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ORIGINAL PAPER

Mianserin affects alarm reaction to conspecific chemical alarm cues in Nile tilapia

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Abstract In this study, I show that mianserin, a chemical with serotonin and adrenoceptor antagonist activities, increases fish vulnerability to a potential predator threat, when prey fish must deal with this threat based on conspecific chemical alarm cues. For that, I evaluated whether mianserin, diluted in the water, influences the behavioral responses of Nile tilapia (*Oreochromis niloticus*) to conspecific skin extract (chemical alarm cues). I found that, while mianserin did not abolished antipredator responses, this drug mitigates some components of this defensive reaction. Thus, a potential decrease in serotonin and adrenergic activities reduces the ability of dealing with predators when perceiving conspecific chemical alarm cues.

Keywords Chemical communication · Stress · Anxiety · Alarm substance · Mianserin

Introduction

In some fish species, putative chemical alarm substances (CAS) are thought to be produced and stored in the epidermal club cells and they are released when the epidermis is damaged, as in an injurious predator attack (Chivers and Smith 1998). In consequence, CAS induce antipredator behavior in conspecific fish, as documented for many fish species: speckled catfish (*Pseudoplatystoma coruscans*—Giaquinto and Volpato 2001; Barreto et al. 2012), convict cichlid (*Amatitlania nigrofasciata*—Alemadi and Wisenden 2002), zebrafish (*Danio rerio*—Speedie and Gerlai 2008; Gerlai 2010), Nile tilapia (*Oreochromis niloticus*—Barreto et al. 2010; Sanches et al. 2015), piauçu fish (*Leporinus microcephalus*—Barbosa-Junior et al. 2012), frillfin goby (*Bathygobius soporator*—Barreto et al. 2014), rainbow trout (*Oncorhynchus mykiss*—Kopack et al. 2015) and several Pomacentridae coral reef fishes (Mitchell et al. 2012; Ramasamy et al. 2015).

Neurotransmitter activity modulates the defensive response to CAS. For instance, fluoxetine (a selective serotonin reuptake inhibitor drug) administration caused an anxiolytic-like effect in fish exposed to CAS, abolishing the antipredator response (see Barbosa-Junior et al. 2012). The psychiatric drug mianserin is a second-generation tetracyclic antidepressant and a potent serotonin (5-hydroxytryptamine [5-HT]) receptor (5-HT2 family) and α2-adrenoceptor antagonist. In fact, in fish, mianserin has biological effects: it causes endocrine disruption (van der Ven et al. 2006). These authors showed that mianserin has an estrogenic activity, disturbing the Hypothalamo–Pituitary–Gonadal (HPG) axis. Regarding defensive

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reactions, there is no study on the effect of mianserin in fish. However, it is plausible to assume this potential effect in fish, since, in mammals, mianserin changes defensive behaviors (Griebel 1993; Griebel et al. 1997).

Based on these above-mentioned evidences, mianserin can be elected as a potential drug that could modulate fish antipredator behavior induced by CAS, due to changes in serotoninergic and adrenergic activities. In this study, operationally, I evaluated whether mianserin, diluted in the water, influences the behavioral responses of Nile tilapia (*O. niloticus*) to conspecific skin extract (CAS). This species was used as experimental model because it displays alarm reaction to CAS (Barreto et al. 2010; Sanches et al. 2015), hence, a suitable animal to test my hypothesis.

Materials and methods

Fish

The Nile tilapia (*O. niloticus* L.) (both sexes, ∼6.7 cm—standard length and ∼10 g—body mass) utilized here were a progeny reproduced in indoor tanks, whose the parental specimens were previously acquired from fishfarms. The tilapia were allocated in two stock tanks (total volume = 310 L, water volume = 280 L, holding density = ∼1.3 g/L). Stock tanks were equipped with biological–mechanical filters and had a continuous flow of water for constant water renewal. Water was kept in ∼26 °C, ammonia and nitrite about <0.5 ppm and <0.05 ppm, respectively, and pH = ∼7.0. I set a light–dark cycle of 12 h: 12 h, controlled by timer with abrupt transition from light to dark and vice versa. The illumination was provided by using artificial fluorescent lamps (day–light type) with about 120 lux at the tanks water surfaces. Fish were fed once a day with commercial fish pellets (32 % of protein) in an amount of ∼5 % of fish biomass. The leftover food and fish feces were sucked by the tank drainage system, avoiding organic matter accumulation.

