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Effect of tactile stimulation in a territorial fish

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Atestamos que **MARCELA CESAR BOLOGNESI**, RA nº: 153194-3, RG nº 43.939.232-9, expedido pela SSP/SP, defendeu, no dia 24/02/2017, a dissertação intitulada ***Effect of tactile stimulation in a territorial fish***, junto ao Programa de Pós Graduação em BIOLOGIA ANIMAL, Curso de Mestrado Acadêmico, tendo sido 'APROVADA'.

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RESUMO

Peixes que apresentam relações de cooperação respondem a estimulação tátil (como massagens) reduzindo o estresse em uma interação entre cliente-limpador. Neste trabalho, nós testamos o efeito da estimulação tátil sobre a resposta ao estresse em um peixe territorial, a tilápia-do-nilo. Nós desenvolvemos um aparato formado de hastes verticais com cerdas de silicone nas laterais, que foi posicionado no meio do aquário, formando uma fileira de hastes. O peixe precisava passar pelas cerdas para acessar a comida (posicionada no lado oposto que o peixe se encontrava no aquário), recebendo assim a estimulação tátil. O estimulador foi eficiente, pois os peixes passaram espontaneamente pelo aparato na ausência de comida. Peixes isolados foram submetidos à estimulação tátil durante 7 dias e, em seguida, destinados a um dos dois tipos de estressor: não social (confinamento) e social (interação agressiva). Cada tratamento teve um controle sem o estimulador tátil. Após serem estressados os peixes aumentaram o número de atravessamentos pelas cerdas, que foi maior após o estresse social, sugerindo que eles buscaram pela estimulação tátil. Além disso, nós observamos que o número de iterações agressivas em duplas de machos diminuiu comparado ao controle. Entretanto, nós não observamos uma diminuição nos níveis de cortisol imediatamente após o estresse não social e social. Nós concluímos que a estimulação tátil aparentemente causa um efeito positivo no bem-estar de peixes territoriais reduzindo a motivação agressiva, mas não reduzindo a resposta imediata ao estresse.

Palavras-chave: Bem-estar animal. Massagem. Cortisol. Confinamento. Comportamento social. Agressividade.

ABSTRACT

Cooperative fish respond to tactile stimulation (like massage) by reducing stress in a cleaner-client interaction. In this work, we tested the effect of tactile stimulation on the stress response of a territorial fish, Nile tilapia. We developed an apparatus formed by vertical sticks with silicone bristles in their sides, which was positioned in the middle of the aquarium, forming a row of sticks. Fish had to pass through bristled sticks to access food (placed in the opposite location of the fish in the aquarium), thus receiving tactile stimulation. The stimulator was efficient because fish pass through the apparatus spontaneously after trials, i.e. in the absence of feed. Isolated fish were submitted to the tactile stimulation during 7 days and afterwards they were assigned to one out of 2 types of stressors: non-social (confinement) and social stress (aggressive interaction). Each treatment had a control without the stimulator apparatus. After being stressed, fish increased the number of crosses in between the bristles, which was higher after social stress, suggesting fish sought for tactile stimulation. In addition, we observed that the number of aggressive interactions in male pairs decreased when compared to control. However, we did not observe a decrease in plasma cortisol levels immediately after stress either for social or non-social stress treatment. We conclude that tactile stimulation does not have an immediate effect on stress, but it reduces aggression in males of Nile tilapia. Therefore, tactile stimulation seems to cause a positive effect in the welfare of territorial fish by reducing aggressive motivation, but not by reducing acute stress responsiveness.

Keywords: Animal welfare. Massage. Cortisol. Confinement. Social behavior. Aggression.

