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# Reduction of affected limb use after noxious stimulus in fish: is it an indication of pain sensation?

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**ABSTRACT.** The mobilization of an organism in response to noxious stimulation varies according to the degree of evolution of the nervous system, and the response, which is considered species-specific, depends on the type of noxious stimulus applied. Knowledge about the specific response of a species to a noxious stimulus is crucial to understanding the evolution of nocifensive responses and to providing information about the signals that can indicate discomfort or pain. The present study evaluated the behavioral and hormonal nociceptive responses of *Oreochromis niloticus* treated by subcutaneous injection of vehicle or formaldehyde 3% in the posterior region of the dorsal fin. Fish receiving formaldehyde injection spent less time with the soft ray dorsal fin moving and more time in a lowered position than vehicle-treated fish, and there was a negative correlation between these two variables in formaldehyde-treated fish. A skin-darkening pattern was identified in fish who died as a result of the noxious stimulus. The speed and distance traveled, plasma cortisol, body and eye darkening, and time spent with the spine ray dorsal fin erect were not significantly different between the treatments. The formaldehyde test activated the nociceptive system in *O. niloticus* and confirmed the hypothesis that the responses presented to this test are species-specific. In addition, the reduction of affected limb use (soft ray dorsal fin), similar to that observed in mammals submitted to the same test, indicates that pain sensation may be present in these animals.

**Keywords:** behavior; cortisol; fish; nociception; pain.

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## Introduction

Nociception comprises the process of detecting stimuli that produce pain through primary sensory neurons known as nociceptors (Julius & Basbaum, 2001). These potentially tissue-damaging stimuli are carried to the central nervous system from the spinal cord and processed into subcortical regions, such as the brainstem and other regions that promote adequate defensive and retrieval responses (for a review see Basbaum, Bautista, Scherrer, & Julius, 2009). Defensive responses can be observed in a wide range of animal groups, from invertebrates, that are capable of reacting to a noxious stimulus, to mammals, and the degree of mobilization of the organism in response to these stimuli varies according to the evolution of the nervous system (for a review see Smith & Lewin, 2009).

Some studies have shown that fish have free nerve endings that resemble the nociceptors present in mammals (Dunlop & Laming, 2005; Roques, Abbink, Geurds, van de Vis, & Flik, 2010; Sneddon, 2002; Sneddon 2003b; Sneddon, Braithwaite, & Gentle, 2003; Sneddon, 2019). They also have pathways for conducting information to the brain (Chandroo, Duncan, & Moccia, 2004; Dunlop & Laming, 2005) and present behavioral and physiological responses when receiving potentially harmful stimuli (Alves, Barbosa Júnior, & Hoffmann, 2013; Reilly, Quinn, Cossins, & Sneddon, 2008; Roques et al., 2010; Roques et al., 2012; Sneddon, 2003a; Wolkers, Barbosa Junior, Menescal-de-Oliveira, & Hoffmann, 2013; Magalhães et al., 2017; Nilsson et al., 2019). These lines of evidence indicate that the processing of nociceptive information, as well as the responses triggered by it in fish, could bear similarities to the mammalian nociceptive system.

Knowledge about nociception in basal vertebrates such as fish is still incipient, and the tests used to evaluate nociception are mostly based on those employed in mammals, in which termal, mechanical and chemical noxious stimuli are applied, such as the use of clips on the fin (Roques et al., 2010), alterations in water temperature (Nilsson et al., 2019), as well as the use of intraperitoneal or subcutaneous injections of acetic acid (Sneddon, 2003a; Sneddon et al., 2003; Costa et al., 2018) and formaldehyde in the fin, tail or lips (Alves et al., 2013; Ide & Hoffmann, 2002; Wolkers et al., 2013; Wolkers, Barbosa Junior, Menescal-de-Oliveira, & Hoffmann, 2015a; 2015b; Magalhães et al., 2017), which is especially interesting because it is a methodology very similar to that applied in mammals (Dubuisson & Dennis, 1977). The formaldehyde test was adapted by Ide and Hoffmann (2002) for the study of nociception in fish and applied in other studies by Alves et al. (2013) and Wolkers et al. (2013, 2015a, 2015b); however, the behavioral nociceptive response using formaldehyde injection was not evaluated in *Oreochromis niloticus*.