Experimental design

My general experimental strategy consisted of 2 × 2 design. For that, mianserin solution or deionized water (drug-vehicle control) was administered by adding

them into water of the experimental aquaria. After 24 h of exposure to mianserin or vehicle, I quantified the responses of fish to CAS (skin extract) or deionized water (extract eluent control) immediately after injection of these stimuli into the aquaria. For complete details of experimental schedule, see Fig. 1. In addition, a two-way ANOVA revealed no difference in fish length or weight among these four experimental conditions (see Table 1 for details).

As previously reported, Nile tilapia displays specific behavioral responses to chemical cues obtained from conspecific skin extract (Sanches et al. 2015), or even conspecific blood (Barreto et al. 2013), when compared to chemical stimuli from heterospecific fish that are phylogenetically distant from tilapia and are, therefore, unlikely to contain the same chemical alarm cues. Thus, a heterospecific chemical cue was not conducted here as control stimulus, because tilapia responses are specific to conspecific cues and not a general response to any new scent.

Forty tilapias ($n = 10$ 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 10 days. The fish were fed daily as described above, and the leftover food was removed. After this period, fish antipredator behavior was recorded for 5 min before chemical stimulus (baseline). Next, a chemical stimulus was injected into the experimental aquaria, and fish antipredator behavior was recorded 10 s later for an additional 5 min (post-stimulus period). A methylene blue dye test demonstrated that the colorant was completely spread throughout the aquarium in ∼8 s. Behavior recording occurred from 11 to 14 h in a random order defined by raffle. During this interval, the plasma level of cortisol in Nile tilapia, a factor that affects alarm reaction when increased (Barreto et al. 2014), is low and it peaks during the early photophase (∼06 h) (Nikaido et al. 2010).

Mianserin

I administered mianserin hydrochloride (CAS number: 21535-47- 7) purchased from Tocris Bioscience (Ellisville, MO, USA). The drug was applied noninvasively by adding them directly into the water of the experimental aquaria. For drug application, 5 mg of mianserin was diluted into 50 mL of distilled water
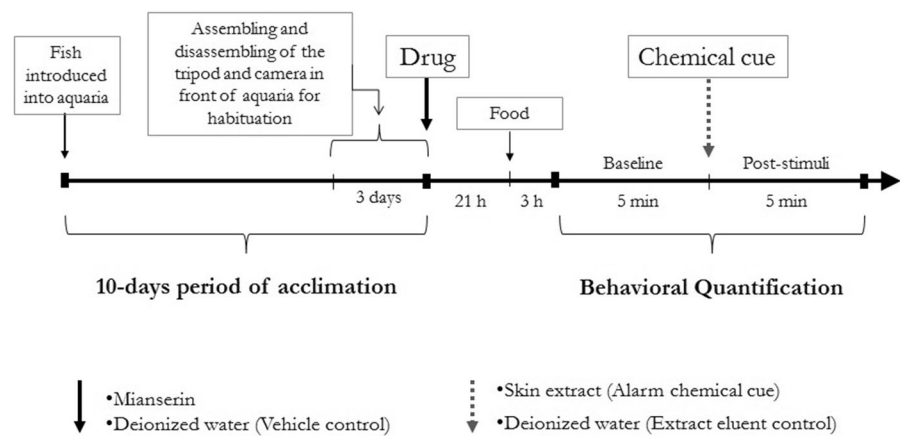
Table 1 Mean ($\pm$ SD; $n = 10$) standard length and body
mass of Nile tilapia *O. niloticus* according to experimental
conditions

Drugs	Chemical stimuli	Biometric variable	
		Length (cm)	Mass (g)
Vehicle	Extract eluent	6.7 $\pm$ 0.3	10.1 $\pm$ 1.4
	Skin extract	6.7 $\pm$ 0.2	9.8 $\pm$ 1.7
Mianserin	Extract eluent	6.6 $\pm$ 0.2	9.6 $\pm$ 1.4
	Skin extract	6.7 $\pm$ 0.3	10.0 $\pm$ 1.6