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1 INTRODUCTION

Several studies have shown the positive effects of tactile stimulation in mammals. This type of stimulation performed as a massage can relieve stress in humans, thus having positive effects on health, reducing cortisol and elevating serotonin levels (FIELD et al., 2005). Petting, another kind of stimulation, decreases heart rate in piglets (TALLET et al., 2014), lambs (COULON et al., 2015) and reduces stress in cattle (PROBST et al., 2012) and dairy cows (SCHMIED et al., 2010). Additionally, this positive effect occurs in cooperative teleost fishes. Soares et al. (2011) demonstrated that tactile stimulation performed by the cleaner fish *Labroides dimidiatus* reduces stress in a coral reef fish, in a cleaner-client interaction.

Positive responses to tactile stimulation can, however, be associated to the animals' natural behavioral repertoire. Physical contact of mother-cub interactions such as mammal grooming (DUNBAR, 2010), and the cleaner-client interactions for coral reef fish (GRUTTER, 1995), are part of the natural behavior of these animals. Conversely, several tactile stimulations are naturally negative, such as the ones coming from agonistic interactions, which may cause injuries, pain and social stress (DAMSGÅRD; HUNTINGFORD, 2012). In this case, it is possible that tactile stimulation means something else for animals whose natural behavior is characterized by the presence of aggressive fights, e.g. animals that fight to defend a territory or to establish a dominance hierarchy, such as cichlid fish.

Cichlid fish are territorial animals, commonly found in fish farming activities, being frequently subjected to stressful situations, such as net catching (ELLIS et al., 2004; ELLIS et al., 2007), overpopulation (BARCELLOS et al., 2004; SCHRAM et al., 2006) confinement (POTTINGER; PRUNET; PICKERING, 1992; POTTINGER, 2010), grading (FLOS et al., 1988) and conditions that increase aggressive interactions, causing high stress level and detrimental effects on fish health, including probability of mortality (HUNTINGFORD et al., 2006; BOSCOLO; MORAIS; GONÇALVES-DE-FREITAS, 2011; BARRETO; BOSCOLO; GONÇALVES-DE-FREITAS, 2015). Although stress is considered an adaptive response to environmental challenges (WENDELAAR BONGA, 1997; ELLIS et al., 2012), if it is intense or cannot be avoided, animals may undergo a chronic stress state (distress). This state is characterized by depression of the immune system (POTTINGER, 2008), reduction of growth rates (FERNANDES; VOLPATO, 1993; ØVERLI et al., 2006), and impairment of reproduction (HUNTINGFORD et al., 2006). Chronic stress also causes neuron destruction (McEWEN; SAPOLSKY, 1995; SØRENSEN et al., 2012) with consequent reductions of the

cognitive ability (WOOD, DESJARDINS; FERNALD, 2011). All those effects are undesirable, either for fish culture and fish welfare.

Most handling methods of fish in rearing environments are still unavoidable, then finding ways of avoiding or relieving any kind of stress are ways of promoting animal welfare. In this sense, positive effects from tactile stimulation could be an easy method to improve fish welfare apart their natural behavior. Anecdotal informations about fish pets indicates that, even for cichlids, tactile stimulation could be a positive interaction because fish seems to choose being touched by owners, who manipulate the fish by doing a kind of massage. Empiric data, however, are unavailable. In this study, we tested whether the positive effect from tactile stimulation can occur in the cichlid Nile-tilapia (*Oreochromis niloticus*, L.), a territorial species of aquaculture interest, and a widespread model to study mechanisms underlying fish behavior. We tested the effect of tactile stimulation on two kinds of stressors, confinement (non-social stressor), commonly present in fish farming (POTTINGER; PRUNET; PICKERING, 1992; POTTINGER, 2010), and aggressive interactions (social stressor), which is part of the cichid's natural behavior, but can be exacerbated in rearing conditions (BOSCOLO; MORAIS; GONÇALVES-DE-FREITAS, 2011; DAMSGÅRD; HUNTINGFORD, 2012). We predicted that tactile stimulation relieves stress by reducing cortisol levels. Also, whether tactile stimulation alleviates stress, fish would search for more stimulus after experiencing stress.

