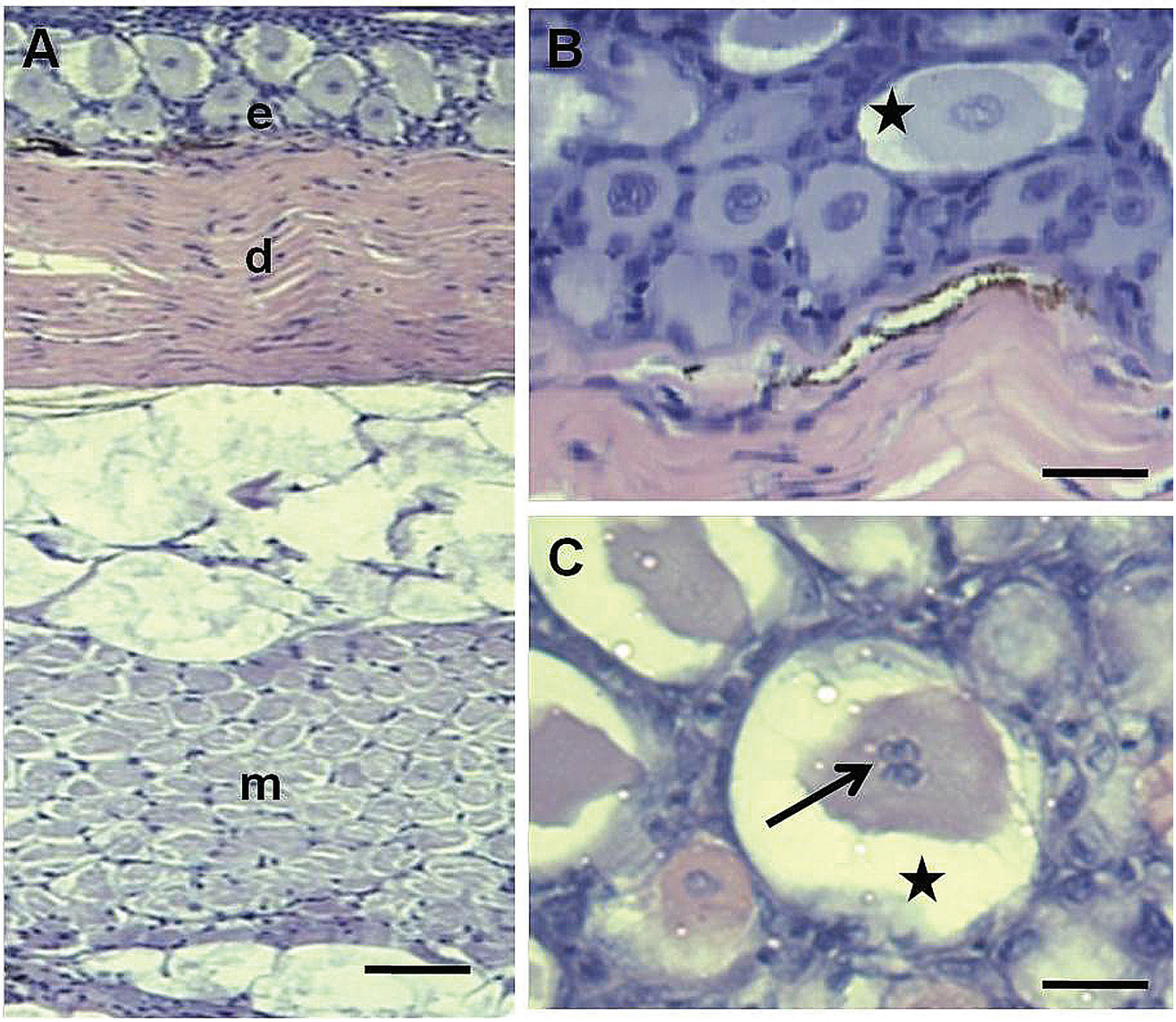

Fig. 2. Epidermis of *R. quelen* under light microscopy, PAS and hematoxylin staining. (A) Broad view showing the different skin layers: epidermis (e), dermis (d), and skeletal muscle (m). Scale bar = 5 µm; (B) club cells within the epidermis (star). Scale bar = 3 µm; (C) larger magnification of a club cell, with the arrow indicating the presence of the two nuclei. Scale bar = 1 µm.

