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Behavioral characterization of the alarm reaction and anxiolytic-like effect of acute treatment with fluoxetine in piauçu fish

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ABSTRACT

In Ostariophysan fish, the detection of the alarm substance liberated into the water as a consequence of an attack by a predator elicits an alarm reaction or anti-predatory behavior. In this study, experiments were performed to: (i) describe and quantitatively characterize the behavioral and ventilatory responses in piauçu fish (*Leporinus macrocephalus*), individually and as part of a school, to conspecific alarm substance (CAS) and; (ii) test the effect of acute fluoxetine treatment on alarm reaction. Histological analysis revealed the presence of club cells in the intermediate and superficial layers of the epidermis. The predominant behavioral response to CAS was freezing for fish held individually, characterized by the cessation of the swimming activity as the animal settles to a bottom corner of the aquarium. Fish exposed to CAS showed decrease in the mean ventilatory frequency (approximately 13%) relative to control. In schools, CAS elicited a biphasic response that was characterized by erratic movements followed by increased school cohesion and immobility, reflected as an increased school cohesion (65.5% vs. −5.8% for controls) and in the number of animals near the bottom of the aquarium (42.0% vs. 6.5% for controls). Animals treated with single i.p. injections of fluoxetine (10 μg/g b.w.) did not exhibit alarm behavior following CAS stimulation. These results show that an alarm pheromone system is present in piauçu fish, evidenced by the presence of epidermal club cells and an alarm reaction induced by CAS and consequently of a chemosensory system to transmit the appropriate information to neural structures responsible for initiating anti-predator behavioral responses. In addition, fluoxetine treatment caused an anxiolytic-like effect following CAS exposure. Thus, the alarm reaction in piauçu can be a useful model for neuroethological and pharmacological studies of anxiety-related states.

1. Introduction

Predation is a strong selective force that shapes many behavioral, developmental and morphological traits in prey animals [1]. Prey animals are able to assess predation risk using environmental cues, which may be visual, chemical, electrical or mechanical in nature [2]. Anti-predator behavior in Ostariophysan fish may be elicited by chemical cues, including alarm substances and predator odors [1–3]. Specifically, Ostariophysan fish possess an alarm pheromone that warns conspecifics about predator activity. Pfeiffer et al. have suggested that the alarm pheromone may be hypoxanthine-3-*N*-oxide, a chemical that is contained in large club cells found in much of the epidermis covering the body of the fish [4,5]. However, hypoxanthine-3-*N*-oxide may not be the only active molecule in the Ostariophysan alarm system since any compound with a nitrogen-oxide functional group can potentially act as an alarm signaling agent [6–8].

The alarm behavior elicited by exposure to the alarm substance, classically termed "Schreckreaktion" (fright reaction) by von Frisch [9], consists of a set of behaviors and physiological responses [10] that may protect fish from nearby active predators. Although it varies among species, the fright reaction may include rapid dashing (fugue), immobility, area avoidance and increased school cohesion [11]. Following exposure to an alarm substance, European minnows (*Phoxinus phoxinus*) show bradycardia [12], whereas the pearl dace (*Semotilus margarita*) and coho salmon (*Oncorhynchus kisutch*) exhibit increased plasma glucose and cortisol levels [13,14]. However, such studies investigating the correlated physiological responses are scarce.

There is substantial evidence in the literature implicating the serotonergic (5-HTergic) system as a mediator of emotional responses in

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animals and humans [15]. 5-HT primarily plays an inhibitory role in the expression of aggression and has been shown to influence the dynamics of agonistic interactions [16]. Fish exhibit the same general relationship between dominance, aggression and 5-HT levels as do other vertebrates, such that dominance and aggression, along with exposition to stressors, reduce the activity of the 5-HTergic system in the central nervous system of fish [16–19].

The present study was undertaken to: (i) describe and quantitatively characterize the behavioral and ventilatory responses of the South American freshwater fish, piauçu (*Leporinus macrocephalus*), to a conspecific alarm substance and (ii) test the effect of acute fluoxetine treatment, a selective 5-HT reuptake inhibitor (SSRI), on its alarm reaction.

2. Methods

2.1. Animals and holding conditions

Experiments were conducted using a total of 96 freshwater piauçu fish, *L. macrocephalus* (ranging from 10 to 12.5 cm in standard length). Piauçu is a Brazilian non-migratory omnivorous fish which occurs in waters with a relatively high oxygen content and can be captured in river channels especially near the vegetation. Animals were raised in captivity and obtained from a local commercial distributor and were acclimated at the laboratory for a minimum of 10 days prior to experimentation. Since juveniles were used and sexual dimorphism is absent, the effects of sex, if any, were ignored. Piauçu fish were held individually (61 animals) or in schools (a total of 35 animals were divided in 7 schools with 5 fish in each) in glass aquaria ($30\times22\times20$ cm and $70\times25\times20$ cm, respectively) containing dechlorinated tap water at 26 ± 1 °C and kept on a 12:12 h light/dark cycle. All aquaria were fitted with a filtration system and contained substrate on the bottom. The animals were fed *ad libitum* once a day with commercial flake food (Nutripeixe AL45, PURINA). Feeding was discontinued 24 h before the experiments [20].