At our knowledge, there are only two studies regarding the positive effects of tactile stimulation in fish (SOARES et al., 2011; SCHIRMER; JESUTHASAN; MATHURU, 2013), which is limited when compared to the knowledge about mammals. Thus, studies about functions and effects involved with tactile stimulation in fish species can help us to understand the evolution of this mechanism in vertebrates.

2 METHODS

2.1 Fish housing

Adult males of GIFT Nile tilapia from the Aquaculture Center of UNESP (CAUNESP) in Jaboticabal, SP, Brazil, were kept in outdoor ponds at the IBILCE, UNESP, São José do Rio Preto. They were selected for the study and taken to the laboratory where they were acclimated for 20 days in polyethylene water tanks (ca. 500 L, 1 fish/10 L) with water at 27°C and 12L:12D light regime (7:00 a.m. to 7:00 p.m.). The fish were fed with ration for tropical fish (28% CP, apparent satiety) twice a day (9:00 a.m. and 3:00 p.m.). The water quality was maintained using biological filters with filtration of 400 L/h, and constant aeration.

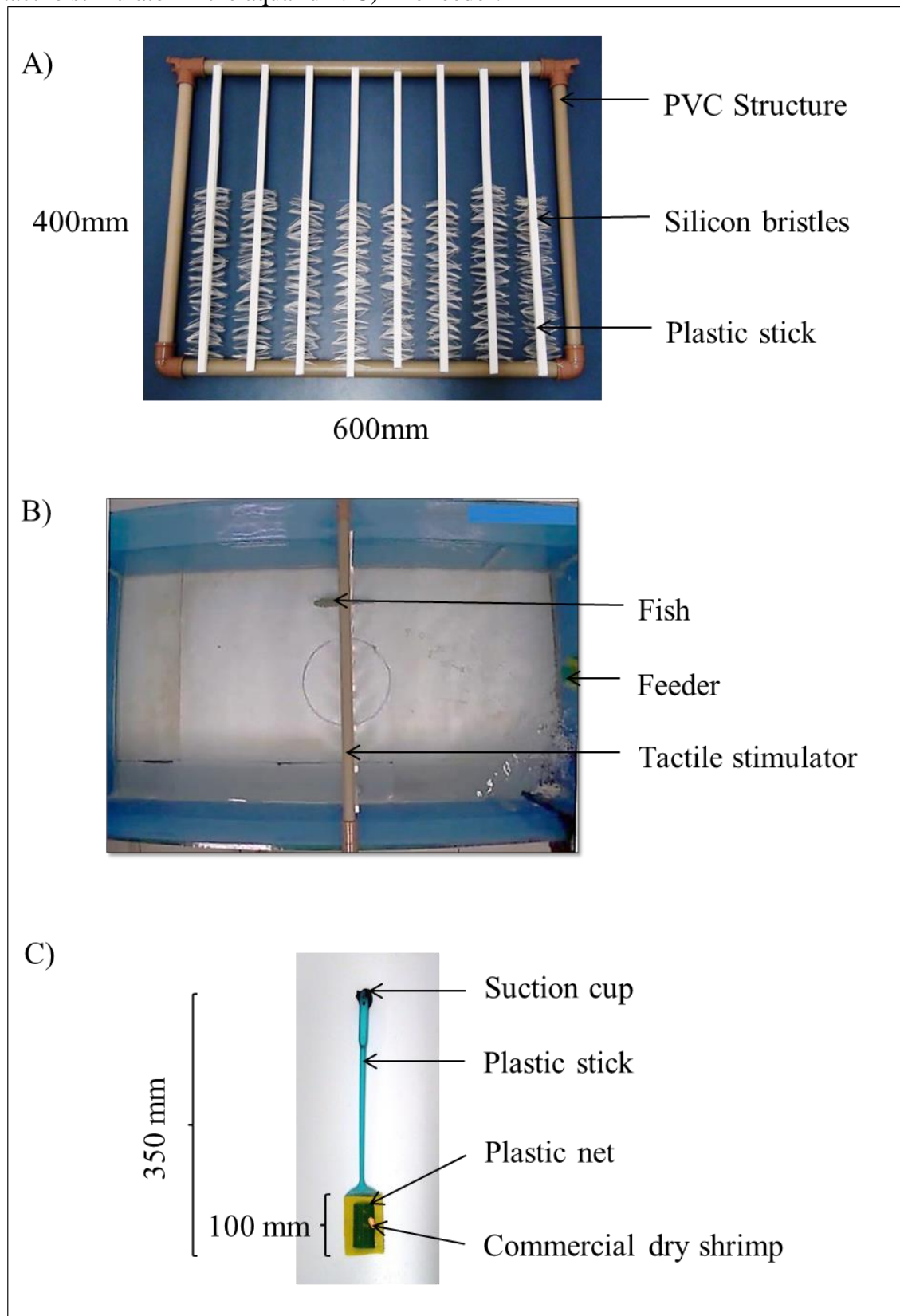
2.2 Experimental Design

Isolated Nile tilapia males were assigned to one out of 2 types of stressors: non-social (confinement) and social stress (aggressive interaction). Each treatment had a control without tactile stimulation. Before, we developed a tactile stimulator apparatus to test our hypothesis.

2.3 Tactile stimulator apparatus