Therefore, it is essential to verify the applicability of the formaldehyde test in other fish species to verify the hypothesis that these behavioral responses are species-specific in order to broaden the knowledge regarding nociception within the vertebrate phylum. In addition, knowledge about the behavioral response to nociception specific to a species is an important tool for the detection of exposure to discomfort and noxious stimuli. The Nile tilapia is a widely farmed species around the world, and the knowledge about the signs of discomfort exhibited by this species is crucial to ensuring the welfare of these fish in captivity. Thus, the objective of this study was to evaluate the behavioral and hormonal nociceptive responses in the fish Nile tilapia (*Oreochromis niloticus*) triggered by subcutaneous injection of 3% formaldehyde in the posterior region of the dorsal fin.

## Material and methods

### Husbandry and set-up

A total of 20 juvenile male Nile tilapia (weight 39.46 ± 5.97 g), approximately 2 months old, were used. The animals were obtained from the Institute of Biosciences, Letters and Exact Sciences (IBILCE) and were kept in stock tanks (1,000 L) with constant aeration and water circulation (6.25 mL $s^{-1}$) until the experiments. Three days before the experiments, they were transferred to individual glass aquaria (~20 L) in a closed system with aerated water (temperature: 26°C ± 1°C; unionized ammonia ($NH_3$): lower than 0.04 mg $L^{-1}$). During the experiment, the water was not replaced to avoid disturbance. The side walls of the aquaria were covered with opaque white paper to isolate fish from visual stimuli of conspecifics in neighboring aquaria. During the acclimation period, the fish were subjected to a light/dark cycle of 12:12h (start 07:00 a.m. and end 07:00 p.m.) and fed daily with pelleted food corresponding to 3% of their biomass. Feeding was stopped 24 hours before the experiments. All of the experiments were conducted at the same time of day (between 7:00 and 10:00 a.m.) to avoid circadian interference.

### Experimental procedures

In this study, subcutaneous injection of 3% formaldehyde was used as a noxious stimulus, replicating the nociceptive formaldehyde test developed in rats and cats (Dubuisson & Dennis, 1977) and adapted for nociceptive studies in fish (Ide & Hoffmann, 2002; Alves et al., 2013). The formaldhyde 3% solution was produced through dilution of formol solution (formaldehyde 37%). A camera (Sony HDR-CX190) was positioned toward the anterior face of the aquarium, and the fish was filmed for 10 minutes (baseline, BL). Immediately after this period, the fish was carefully removed from the aquarium using a nylon net and received a subcutaneous injection of 20 µl of vehicle or 3% formaldehyde solution in the posterior region of the dorsal fin (vehicle, n = 8, formaldehyde, n = 12). After injection administration, the fish was returned to the aquarium and filmed for 10 minutes (post-stimulus, PE). Then, the fish were removed from the aquarium and anesthetized by immersion in water containing benzocaine (100 mg $L^{-1}$), and their blood was withdrawn from the caudal vein with a heparinized sterile syringe. The blood was centrifuged (3,000 rpm for 10 minutes at 4°C), and the plasma obtained was used to determine the cortisol concentration (kit DRG® Cortisol ELISA - EIA-1887). The fish were randomly assigned to vehicle or formaldehyde injection prior to the beginning of the experiment.

For behavioral analysis, locomotor activity, position of the spine rays of the dorsal fin and the soft rays of dorsal fin, and movement of the soft ray of the dorsal fin were evaluated by examining the recording. Data are presented as the difference (Δ) between the values after (post-stimulus, PS) and before (baseline, BL) methodological interventions (Δ = PS-BL). Locomotor activity (traveled distance) was analyzed with EthoVision XT 7.1 software (Noldus Information Technology, Wageningen, NL). The position of the dorsal fin was determined manually by measuring the time the fish kept the spine dorsal fin erect and the soft dorsal fin lowered. The duration for which the soft dorsal fin performed wave movements was also timed. The position of the dorsal fin is presented in Figure 1. The experimenter was blind to the treatment during the analysis, and a reliability test for the video analysis was performed.