Both length and weight of fish were considered normally
distributed and homoscedastic according to Kolmogorov–
Smirnov test and Cochran's C test, respectively. Thus,
parametric statistics were conducted and I found no
significant difference for these biometric variables among
experimental conditions as revealed by two-way ANOVA
(lower P value was $p > 0.405$). Deionized water was either
drug vehicle or extract eluent

and 5 mL of this solution was injected into the aquaria
water (total water volume = 20 L). Thus, the con-
centration reached was 5 µg of mianserin/L of aquar-
ium water. Mianserin can cause endocrine disruption
at low concentration in water (25 µg/L) in zebrafish
(van der Ven et al. 2006). Here, to investigate the
potential adverse effects of and mode of action by
aquatic exposure to mianserin in fish behavior, I
sought to assess a concentration lower than the
successfully used by van der Ven et al. (2006). In
addition, zebrafish acutely exposed to fluoxetine
diluted in water (1 µg/L), a substance that also
interferes with serotonin activity, have changes in
stress responses (Abreu et al. 2014). This concentra-
tion was also utilized herein as a parameter for
conducted my procedures.

Chemical cues

The skin extract was prepared based on Brown et al.
(2004). For this, I used three tilapias coming from the
same stock tanks as those fish used in this experiment.
The fish were sacrificed by spinal cord section without
the use of anesthetics to avoid chemical interference.
Then the fish skin was carefully removed, delimiting a
trapezoidal area on both sides with the aid of a tweezer
and a scalpel, and immediately placed into 50 mL of
deionized water. The skin was homogenized and then
filtered using glass wool placed in a glass funnel to
remove the remaining fragments of tissue. The final
volume was adjusted with addition of deionized water,
and the final 'concentration' of tilapia skin extract was
2.6 mm^2 of skin/mL of deionized water (Barreto et al.
2010). I placed extracts into beakers and kept them
chilled by using frozen gel packs inside a Styrofoam
box for immediate use during the trial. The eluent of the
extracts, used as a chemical stimulus control, was kept
under identical conditions. A volume of 5 mL of skin
extract or eluent was applied to tests aquaria using a
syringe and during this procedure fish could not see the
investigator (Sanches et al. 2015). The chemical stimuli
were injected at the water surface just above the bubbles
produced by the air compressor to facilitate dispersion.

Fish behavior

The behavior was quantified by analyzing the videos
without knowing which treatment each fish had been
submitted. As indicators of antipredator responses, I
quantified the swimming activity, the time spent near
the tank bottom, the frequency of bristling of the

dorsal fin, the frequency of dashes and the frequency and duration of episodes of freezing.

To assess the swimming activity, I put a grid design on the back wall of the aquarium that had nine identical quadrants and quantified the frequency of quadrants changes (Barreto et al. 2010; Miyai et al. 2016). The presence of an animal in one quadrant was scored considering the bigger part of a fish body that was inside a quadrant. When it was not evident, I kept as reference point the head region. The evaluation of the presence of fish in quadrants over time allowed us to estimate the percentage of time fish spent in each of part of the aquarium (near surface or near bottom or in the middle of water column). I also considered here the time spent near the bottom, as it is a fish defensive response (Speedie and Gerlai 2008).

The Nile tilapia has spines in the dorsal fin. In context of confrontation, when paired with a conspecific competitor (Barki and Volpato 1998) or a predator (Freitas and Volpato 2008), they tend to keep dorsal fin bristled, consequently, dorsal spines as well. The frequency of bristling of the dorsal fin (FBDF) was binary quantified. When the position of the fin was completely bristled, I scored one (1), on the contrary zero (0). It was done every 10 s during the two intervals of 5 min of observation (before and after injection of chemical stimuli), totaling 30 observations in each period. If the dorsal fin was only partially bristle, I scored zero (0), because in this position, the spine does not form an angle (ideal angle = $\sim 90°$ considering the longitudinal body axis of tilapia) that could act as a defense weapon in the advent of predator bite.