We developed an apparatus formed by a rectangular PVC (polyvinyl chloride) structure, filled with vertical sticks bordered by silicone bristles (Fig. 1A). We chose the silicone bristles because they are soft, making difficult to remove mucus from skin and cause injuries on the fish's body. The apparatus was positioned in the center of the aquarium so that fish had to pass through bristled sticks to access food (placed in the opposite location of the fish in the aquarium), thus receiving tactile stimulation (Fig. 1B).

Figure 1. A) Photo of the tactile stimulator apparatus. The silicone bristles were placed to the limit of the water level in the aquarium, so they are up to half the sticks. B) Position of the tactile stimulator in the aquarium. C) The feeder.



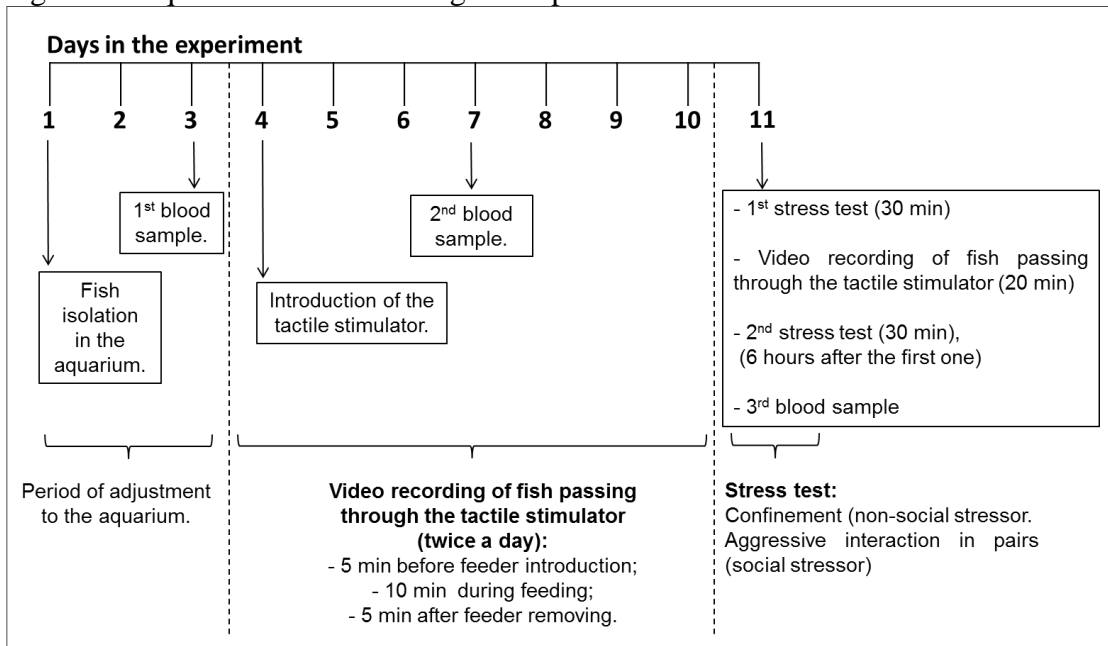
To ensure the contact of the fish with the stimulator, the animals received food on the opposite side to which they were in the aquarium. The animals were fed dry shrimp (commercial food for ornamental fish) twice a day (9:00 a.m. and 3:00 p.m.). The food was fixed in a feeder (plastic structure where the food was trapped; Fig. 1C) manipulated by the observer and introduced at one end of the aquarium (Fig. 1B). In the treatment without tactile stimulation (control) the feeder was placed on the opposite side to what the fish was to a midline of the center of the aquarium. Animals that did not feed were withdrawn from the study.