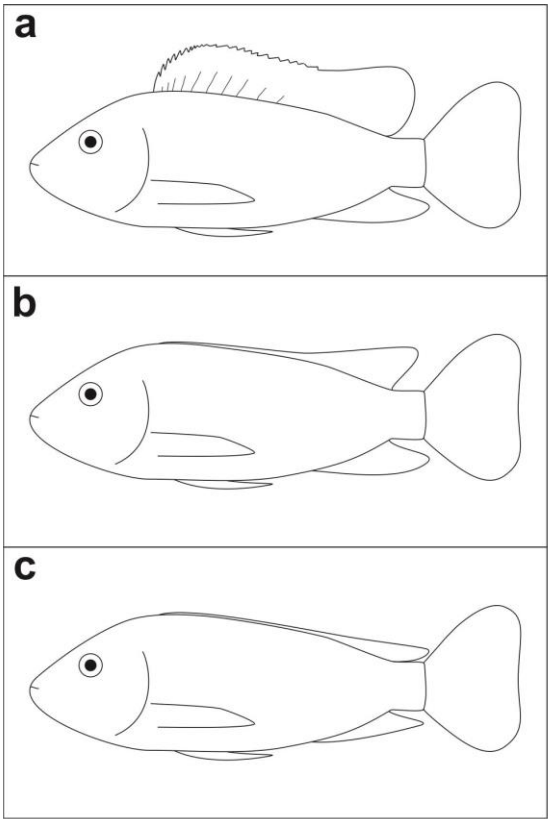

**Figure 1.** Schematic drawing of the *Oreochromis niloticus* dorsal fin position. (a) Spine dorsal and soft dorsal fin erected; (b) Spine dorsal fin lowered with soft dorsal fin lifted; and (c) Soft dorsal fin lowered.

Skin and eye darkening was also evaluated from the recordings, and the data are presented as the variation between PE and BL ($\Delta = PE - BL$). Skin darkening was analyzed using ImageJ (National Institutes of Health, United States) imaging software, designed for multidimensional scientific images. Initially, a selection of the animal's body area was performed with the freehand selections tool, and then the selected area was analyzed to obtain a black-and-white linear-scale measurement where 0 equals black and 255 represents white. The mean value was obtained by selecting the measure option from the analyze menu. Skin darkening was analyzed from the side (lateral) of the fish facing the camera. For eye darkening determination, a methodology adapted from Volpato, Luchiari, Duarte, Barreto, and Ramanzini (2003) and Freitas, Negrão, Felício, and Volpato (2014) was used. The darkened area of the fish's eye sclera as a percentage of the total was estimated from the eye on one side, based on the images captured inthe videos. The images were collected between minutes 2 and 8 of BL and PE, in order to avoid the effect caused by the experimenter approaching to begin filming.

## Statistical analysis

Data were submitted to the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity of variances to determine the use of parametric or non-parametric tests. The distance traveled, speed, cortisol, body darkening, and time spent with the soft ray dorsal fin lowered showed a normal distribution and homogeneity of variances and were submitted to the *t*-test. Time spent with the spine dorsal fin erect, duration of wave movement of soft ray dorsal fin and eye darkening were not normally distributed and were submitted to the Mann-Whitney test. Spearman correlation analysis was performed between the time with the soft ray of dorsal fin lowered and its wave motion and between the cortisol levels and the behavioral variables. Results are presented as mean ± standard error or median/25%/75%/maximum/minimum. The level of significance adopted in all tests conducted was 5%.

## Ethical note

This research was conducted in accordance with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation - Brazil (CONCEA – *Conselho Nacional de Controle da Experimentação Animal* - Brazil) and was approved by the Ethical Committee for Animal Research from the *Faculdade de Ciências Agrárias e Veterinárias*, Jaboticabal campus, *Universidade Estadual Paulista* (UNESP) (Case nº 18.897/16).

## Results

During the experiments, 5 (41%) of the 12 fish submitted to subcutaneous injection of formaldehyde 3% died after receiving the noxious stimulus, and their data were excluded from the statistical analyses. None of the fish submitted to subcutaneous vehicle injection died during the experiment.