A fast swimming in an escape route is considered a defensive maneuver, but in an aquarium that is not possible. However, we can see rapid and unplanned swim (dashing) from one side to another and from the bottom to the surface and vice versa (Giaquinto and Volpato 2001). Here, I also quantified the frequency of dashes as a defensive reaction.

Another defensive response in fish exposed to alarm substance is a freezing reaction (Lawrence and Smith 1989). I considered an animal was freezing when it is directed to the tank bottom, touching the gravel and the vertical wall of the aquarium (but generally they went to aquaria corners), and stood still, with little or no movement of the dorsal and caudal fins (Lawrence and Smith 1989). I recorded both frequency and duration of freezing episodes.

Statistical analyses

Normality and homoscedasticity were assessed by utilizing Kolmogorov–Smirnov test and Cochran's C test, respectively. The data of time at the bottom and FBDF reached these premises. The data of frequency and duration of freezing episodes and frequency of dashes achieved these premises only after log-transformation of the values [transformed value = log10 (value + 2)]. In the case of swimming activity, data were considered neither normally distributed nor homoscedastic even after square root or log-transformation. However, I reached these premises by subtracting baseline from post-stimulus values of each individual (Δ = post-stimulus−baseline). The data of swimming activity, freezing frequency and duration, and frequency of dashes were analyzed by two-way ANOVA, having drugs (mianserin or vehicle) and chemical cues (skin extract or extract eluent) as independent categorical factors. For time spend near the tank bottom and FBDF, I had the same categorical factors, but data were compared by a three-way mixed ANOVA, once I included time (baseline or post-stimuli—after chemical cues exposure) as repeated measure. When necessary ANOVAs were complemented by post-hoc test of Duncan. Significant statistical differences were considered when $p < 0.05$.

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.

Results

Swimming activity

I found a significant effect of chemical cues on swimming activity ($F_{(1;36)} = 6.54$; $p = 0.0149$), but no effect of the drug ($F_{(1;36)} = 0.25$; $p = 0.62$) or interaction between these factors ($F_{(1;36)} = 0.005.54$; $p = 0.94$). This finding denotes that skin extract induced swimming activity to decrease compared to fish exposed to extract eluent (Fig. 2).

Time spent near the aquaria bottom

I found no significant effect of drugs, chemical cues or time on the time at the bottom (Fig. 3a). In addition, no significant interaction among these variable was also revealed by ANOVA (Fig. 3b).

The frequency of bristling of the dorsal fin

I found no significant effect of drugs, chemical cues or time on FBDF and also no significant interaction among them (Fig. 3b). However, I conducted an extra analysis considering the fish that received vehicle and were exposed to skin extract by using Wilcoxon matched pairs test. This test revealed a significant increase in FBDF ($Z = 2.20$; $p = 0.0277$) induced by skin extract (Fig. 3b).

Frequency and duration of freezing episodes

I found a significant effect of chemical cues for both frequency ($F_{(1;36)} = 12.79$; $p = 0.0010$; Fig. 4a) and duration ($F_{(1;36)} = 13.36$; $p = 0.0008$; Fig. 4b) of freezing episodes. For both variables, an increased level was observed in response to skin extract compared to extract eluent, independently of the drug that fish were exposed.
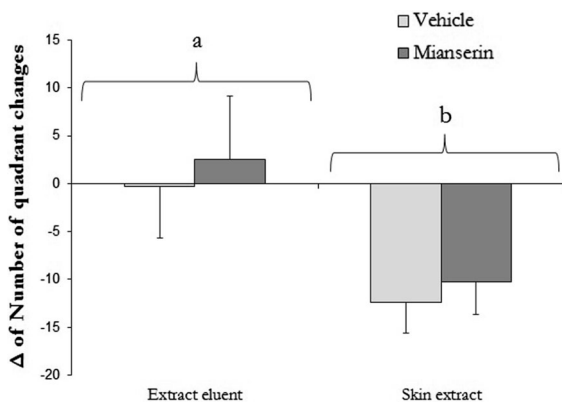

