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Cortisol influences the antipredator behavior induced by chemical alarm cues in the Frillfin goby

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ABSTRACT

We evaluated the effect of increased plasma cortisol levels on fish antipredator behavior induced by conspecific chemical alarm cues. The experimental model for the study was the Frillfin goby *Bathygobius soporator*. We first confirmed that the alarm substance induces typical defensive antipredator responses in Frillfin gobies and described their alarm substance cells (epidermal 'club' cells). Second, we confirmed that intraperitoneal cortisol implants increase plasma cortisol levels in this species. We then demonstrated that exogenous cortisol administration and subsequent exposure to an alarm substance decreased swimming activity to a greater extent than the activity prompted by either stimulus alone. In addition, cortisol did not abolish the sheltering response to the alarm chemical cue even though it decreased activity. As predators use prey movements to guide their first contact with the prey, a factor that decreases swimming activity clearly increases the probability of survival. Consequently, this observation indicates that cortisol helps improve the antipredator response in fish.

Introduction

Most vertebrates respond to stressors by increasing the secretion of the glucocorticoid hormones, cortisol and corticosterone. This hormonal response represents the principal physiological response to stress in these animals (Sapolsky et al., 2000; Wingfield, 1994), including fish (Barton, 2002; Huntingford et al., 2006; Koakoski et al., 2013; Wendelaar-Bonga, 1997). These hormones stimulate responses that can increase the probability of survival by improving the body's readiness to address the stressor. Responses include an increase in plasma glucose (Barton, 2002; Wendelaar-Bonga, 1997) and enhanced analgesia (Alves et al., 2013; Wolkers et al., 2013). Certain behaviors and functions not crucial to immediate survival can be concomitantly suppressed by a stressor, such as parental investment (Thierry et al., 2013), reproduction (McConnachie et al., 2012), feeding (Barreto et al., 2013; Ferrari et al., 2012; Giaquinto and Volpato, 2001) and memory (Barreto et al., 2006; Moreira et al., 2004).

During predator–prey interactions, the prey's life is clearly at risk. The perception of a predator by the prey can accordingly elicit stress responses (Hawlena and Schmitz, 2010). In fish, interactions with a predator may induce an increase in plasma cortisol levels (Soares et al., 2012). The visual perception of a predator induces an increased ventilation rate (Barreto et al., 2003) and might (Barcellos et al., 2007; Kagawa and Mugiya, 2000) or might not (Barcellos et al., 2010) increase the prey's cortisol levels. The direct perception of a predator due to its odor can elicit an increase in cortisol levels (Rehnberg and Schreck, 1987). Cortisol levels may also increase due to the indirect perception of the predator when the prey detects a conspecific chemical alarm (Rehnberg and Schreck, 1987; Rehnberg et al., 1987; Toa et al., 2004) and disturbance cues (Barcellos et al., 2011; Oliveira et al., 2013). Changes in ventilatory responses have also been reported in fish exposed to conspecific chemical alarm cues (Barbosa Junior et al., 2010; Barreto et al., 2010, 2012; Gibson and Mathis, 2006).

Early detection of a predator has important implications for prey survival because it allows prey animals to anticipate a potential predator attack and to employ antipredator responses accordingly. In this context, it is plausible to suppose that cortisol could facilitate components of antipredator behavior in fish because cortisol prepares the body to perform behaviors that address stressors. In fact, an increase in circulating corticosterone levels has been found to enhance antipredator responses in tree lizards (*Urosaurus ornatus*) during a simulated encounter with a caged predator, the collared lizard (*Crotaphytus nebrius*), without altering the prey's behavioral repertoire (Thaker et al., 2009).

For the first time in fish, we evaluated the hypothesis that increased plasma cortisol levels influence antipredator behavior using the Frillfin goby *Bathygobius soporator* as an experimental model. To induce defensive antipredator responses, we exposed the fish to an alarm substance

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(conspecific skin extract). In fish belonging to the superorders Ostariophysi and Acanthopterygii (Perciformes), putative chemical alarm cues are thought to be produced and stored in the epidermal club cells and released when the epidermis is damaged, as in an injurious predator attack (Chivers and Smith, 1998). Gobies (Perciformes) display antipredator responses to these chemical cues (Smith, 1989; Smith et al., 1991). Thus, the Frillfin goby is a suitable model for our study. Prior to experimentally testing our hypothesis, we confirmed that the conspecific skin extract induced antipredator behavior in this species. Furthermore, we described the epidermal 'club cells' of this goby species.

Materials and methods

Animal welfare statement

This study complied with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the School of Medicine of Ribeirão Preto of the University of São Paulo Ethics Committee on Animal Research (CETEA), Protocol no. 010/2006.