The distance traveled (t = 0.105, GL = 13, P = 0.918, Table 1), plasma cortisol (t = −1.937, GL = 13, P = 0.075, Table 1), body darkening (t = 0.500, GL = 13, P = 0.625, Figure 2), eye darkening (U = 25.500; P = 0.779, Figure 2), and time spent with the spine dorsal fin erect (U = 25.500; P = 0.779, Figure 3) did not present significant differences between the fish treated with subcutaneous injection of vehicle or formaldehyde.

**Table 1.** Locomotor activity and plasma cortisol of *Oreochromis niloticus* subjected to subcutaneous injection of formaldehyde 3% in the dorsal fin.

| Treatment/Variable | Vehicle | Formaldehyde |
|---|---|---|
| Distance (cm) | 482.38 ± 550.10 | 407.04 ± 433.86 |
| Plasma cortisol (mg dL$^{-1}$) | 54.88 ± 9.34 | 88.22 ± 15.03 |

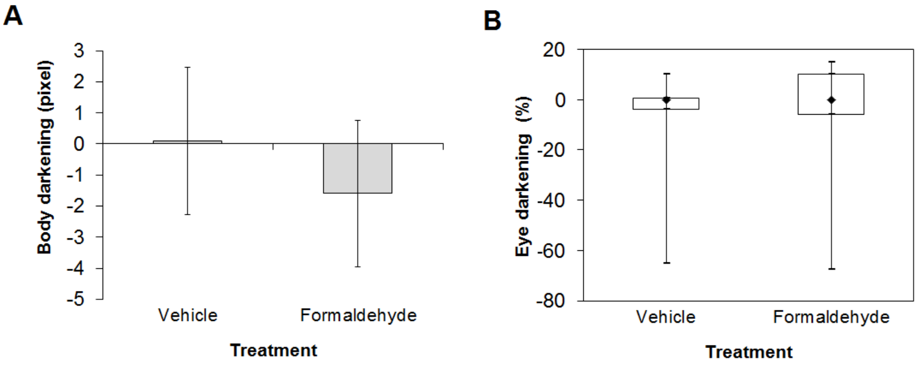

**Figure 2.** Body darkening (A) and eye darkening (B) of *Oreochromis niloticus* subjected to vehicle injection (n = 8) or formaldehyde 3% (n = 7). The data are presented as the difference (Δ) between baseline and post-stimulus. In A are presented mean ± standard error (*t*-test). In B ♦ indicates the median (Mann-Whitney).

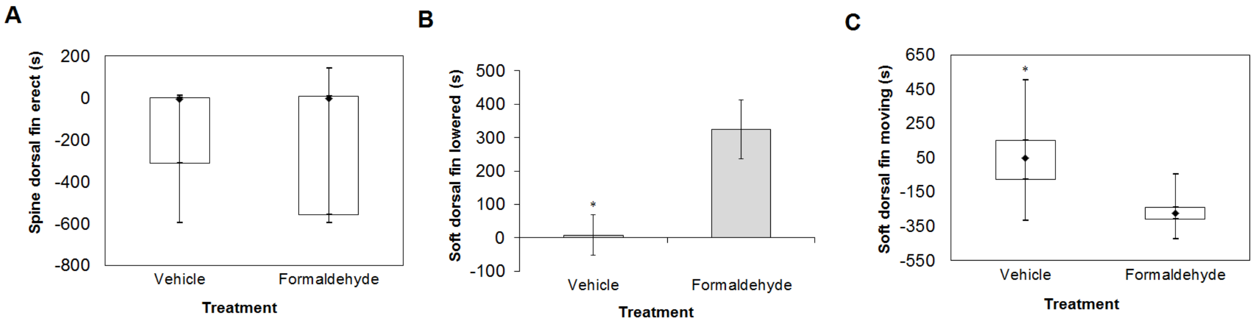

**Figure 3.** Dorsal fin position of *Oreochromis niloticus* subjected to vehicle injection (n = 8) or formaldehyde 3% (n = 7). (A) Time spent with the spine dorsal fin erect; (B) Time spent with the soft dorsal fin lowered; (C) Time spent with the soft dorsal fin moving. The data are presented as the difference (Δ) between baseline and post-stimulus. In A and C ♦ indicates the median (Mann-Whitney). In B are presented mean ± standard error (*t*-test). * Indicates significant difference.