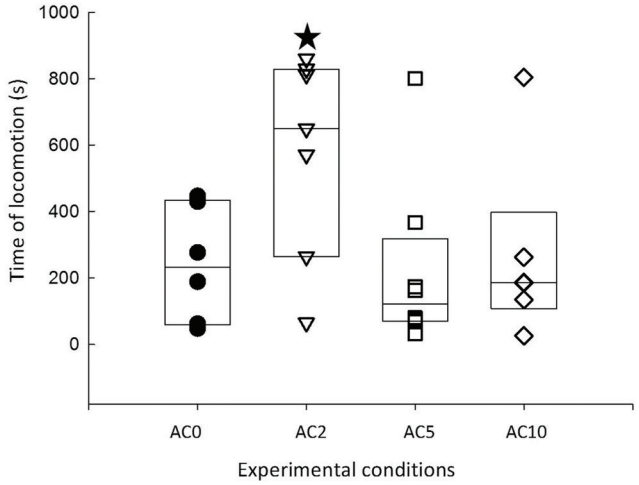

Fig. 3. Locomotion displayed by *Rhamdia quelen.* Symbols represent raw data, and boxes represent medians and 25-75% percentiles in fish exposed to skin extract of conspecifics (AC2, AC5, AC10). The star indicates statistical difference when compared to controls (AC0). Individuals who remained still were not included in the analysis.

60% of the fish dispersed in the final minutes of recording for AC2, AC5, and AC10 (Fig. 4A). According to Fisher's exact test, fishes exposed to the skin homogenate at 2% had higher dispersion between 9 and 12 min, when compared to controls (close to significant effect, P = 0.057) (Fig. 4A).

Dispersion area is displayed in Fig. 4B: lower values correspond to a smaller area explored by the fish during the evaluated period. Although the same response profile was detected for all experimental groups, with a reduction and posterior increase in the area explored, again, only the group exposed to AC2 presented an increase in the area of dispersion (P = 0.036) compared to the AC0.

Osmo-ionic homeostasis and stress indicators. Exposure of the jundiás to the alarm substance for 15 min did not affect either their extracellular osmo-ionic homeostasis or levels of stress indicators (plasma glucose and cortisol) by any of the three concentrations used (Table 1).

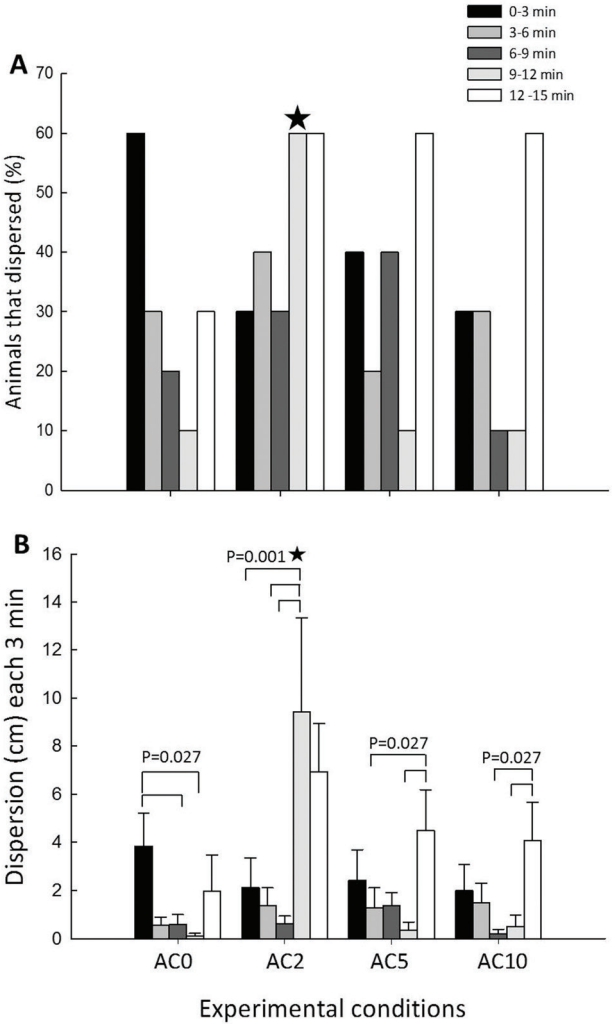

Fig. 4. (A) Percentage of fish that dispersed and (B) dispersion area along consecutive time windows of 3 min of recording. Lines correspond to differences along a time series for a same experimental condition. Stars indicate differences between the extract-exposed (AC2, AC5, AC10) and control groups (AC0).

Table 1. Plasma parameters evaluated in *Rhamdia quelen* exposed to different concentrations of the skin homogenate containing the alarm substance from conspecifics. Data displayed as mean ± s.e.m., n= 9-10. There were no differences between any of the values of the experimental groups and the values of the control group.