Animals and holding conditions

Specimens of Frillfin goby *Bathygobius soporator* (Valenciennes, 1837) were collected from the estuary of the Itanhaem River in Itanhaem city, São Paulo state, Brazil. The fish were collected using funnel traps baited with shrimp. The traps were sunk into the estuary water near rocks. After a few minutes, we withdrew the traps from the water and the captured Frillfin gobies were carefully placed into plastic lidded buckets filled with seawater. This procedure was repeated several times to reach a suitable sample size. The bucket water received constant aeration from the field to the laboratory using air stones connected to battery-operated aquarium air pumps by plastic tubing. No mortality was observed. After collection, the fish (mean length ± SD (cm); 10.6 ± 1.4; ranging from 9.0 to 13.3 cm) were acclimated for 15 days before performing the experimental procedures in plastic stock tanks (12 fish/70 L water; stock density = 1.0 g/L of water). The tanks were maintained at constant room temperature (~24 °C) using air conditioning equipment. The water was constantly aerated by air pumps connected to the air stones via nontoxic silicone tubing and with biological and mechanical filtrations. The fish were exposed to a natural ~12/12 light/dark cycle and indirect illumination through lab windows. The fish were fed once daily with commercial feed for bottom-dwelling carnivorous fish [36% protein; Poytara carnivoros de fundo® (Bottom-dwelling carnivorous fish chow), Poytara LTDA, Araraquara — SP, Brazil] at a quantity that was equivalent to 4% of their body weight. Another fish species, the platy *Xiphophorus maculatus*, was also used (see below; Günther, 1866). The platies were only used as a control stimulus, and they were obtained from a fish dealer 72 h before experimentation. The platies' body length (mean ± SD) was 4.0 ± 0.5 cm. The platies were maintained in similar conditions to the Frillfin goby, except for the water; the platies were housed in dechlorinated freshwater instead of seawater and were fed with commercial fish flakes (TetraMin Tropical Crisps®, Melle, Germany).

Chemical stimuli

The skin extract was prepared as described by Giaquinto and Volpato (2001). The skin extract donor fish were sacrificed by cephalic contusion without the use of anesthetics to prevent interference from chemical odorants. Twenty-five superficial skin incisions were then made on both sides of the animal over the entire body. Because of the size difference between the species, we used three platies for each Frillfin goby. The fish (one goby or three platies) were placed in a beaker with 200 ml of chilled distilled water (extract eluent) and carefully swirled for 5 min. The mixture was filtered through 185 mm filter paper (40 Whatman) to remove any remaining tissue fragments. The remaining solution was kept on ice during the experimental procedures. A 10-ml volume of the filtrate was applied to the tank containing the test fish using a syringe. The extract was carefully injected onto the surface of the water from behind a curtain (based on Gibson and Mathis, 2006). The volume of filtrate injected into experimental aquaria was based on a pilot study. In brief, we quantified the opercular beat rate (OBR) of the Frillfin goby 3 min before and after stimulus exposure and found that 10 ml was a suitable volume to use in this study (mean ± SD; OBR before stimulus = 45.2 ± 5.2 beats/min and OBR after stimulus = 74.7 ± 17.2 beats/min; paired Student's *t* test, n = 7, $t_6 = 3.99$ and $p = 0.0072$).

Experiment 1: club cells and the effects of skin extracts on Frillfin goby behavior

The basic strategy of this experiment was to evaluate the behavioral response of fish exposed to conspecific skin extracts (alarm substance) compared to fish exposed to skin extracts from an allopatric heterospecific fish, the platyfish *X. maculatus* (control chemical cue). This heterospecific chemical cue was used to test if the response to Frillfin goby skin extract was specific to Frillfin goby or a general response to chemical stimuli from injured fish, as platies are phylogenetically distant from gobies and are therefore unlikely to contain the same chemical alarm cues (based on Larson and McCormick, 2005; Manassa et al., 2013). In addition, distilled water was used as the extracts eluent control. Thirty-nine Frillfin gobies (n = 13 for each condition) from the stock population were randomly selected and housed in individual glass aquaria (40 × 23 × 25 cm; 1 fish/aquarium). The fish were acclimated to the conditions of the experimental aquaria for five days. The fish were fed daily as described above, and the leftover food was removed. After this period, fish antipredator behavior was assessed for 3 min before stimulus (baseline). Next, a chemical stimulus was injected into the experimental aquaria, and fish antipredator behavior was assessed 15 s later for an additional 3 min (post-stimulus period). A methylene blue dye test demonstrated that the colorant was completely spread throughout the aquarium in <15 s.

Antipredator behavior

The behavior was quantified using videotape analysis, and the observer was blinded to the treatment. The behaviors evaluated were swimming activity and sheltering. To assess swimming activity, we drew a grid with nine quadrants of identical area on the rear walls of the experimental aquaria (40 × 20 × 30 cm) and quantified the frequency of quadrant changes (Barreto et al., 2010). Sheltering was quantified in a binary manner by inspecting the position of the fish relative to the shelter (inside or outside) every 10 s during both 3-min observation periods (before and after chemical stimuli injection), totaling 18 observations per period. Each experimental aquarium was equipped with a shelter consisting of a 10-cm^2 tile supported by four transparent plastic rods that were 3 mm in diameter and 4 cm tall, located at the right rear wall corner of each aquarium. Additionally, the experimental aquaria were equipped with air stones, each of which was connected to an air pump by nontoxic silicone tubing for constant aeration. The water temperature was ~24 °C, and the pH was approximately 8.0. The concentrations of nitrite and ammonia were lower than 0.5 and 0.01 mg/l, respectively. The Frillfin goby is a euryhaline species that experience daily variation in water salinity (brackish to seawater and *vice versa*) due to daily flood of seawater or river water in high tide and low tide, respectively. Hence, held the gobies in artificial seawater conferred a suitable salinity in this study. Artificial sea water was used to avoid any environmental scent that might be present in natural seawater. The photoperiod and illumination were natural.