The position and wave movement of the soft ray of the dorsal fin showed significant differences between the treatments (position, t = -3.025; GL=13; P = 0.010; movement, U = 49.000; P = 0.014, Figure 3). Fish treated with formaldehyde remained with the soft ray of the dorsal fin lowered and stationary for longer than vehicle-treated animals. There was a negative correlation between these two variables (ρ = -0.576; P = 0.0241).

During the behavioral analysis, fish which received subcutaneous injection of formaldehyde and died before the end of the post-stimulus filming (n = 5) showed a change in the color pattern in the moments that preceded death, with five phases as described in Figure 4. These changes did not occur in any of the vehicle-treated fish or in formaldehyde-treated animals that survived the experimental period.

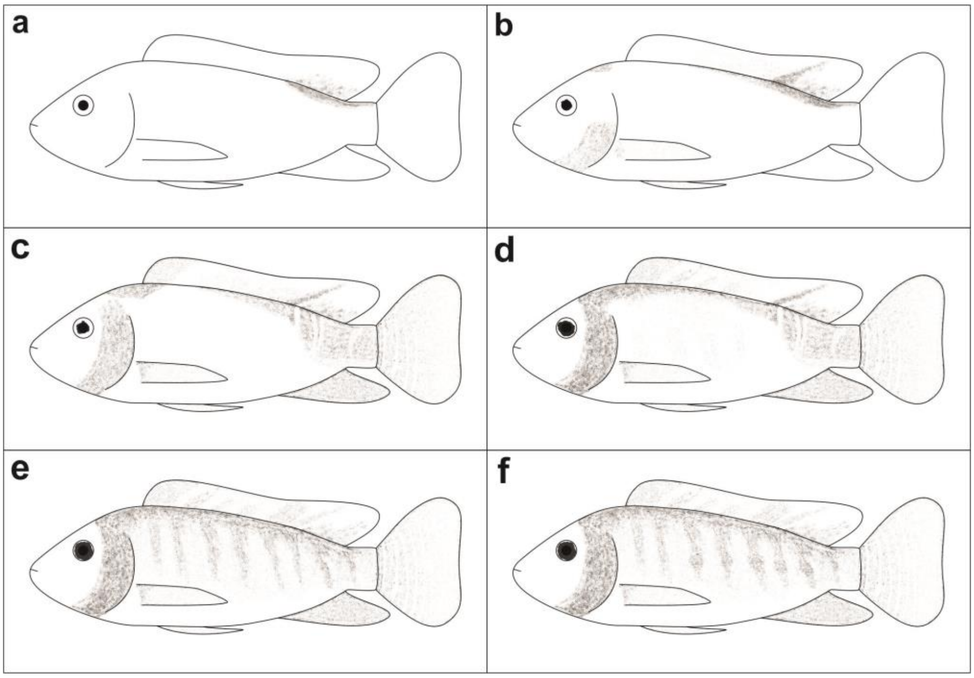

**Figure 4.** Schematic drawing of the skin darkening pattern in the *Oreochromis niloticus* that died during the experimental procedures after subcutaneous injection of 3% formaldehyde in the posterior region of the dorsal fin. (a) onset of skin darkening in the dorsal region near the injection site; (b) onset of skin darkening in the opercular region and superior to the operculum and continuous darkening of the dorsal region; (c) onset of darkening of the skin in the caudal region and continuous darkening of the opercular and dorsal regions; (d) continuous darkening of the skin in the dorsal, opercular and caudal regions; (e) appearance of stripes in the middle of the body or (f) appearance of stripes and overlapping spots, the first being more common in the experimental group.

## Discussion

The results indicate that subcutaneous injection of formaldehyde 3% in the posterior region of the dorsal fin caused a local effect observed as a decrease in wave movements of the soft ray of the dorsal fin and its lowered positioning, corroborating the conclusion of Wolkers et al. (2013) that behavioral and physiological responses to the noxious stimulus are species-specific. Moreover, the reduction of movement of the affected limb (soft caudal fin) is a response also observed in mammals (Dubuisson & Dennis, 1977), showing an analogy to the behavioral pattern front of a noxious stimuli.