	Concentration of skin homogenate			
Parameters	**Control**	**2%**	**5%**	**10%**
Osmolality (mOsm/kgH2O)	280±5.7	277±3.4	280±5.2	284±5.5
Chloride (mM)	124±2.4	126±1.9	123±1.8	125±2.5
Sodium (mM)	150±1.7	146±1.8	144±1.9	145±1.3
Magnesium (mM)	1.02±0.05	1.05±0.03	1.00±0.06	1.05±0.02
Potassium (mM)	3.6±0.3	3.8±0.3	3.4±0.2	3.8±0.2
Glucose (mg/dL)	43±6.1	39±3.6	41±5.9	42±6.9
Cortisol (ng/mL)	59±12.2	58±8.7	68±15.5	70±11.7

Discussion

Morphological, physiological, and behavioral data presented here for *R. quelen* provide evidence of the presence of alarm cue and its use as a chemical signal for this catfish. However, the exposure to different concentrations of this substance for 15 minutes did not represent a source of stress.

Alarm substances - first named "*Schreckstoff*", fear or fright substance in German - were described by Karl Ritter von Frisch in 1942, who observed that European minnows (*Phoxius phoxinus*) with skin injury evoked escape responses in conspecifics (Stensmyr & Maderspacher, 2012). Since then, many fish species have been studied focusing on the identification of skin club cells, which produce the alarm substance and the physiological and behavioral effects of these substances. Alarm cells are typically described in fish of the superorder Ostariophysi, where the number of species studied is variable among its five orders. Reviewing the literature we found no studies on the Gonorynchiformes order; in Cypriniformes, studies focus primarily on four species: *Danio rerio* (*e.g.*, Suboski *et al*., 1990, Parra *et al.*, 2009; Wisenden *et al*., 2010); *Margariscus margarita* (*e.g.*, Rehnberg *et al.*, 1987); *Pimephales promelas* (*e.g.*, Lawrence & Smith, 1989; Chivers & Smith, 1994; Wisenden & Smith, 1997; Wisenden & Smith, 1998; Wisenden & Thiel, 2002; Wisenden & Barbour, 2005; Chivers *et al.*, 2007; Carreau-Green *et al.*, 2008; Jung & Tonn, 2011), and *Pseudorasbora parva* (*e.g.*, Sunardi-Takashi & Manatunge, 2007). In Gymnotiformes, there are reports of the presence of alarm cells, but without direct evidence of alarm substances (*e.g.*, Smith, 1992; Wisenden & Barbour, 2005). In Characiformes, which features the largest number of species assessed in this context, alarm responses to alarm cells substances have been reported, for example, in *Brycon amazonicus* (Honda *et al.*, 2008); *Brycon cephalus* (Ide *et al.*, 2003); *Gymnocharacinus bergi* (Cordi *et al.*, 2005); *Leporinus macrocephalus* (Alves *et al.*, 2013); *Leporinus piau* (Barbosa Júnior *et al.*, 2010); *Mimagoniates lateralis* and *M. microlepis* (Duboc, 2007), and *Piaractus mesopotamicus* (Jordão & Volpato, 2000). In the Siluriformes, the second order most studied in this context, six species have been evaluated: *Arius felis* (*e.g.*, Smith 2000), *Clarias gariepinus* (*e.g.*, Guerra *et al.*, 2006; van de Nieuwegiessen *et al.*, 2008; van de Nieuwegiessen *et al.*, 2009), *Ictalurus punctatus* (*e.g.*, Chapman & Johnson, 1997; Valentic & Caprio, 1994), *Pimelodella lateristriga* (*e.g.*, Damasceno *et al.*, 2012), *Pseudoplatystoma corruscans* (*e.g.*, Giaquinto & Volpato, 2001). However, only two studies were found about the role of epithelial alarm cells on behavioral responses in jundiá, *R. quelen*. Kochhann *et al.* (2009) demonstrated that the number of line crossings and feeding bites significantly decreased in response to skin extract, whereas the time spent hidden in the shelter significantly increased in jundiá larvae. Weber *et al.* (2012) suggested that naive juveniles never exposed to predators are able to identify predators and skin extract from conspecifics by odors.

We have here demonstrated that club cells are indeed present in high density in the skin of the jundiá, that these cells are large and rounded, and located in the middle of the stratified epithelium. Interestingly, club cells of *R. quelen* are binucleated, as has been found for other catfishes (*e.g.*, Chapman & Johnson, 1997; Smith, 2000; Guerra *et al.*, 2006; Damasceno *et al.*, 2012) suggesting an intense cell metabolic activity; the significance of this finding, however, remains to be clarified.