2.2. Conspecific alarm substance

Conspecific alarm substance (CAS) was obtained by sacrificing ten juvenile piauçu fish via blows to the head and then removing skin fillets from both sides of the body. Approximately 4 cm^2 of skin was homogenized in 10 mL of distilled water (DW) at 29,000 rpm for 1.5 min (Ultra Stirrer Homogenizer, Ultra380). The homogenate was filtered to remove scales and remaining tissues. The CAS aliquots were immediately frozen and stored at −20 °C until required. Alarm substance was injected into the aquarium water with a syringe connected to a polyethylene tube. The mean time for the introduction of the tube, injection and diffusion of the substance into the aquarium water was approximately 3 s, 5 s and 6 s, respectively.

2.3. Histological analysis of the epidermis

Ten piauçu fish were sacrificed by immersion in tricaine methasulfonate (MS222 0.2 g/L; Sigma, St. Louis, MO), and skin fillets were removed from both sides of the body and preserved in 4% formaldehyde in phosphate buffer 0.1 M. These samples were dehydrated through a standard ethanol series to 100% ethanol, cleared in xylenes, embedded in paraffin and sliced into 7 μm sections. Sections were deparaffinized, stained with periodic acid-Schiff's reagent (PAS) and counterstained with Harris hematoxylin [11,21]. Slides were observed and photographed using a microscope Leica DM5500 B equipped with a digital color camera Leica DFC290. The software Leica Application Suite 3.6 was used for morphometric analysis.

2.4. Drug and administration procedure

Fluoxetine hydrochloride (N-Methyl-3-[(4-trifluoromethyl) phenoxy]-3-phenylpropyla mine hydrochloride; Tocris Bioscience, St. Louis, MO) was dissolved in teleost Ringer's solution (saline) one day before the experiments and stored at 4 °C. Intraperitoneal (i.p.) injections of the saline or fluoxetine were made using as a reference the midline of the ventral surface of the fish, 5–10 mm anterior to the pelvic girdle using a 1 mL insulin syringe and a 28.5 G needle. We chose to use 10 μg/g body weight (b.w.) based on success with this dose in previous behavioral studies that assessed the acute effect of fluoxetine treatment in fish [15,18]. The volume injected ranged from 0.12 to 0.16 mL according to body weight. The fluoxetine used in this study is a mixture of the R- and S-isomers.

2.5. Experimental procedures

2.5.1. Behavioral responses in solitary fish

A total of 16 animals were maintained individually in glass aquaria ($30\times22\times20$ cm) and divided in two groups: control animals ($n=9$) exposed to 1 mL of DW and; experimental animals ($n=9$) exposed to 1 mL of CAS. Behavior and locomotion (to be described below) were assessed during two consecutive observation periods (baseline and post-stimulus) of 10 min each.

2.5.2. Behavioral responses in school

A total of 35 animals were divided in 7 schools with 5 fish in each school. The distance of each school member to the center of the school (school cohesion) and the number of animals near the bottom were analyzed at scan intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min examining isolated video frames (scan sampling) for each consecutive observation periods: baseline; introduction of 1 mL of DW and; introduction of 1 mL of CAS into the aquarium.

2.5.3. Ventilatory responses

A total of 7 animals were used in this experiment. To measure the ventilatory frequency (VF), fish were individually placed in a cuvette ($31\times5\times5$ cm) containing aerated freshwater for 1 h to acclimate to the experimental environment. The VF is expressed in beats/min and was calculated by visually counting the time necessary for twenty successive opercular or buccal movements to occur [adapted from 23]. Counting of opercular/bucal movements was done minute by minute during 10 min of each consecutive observation period: baseline; introduction of 0.1 mL of DW and; introduction of 0.1 mL of CAS into the cuvette. The VF was normalized for each condition (DW or CAS conditions) and is represented as the means of the delta values (difference between post-stimulus and baseline values) expressed in percentages with baseline value set at 100%.

2.5.4. Effects of acute fluoxetine on the alarm reaction

A total of 16 animals were divided in two groups: 8 animals were treated with a single i.p. injection of saline and 8 animals received fluoxetine hydrochloride (10 μg/g b.w). One hour after the i.p. injection, behavior and locomotion were assessed during three consecutive observation periods (baseline; introduction of 1 mL of DW and; introduction of 1 mL of CAS into the aquarium) of 10 min each.

2.6. Behavioral responses and quantitative evaluation of locomotion

Behavioral experiments were conducted between 11 am and 1 pm. During the experiments, fish were monitored by a VHS video camera placed in front of the aquarium. Behavioral responses to CAS in solitary fish were assigned to one of the following five categories according to the ethogram described by Lawrence and Smith [21]: (1) increase, defined by rapid swimming activity; (2) slowing, characterized by decreased locomotion occasionally interrupted by bursts