The behavioral response to subcutaneous formaldehyde injection observed in *O. niloticus* in the present study differs from that reported for *L. macrocephalus* submitted to the same type of noxious stimulation. In *L. macrocephalus* a significant increase in speed and distance travelled in response to subcutaneous injection of formaldehyde (Alves et al., 2013; Wolkers et al., 2013) was observed, as well as an erratic pattern of swimming (Wolkers et al., 2013). The study by Ide and Hoffmann (2002) with *O. niloticus* showed that the stress caused by subcutaneous formaldehyde injection (2 and 3%) promotes immobility. Another study with *Danio rerio* also showed reduction in locomotor activity after formaldehyde (0.1%) injection in the tail and the lips (Magalhães et al., 2017). In the present study, fish that received noxious stimulation by subcutaneous injection of formaldehyde did not show significant changes in the locomotion pattern, and erratic swimming behavior was not observed. Despite being conducted in the same species, the present study differed from that of Ide and Hoffmann (2002) with respect to the injection location (subcutaneous in the posterior region of the dorsal fin and in the pectoral muscles, respectively), and this discrepancy in the locomotion pattern could be attributed to the location of formaldehyde injection and the importance of the particular area to locomotion. Studies using other fish species and different nociceptive tests have also demonstrated diverse behavioral responses. *Oreochromis mossambicus*, another cichlid, displayed a significant decrease in swimming activity for at least 3 hours when submitted to an electrical stimulus (Roques et al., 2012), a behavior not observed in *O. niloticus* in this study. Treatment with acetic acid at 10% promoted a significant reduction in locomotor activity in *Danio rerio* (Correia, Cunha, Scholze, & Stevens, 2011), and in *Oncorhynchus mykiss*, administration of 0.1 mL of the same acid caused anomalous behaviors such as rubbing of the lips in the gravel and against the sides of the tank, and a delay in returning to food intake (Sneddon, 2003a). In *Gadus morhua*, a reduction in the use of the shelter was observed, with the fish remaining for longer near the bottom of the aquarium and presenting a slight delay in the recovery of the opercular beats when treated with capsaicin 1% (Eckroth, Øyvind, Sneddon, Bichão, & Døving, 2014). Together, these studies show that the behavioral response in the face of a noxious stimulus shows great variability across fish species.

In the present study, subcutaneous injection of formaldehyde in *O. niloticus* promoted changes in the positioning and movement of the soft ray of the dorsal fin, a region that constitutes the final portion of the dorsal fin, located after a more rigid region (Geerlink & Videler, 1974). This fin is important for giving greater stability to the fish body during swimming (Lauder & Madden, 2007). Changes in the locomotor pattern and fin positioning were not observed in studies that applied the same noxious stimulus in *L. macrocephalus*. It is possible that the absence of this response in *L. macrocephalus* is related to the injection site, since, in this species, subcutaneous formaldehyde injection was applied in the adipose fin region, a fin greatly reduced or absent in cichlids such as *O. niloticus*. The change in the dorsal fin position and movement observed here may be an attempt to reduce utilization of the affected region in order to prevent intensification of the pain sensation. In fact, in studies with rats and cats, injection of 5% formaldehyde into one of the front paws caused a decrease in the use of the affected limb and was characterized by paw elevation as a response to pain (Dubuisson & Dennis, 1977). However, more studies are needed to elucidate this similarity and the physiological mechanisms activated as a result of formaldehyde injection in the dorsal fin and other areas of *O. niloticus*.

In cichlids, skin color is related to social communication, competition, partner choice, foraging, and predation (Maan & Sefc, 2013). In addition, fish may exhibit color patterns that are associated with different types of behavior (Baerends & Baerends-Van Roon, 1950; Baldaccini, 1973; Hulscher-Emeis, 1992; Lanzing & Bower, 1973; Potts, 1974). In our study, a darkening pattern was identified in fish that died from subcutaneous injection of formaldehyde. This darkening pattern observed resembled that described for juveniles of *Tilapia mossambicus* (*Oreochromis mossambicus* in current nomenclature) when they are aroused and frightened (Lanzing & Bower, 1973). The vertical stripes that stand out in the final phase of the pattern presented here also appear in *Tilapia natalensis* (Baerends & Baerends-Van Roon, 1950) and *Tilapia zillii* (Hulscher-Emeis, 1992) when they are under threat. In *Crenilabrus melops*, there is an appearance of vertical stripes of dark brown coloration on the aggressors in intense aggressive encounters; a similar situation is observed in male courtship behavior, in situations where fish are submitted to fear with no possibility of escape, caused by dominant threats, for example, and when they are disturbed (Potts, 1974). The appearance of black streaks, also associated with stress, was described by Ide and Hoffmann (2002) in *O. niloticus* submitted to subcutaneous injection of 2% and 3% formaldehyde.