Fig. 2 Mean ($\pm$SD; $n = 10$) of changes in swimming activity of Nile tilapia *O. niloticus* treated with mianserin or vehicle and exposed to chemical alarm cue or eluent. A two-way ANOVA revealed a significant effect of chemical cues on swimming activity ($F_{(1;36)} = 6.54$; $p = 0.0149$), but no effect of the drug ($F_{(1;36)} = 0.25$; $p = 0.62$) or interaction between these factors ($F_{(1;36)} = 0.005.54$; $p = 0.94$). *Different letters* denote a statistical significant difference
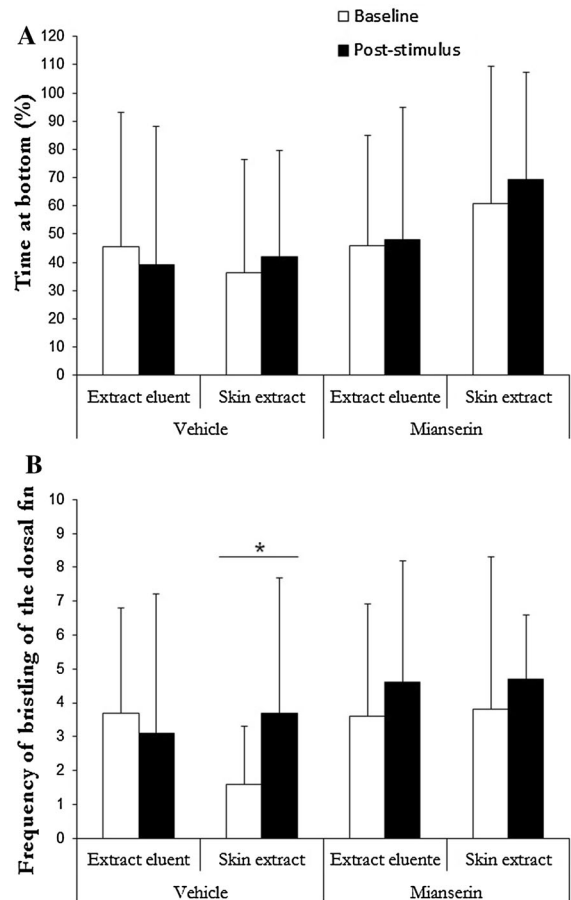

Fig. 3 Mean ($\pm$SD; $n = 10$) time at the bottom (**a**) and FBDF (**b**) of Nile tilapia *O. niloticus* treated with mianserin or vehicle and exposed to chemical alarm cue or eluent. A three-way mixed ANOVA showed no significant effect of drugs, chemical cues, time or interactions between these factors on the time at the bottom and FBDF. An extra analysis considering the fish that received vehicle and were exposed to skin extract revealed a significant increase (*) in FBDF (Wilcoxon matched pairs test; $Z = 2.20$; $p = 0.0277$) induced by chemical alarm cue (**b**)