2.4 Non-social stress experiment

Isolated Nile tilapia males underwent two treatments: with tactile stimulation and without tactile stimulation (15 replicate each). In the tactile stimulation treatment the fish were isolated in the test aquarium for 10 days, in which they received food twice a day with a feeder on the opposite side to which they were in the aquarium (Experimental schedule is summarized in the Fig. 2). The first three days were the time for fish adjustments to the aquarium. On the day 4 the tactile stimulator was inserted in the center of the aquarium and remained there until the end of experiment. Fish was recorded twice a day to quantify the number of crossings through the stimulator; 5 min before, 10 min during and 5 min after the introduction of the feeder. Blood samples were taken on the 3rd and 7th days for evaluation of a baseline plasma cortisol levels.

On the morning of the day 11, the animals were submitted to the confinement stress, which consisted of 90% reduction of the aquarium space by an opaque plate, confining the animal to one end of the aquarium for 30 min (e.g. BARRETO et al., 2009). This is a potent stressor that was already tested for Nile tilapia (BARRETO et al., 2009; MOREIRA; VOLPATO, 2004). After the confinement, the plate was removed and the fish were video-recorded (20 min) to quantify the number of crossings through the stimulator. The stressor was applied again 6 hours later, followed by blood collection for plasma cortisol evaluation. In the control treatment the animals underwent the same procedure as the previous treatment, but without the presence of the stimulator.

Figure 2. Sequence of events during the experiment.



2.5 Social stress experiment

Nile tilapia males underwent two treatments: with tactile stimulation and without tactile stimulation (14 replicate each). In tactile stimulation treatment, the fish underwent exactly the same procedure described in the first experiment, including feeding, recording and blood collection (Fig. 2). On the morning of day 11, the animals were submitted to social stress. Two individuals were removed from their aquaria and were paired in a new aquarium (40 x 30 x 40 cm ca. 48 L) to avoid residence effect (e.g. HUNTINGFORD; LEANIZ, 1997). Aggressive interaction was video recorded for 30 min, while fish remained paired. Two fish of the same treatment were used as they went through the same procedures with respect to the tactile stimulator (absence or presence of tactile stimulation). Then, each fish returned to its original aquarium and was video-recorded (20 min) to quantity of the number of crossings through the stimulator. The fish were paired again 6 hours after the first pairing, the aggressive interaction was video recorded for 30 min and a blood collection was done for plasma cortisol assay. Different individuals (from the first encounter) were paired to avoid the effect of the previous experience (e.g. HSU; WOLF, 1999). In the control treatment, the animals underwent the same procedure, but without the presence of the stimulator apparatus.

2.6 Cortisol assay

The animals were anesthetized by immersion in Benzocaine (0.09 g.L^{-1}). Blood were collected by hypodermic needles and heparinized syringes. The capture and anesthesia of each fish lasted less than 2 minutes to avoid interference with the results (e.g. POTTINGER, 2008). The blood was collected by puncture of the caudal vein, taking care not to exceed 2 minutes in this procedure. The blood was centrifuged at 3,000 rpm for 10 min and the plasma was frozen at -20°C for further cortisol assay. The cortisol was measured by the ELISA - Enzyme Linked Immunosorbent Assay, using commercial kits (IBL - Immuno Biological Laboratories, Hamburg, Germany).