Although cortisol levels in the present study did not show a significant increase in fish submitted to subcutaneous injection of formaldehyde compared to those treated with vehicle, it is important to note that the fish that died during the experiment, which were possibly the most responsive to the stress caused by the noxious stimulus, could not have their blood sampled, therefore it was not possible to evaluate their cortisol levels. Furthermore, the time course of cortisol release could be related to the absence of significant cortisol increase in the present study. Sanches, Miyai, Pinho-Neto, and Barreto (2015) described that a significant increase in cortisol levels could be observed 15 minutes after a stressful situation in *O. niloticus,* while in the present study the blood was sampled only 10 minutes after the noxious stimulus. However, considering that the skin darkening pattern presented by these fish is related to extreme stress situations, this result suggests that fish that received subcutaneous formaldehyde injection developed physiological responses associated with those triggered by fear and predation, indicating that the stimulus triggered defensive behavior in this species.

Some studies have shown a positive correlation between cortisol levels and skin darkening (larvae of *Solea senegalensis:* Ruane, Makridis, Balm, & Dinis, 2005; in subordinate fish submitted to acute social stress in *O. niloticus*: Templonuevo & Cruz, 2016). Fish skin darkening can be caused by physiological changes, more specifically, by the translocation (aggregation and disaggregation) of melanosomes within the melanophores (Leclercq, Taylor, & Migaud, 2010) and has been correlated with stressful situations. We did not observe a correlation between skin darkening and cortisol levels, which could suggest that these parameters are not associated. However, as discussed earlier, the time between the injection and the sampling of blood in this study may not have been sufficient to allow a significant and detectable increase in cortisol levels. In *O. mykiss* submitted to social stress, skin darkening was also not correlated with cortisol levels; however, the authors suggest that skin darkening is associated with an increase in alpha-melanocyte-stimulating hormone (α-MSH) levels induced by activation of the hypothalamic-pituitary-interrenal (HPI) axis (Höglund, Balm, & Winberg, 2000). The pituitary polypeptide proopiomelanocortinis (POMC) is the precursor of both α-MSH and adrenocorticotropic hormone, and activation of the HPI axis can result in the release of α-MSH (for a review see Sloman, 2011) one step earlier in the hormonal cascade, i.e., earlier than a significant increase in cortisol levels could be detected. These results indicate that skin darkening may or may not be directly associated with the physiological stress response (increase in cortisol levels) and that this may vary between species and even between applied stressors.

The present study did not demonstrate a significant effect of exposure to a noxious stimulus on eye darkening. In addition, no correlation was observed between plasma cortisol levels and darkening of the eyes. Although in some species of fish, including *O. niloticus*, eye darkening has been related to situations of social (Suter & Huntingford, 2002; Volpato et al., 2003; Miyai et al., 2011) and non-social (Freitas et al., 2014) stress, those studies also did not show a correlation between this parameter and the activation of the HPI axis, and it is possible that mechanisms other than the stress response are responsible for the darkening.

## Conclusion

The results of the present study show that the formaldehyde test activated the nociceptive system in *O. niloticus*, triggering behavioral responses associated with protection of the damaged tissue and indicating activation of the defense system. In addition, the reduction of use of the affected area, the soft ray of the dorsal fin, due to subcutaneous injection of formaldehyde, similar to that observed in mammals submitted to the same stimulus, indicates that these animals may be capable of pain perception. The results also confirm the hypothesis that responses to this noxious stimulus are species-specific and corroborate existing knowledge about nociception within the vertebrate phylum. Further studies are needed to evaluate which physiological mechanisms are activated as a result of formaldehyde injection in this species.

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