Animals exposed to alarm substances can exhibit biphasic behavioral responses, displaying absence of locomotion (including freezing) and intense swimming activity (including erratic movement). Ide *et al.* (2003) observed that *Brycon cephalus* exposed to skin extract of conspecifics presented a brief initial phase of dashing or very rapid swimming, followed by a long-lasting period of immobility. Other reports in the same direction were described by Valentic & Caprio (1994) working with channel catfish *Ictalurus punctatus*, and Giaquinto & Volpato (2001), working with pintado catfish *Pseudoplatystoma corruscans*. In this study an opposite pattern was demonstrated - most of the fishes remained still on the bottom in the first minutes after alarm cue exposure and presented some swimming activity in the final observation periods. As the alarm substance was introduced close to the aeration stone, there isn't the possibility that it took many minutes to diffuse through the whole aquarium and reach the fish. We suppose that adult *R. quelen* probably uses a different behavioral strategy, showing immediate freezing and exploration of the environment later on.

In the control group, none of the animals displayed vertical movement, along the *y* axis, during the 15 min of observation and recording. This is compatible with the demersal habit of this catfish. In fish exposed to different concentrations of skin homogenates, however, half of AC2 group moved along the *y* axis after 10 min, and 40% in the other two groups (AC5 and AC10) did this after 12 and 13 min, respectively. Thus, the responses shown here by *R. quelen* suggest that the alarm substance elicit the typical anti-predatory responses in all concentrations employed. Interestingly, the most obvious response occurred to the lowest homogenate concentration tested (2%), suggesting a correlation with the dose of skin extract used. We can suppose that lower alarm substance concentrations in the environment are related to bigger distances from the potential predator. In this situation, the increased locomotor activity showed here could be associated with reduced danger of predation. However, the meaning of this pattern of response should be further tested, with additional doses.

Plasma levels of ions and glucose obtained in this study are in agreement to those already reported for the species in non-stressed conditions, except for magnesium values that were below previous data in the literature for the species (1.15-1.40 mM) (Borges *et al.*, 2004). Cortisol levels obtained from the control group were similar to those reported previously by Barcellos *et al.* (2012) for the same species. As none of the assayed experimental groups differed from controls in any of the biochemical parameters quantified, these results corroborate our hypothesis that short-time exposure to alarm cues does not promote acute stress in fishes. Controversial data has been described for different species. Tierney *et al.* (2006) tested three different skin extract concentrations in coho salmon (*Oncorhynchus kisutch*) and obtained significant increase in cortisol over control 30 minutes following exposure. On the other hand, Toa *et al.* (2004) find no cortisol response of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed for 1 hour to conspecifics skin extracts. Ide *et al.* (2003) also did not find any effect of conspecifics alarm substance on plasma cortisol and glucose in *Brycon cephalus* after 30 minutes of exposure. However, 15 min of exposure to the alarm substance raised plasma cortisol levels in *Semotilus margarita* (Rehnberg *et al.*, 1987).

This is the first report analyzing a possible cortisol response in *R. quelen* submitted to skin extracts. Minutes of exposure seem like a more realistic approach to simulate the release of alarm substances. In the environment, the volume of water in which the alarm cue is diffused contributes to the rapid disappearance of the stimulus, what can also explain the fact that minutes of exposure did not promote stress. In cultivation systems, on the other hand, handling and management techniques may harm the skin of the fish and cause the release of alarm substances from the club cells of several individuals. This may promote the exposure of conspecifics to the alarm substance for a much longer time, thus putatively eliciting stress responses. Studies considering these issues in jundiá cultivation tanks could be profitably explored.

Acknowledgments

Authors wish to gratefully acknowledge the Pontifícia Universidade Católica do Paraná (LAPEP-PUC) for supply of fish and Mr. L. P. Bastos in obtaining and processing the fishes in the laboratory. Authors also wish to thank CNPq for the receipt of a Doctorate fellowship by L. R. Souza-Bastos, and a research grant by C. A. Freire. The donation of laboratory equipment and reagents by DAAD and CNPq to C. A. Freire are also gratefully acknowledged. Authors are also grateful to L. F. Duboc da Silva for very useful discussion of the data and S. Grötzner and I. A. Santos for help in preparing the material for microscopy.

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Submitted May 27, 2013

Accepted November 7, 2013 by Bernardo Baldisserotto

Published March 31, 2014