Frequency of dashes

The most important result was observed for dashing response. I found a significant interaction between drugs and chemical cues ($F_{(1;36)} = 4.55$; $p = 0.0399$; Fig. 5). Fish exposed to skin extract displayed a higher frequency of dashes than those exposed to extract eluent, but this effect was weaker for fish treated with mianserin, indicating that this drug mitigates dashing in the Nile tilapia.
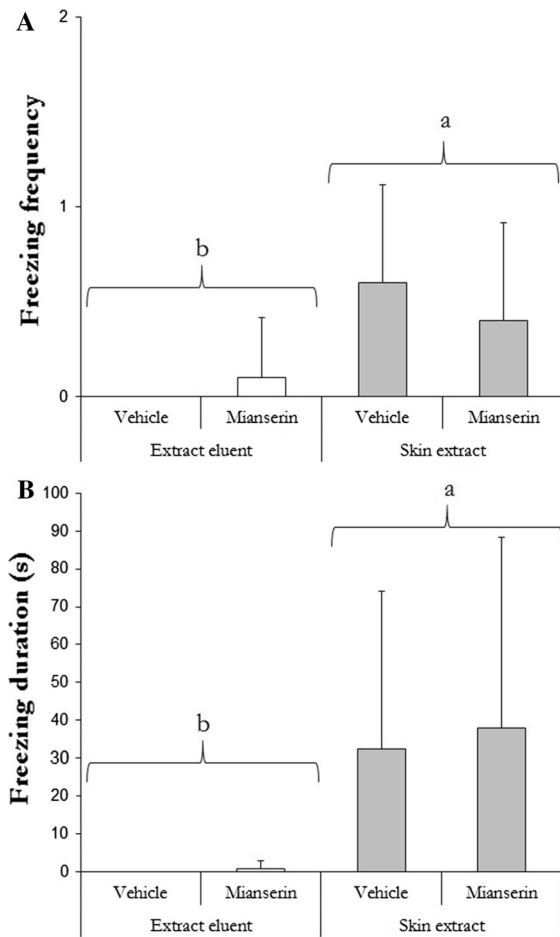
Fig. 4 Mean (±SD; $n = 10$) of freezing frequency (**a**) or duration (**b**) of Nile tilapia *O. niloticus* treated with mianserin or vehicle and exposed to chemical alarm cue or eluent. A significant effect of chemical cues for both frequency ($F_{(1;36)} = 12.79$; $p = 0.0010$; **a**) and duration ($F_{(1;36)} = 13.36$; $p = 0.0008$; **b**) of freezing episodes was showed by two-way ANOVA. For both variables, an increased level was observed in response to skin extract compared to extract eluent, independently of the drug (mianserin or vehicle) that fish were exposed. *Different letters* denote a statistical significant difference

Fig. 5 Mean (± SD; $n = 10$) of dashes frequency of Nile tilapia *O. niloticus* treated with mianserin or vehicle and exposed to chemical alarm cue or eluent. A significant interaction between drugs and chemical cues ($F_{(1;36)} = 4.55$; $p = 0.0399$) was revealed by two-way ANOVA. Fish exposed to skin extract displayed a higher frequency of dashes than those exposed to extract eluent, but this effect was weaker for fish treated with mianserin, indicating that this drug mitigates dashing in the Nile tilapia. *Different letters* denote a statistical significant difference

Discussion

The administration of mianserin modified the expression of alarm reaction to conspecific alarm substance (CAS) in the Nile tilapia. Fish exposed to mianserin still present decreased activity and freezing reaction in response to CAS. They decreased, however, the expression of dashing and were not prompt to increase the bristling of dorsal fin spines. A modification in fish antipredator behavior can consequently influence fish
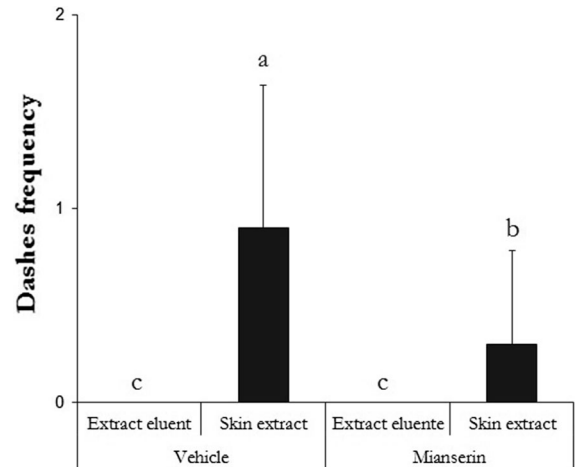
ability to deal with threats arising from potential predators. Thus, a potential decrease in serotonin and adrenergic activity, due to mianserin effects, decrease the ability of dealing with predators when perceiving the CAS. Mianserin is a potent serotonin—5-HT receptors (mainly 5-HT2A and 5-HT2C)—and α2-adrenoceptor antagonist. The 5-HT2A and 5-HT2C receptors have been identified in fish brain (Norton et al. 2008; Schneider et al. 2012), including the diencephalon, where hypothalamus is located (Mager et al. 2012). The hypothalamus is a brain structure linked to defensive reactions in fish (Winberg et al. 1993; Wolkers et al. 2015). The α2-adrenoceptors are G-protein-coupled receptors and are found in fish brain, mediating many of the physiological effects of norepinephrine and epinephrine (Ruuskanen et al. 2005), a system linked to defensive reactions ('fight or flight') (Perry and Bernier 1999). Based on these initial evidences, future studies must address the effects of more specific drugs to continue to elucidate the neural mechanisms underlying the alarm reaction to CAS in fish and potential influences of environmental presence of psychoactive drugs.

The administration of mianserin did not abolish the ability of fish to display a decrease in swimming

activity by exposure to CAS. The freezing component of antipredator behavior was also not abolished or decreased in the tilapia, and it occurred at the same magnitude (duration) of fish untreated with mianserin. Thus, mianserin-treated fish still display defensive reaction when detecting CAS. However, other components of antipredator behavior were modified and can negatively affect the way tilapia must deal with predators. In this context, they had a decreasing in dashing frequency, a response related to escape maneuvers. This finding suggests that fish exposed to mianserin have a reduced capability to flee. In addition, I only detected an increase in bristling of the dorsal fin spines in fish exposed to vehicle and alarm substance. Fin bristling is a way of positioning tilapia weaponry in an angle ($\sim 90°$) that could hurt a predator's mouth when it is biting a tilapia, allowing them to escape (Freitas and Volpato 2008). Therefore, mianserin-treated fish did not show an anticipatory properly positioning of their weaponry for a potential use.

The physiological state of a fish can affect alarm response (Giaquinto and Volpato 2001; Barbosa-Junior et al. 2012; Barreto et al. 2014) and potentially influence ecological processes linked to food webs, because the characters related to foraging and feeding are crucial to these processes. Among them, the use of predatory signals to detect and respond to them appropriately has strong relevance for prey survival. The movement of the prey is used as a key stimulus for numerous types of predators to orient their first contact with the prey (Lima and Dill 1990; Burrows 1994; Burrows and Gibson 1995). Therefore, reducing the activity and/or displaying freezing can be considered a way to reduce the susceptibility of the prey to be detected by predators (Houtman and Dill 1994; Gyssels and Stoks 2005). We can consider, therefore, that a factor that induces decreased activity and/or increases the frequency and/or duration of freezings improves the survival probability of a prey. As an example, frillfin goby *B. soporator* treated with cortisol increase immobility and the strategy of refuging in burrows in response to CAS (Barreto et al. 2014). In this context, cortisol appears to induce an adaptive response to potentially increase prey capability of defense. In this study, fish were able to decrease their activity in response to CAS, as well as, were capable of exhibiting freezing. However, mianserin did not improve these components of the

antipredator behavior, because they just were not abolished. In this context, there is a cost–benefit relationship of remaining immobile (or less active) or fleeing. In fact, the latency to escape after immobility decreases as the probability of success of a predator attack increases (Martin et al. 2009). The display of fast swimming in an escape route (dashing) is required to evade an unavoidable attack of a predator. As mianserin decreased the expression of dashings and there was not an increase in dorsal fin bristling exposing the spines, which could be an effective defense to an inevitable bite of a predator, I conclude that this drug increases the fish vulnerability to a predator attack.

As shown herein, mianserin can affect fish response to CAS; hence, future investigations must address potential effect of this drug in trace levels, in order to elucidate whether the environmental concentrations of mianserin could represent an environmental problem, as have been shown for other drugs (Brodin et al. 2013). In fact, mianserin has been found in trace levels in aquatic environment: in sewage effluents tested in Sweden was present at a concentration in the order of 10 ng/L (Fick et al. 2010) and one of 0.9 ng/L was detected in rivers in Poland (Giebultowicz and Nalecz-Jawecki 2014). In addition, a Europe-wide study identified average concentration of mianserin in wastewater effluents to be 1.5 ng/L (European Union, 2012—http://publications.jrc.ec.europa.eu/repository/bitstream/JRC76400/lb-na-25563-en.pdf). This effect might be still potentially worse, because some psychoactive drugs diluted in the water (such as diazepam, fluoxetine, risperidone and buspirone) were found to be attractive to the fish (Abreu et al. 2016) and, in light of that fact, fish could exposed oneself in areas with chemicals that would change negatively its behavior.

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