2.7 Aggressive interaction

In second experiment, the fish were individually identified by red elastomer (VIE tags), inserted under 2 or 3 scales on each side of the body. Aggressive interactions were quantified based on FALTER (1983) and CARVALHO et al. (2013) descriptions. Aggressive behavior was labeled as attacks and displays. Attacks are the interactions with physical contact and greater energy expenditure (as nipping, mouth fight and undulation), and the displays are interactions without physical contact and less energy expenditure (ROS; BECKER; OLIVEIRA, 2006) (as threat, lateral fight, chase, and circling).

2.8 Experimental details

Before isolation the animals were anesthetized by immersion in benzocaine (0.03 g.L^{-1}), weighted and sized. The mean (\pm S.E.) standard length and weight of fish were respectively: Non-social experiment – Treatment with tactile stimulation: $11.44 \text{ cm} \pm 0.92 \text{ cm}$; $47.26 \text{ g} \pm 11.15 \text{ g}$; Control treatment: $11.74 \text{ cm} \pm 0.90 \text{ cm}$; $51.33 \text{ g} \pm 10.62 \text{ g}$. Social stress experiment: – Treatment with tactile stimulation: $11.6 \text{ cm} \pm 0.57 \text{ cm}$; $54.27 \text{ g} \pm 9.55 \text{ g}$; Control treatment: $10.79 \text{ cm} \pm 0.33 \text{ cm}$; $45.77 \text{ g} \pm 5.23 \text{ g}$.

The animals were observed in glass aquaria (120 x 60 x 40 cm, containing 140 L of water, because this quantity was enough for a single fish) coated with blue plastic to avoid visual contact with animals from neighboring aquaria, and because the blue color is less stressing for Nile tilapia (MAIA; VOLPATO, 2013). Video-recording was done by cameras placed above the aquaria that send the records to a computer in an adjacent room.

Abiotic variables were controlled. The photoperiod was set to 12L:12D (from 7:00 a.m. to 19:00 a.m.) and temperature to 27° C. The water quality was monitored by commercial kits and electronic devices: (mean \pm SE). Ammonia (0.025 ± 0.019 ppm); Nitrite (0.125 ± 0.072 ppm); pH (7.36 ± 0.11).

2.9 Statistical analysis

Data were tested for outliers by Grubbs test and those found were replaced by the mean (COUSINEAU; CHARTIER, 2010). The data were assessed for normality by Kolmogorov-Smirnov test and homoscedasticity by Fmax (LEHNER, 1996). The number of crossings from experiment 2 was transformed by $\log(x + 1)$ to fit to parametric assumptions. In both social and non-social experiments, mixed model ANOVA was used to compare between (with tactile stimulations *vs.* control) and within treatments as follows: - The number of crossings/min in the two observed periods (periods with and without feed); - The number of attacks and displays in the two social stress sessions; - Plasma cortisol concentration in the 3rd, 7th and 11th days. Fisher-LSD or Tukey-HSD were applied as a post hoc test. The number of crossings before and after stress application was also compared by paired t-test. Correlations between the number of crosses and the cortisol level were checked by Pearson's correlation test.

3 ETHICAL NOTE

This work is in accordance with the Ethical Principles on Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA / Brazil) and was approved by the Committee on Ethics in Animal Use, IBILCE, UNESP, São José do Rio Preto, permit #129/2016. It also follows the Animal Behavior Society guideline for using of animals in research (2012).

4 RESULTS

4.1 Non-social Stress Experiment

A different number of crossings per minute through the tactile stimulator was found before stress (from day 4 to 10) (ANOVA for repeated measures, $F_{(2,12)} = 32.73$, $p < 0.0001$, Table 1). Fisher-LSD test showed an increased number of crossings during the presence of feed when compared to periods before and after feed ($p < 0.0001$).

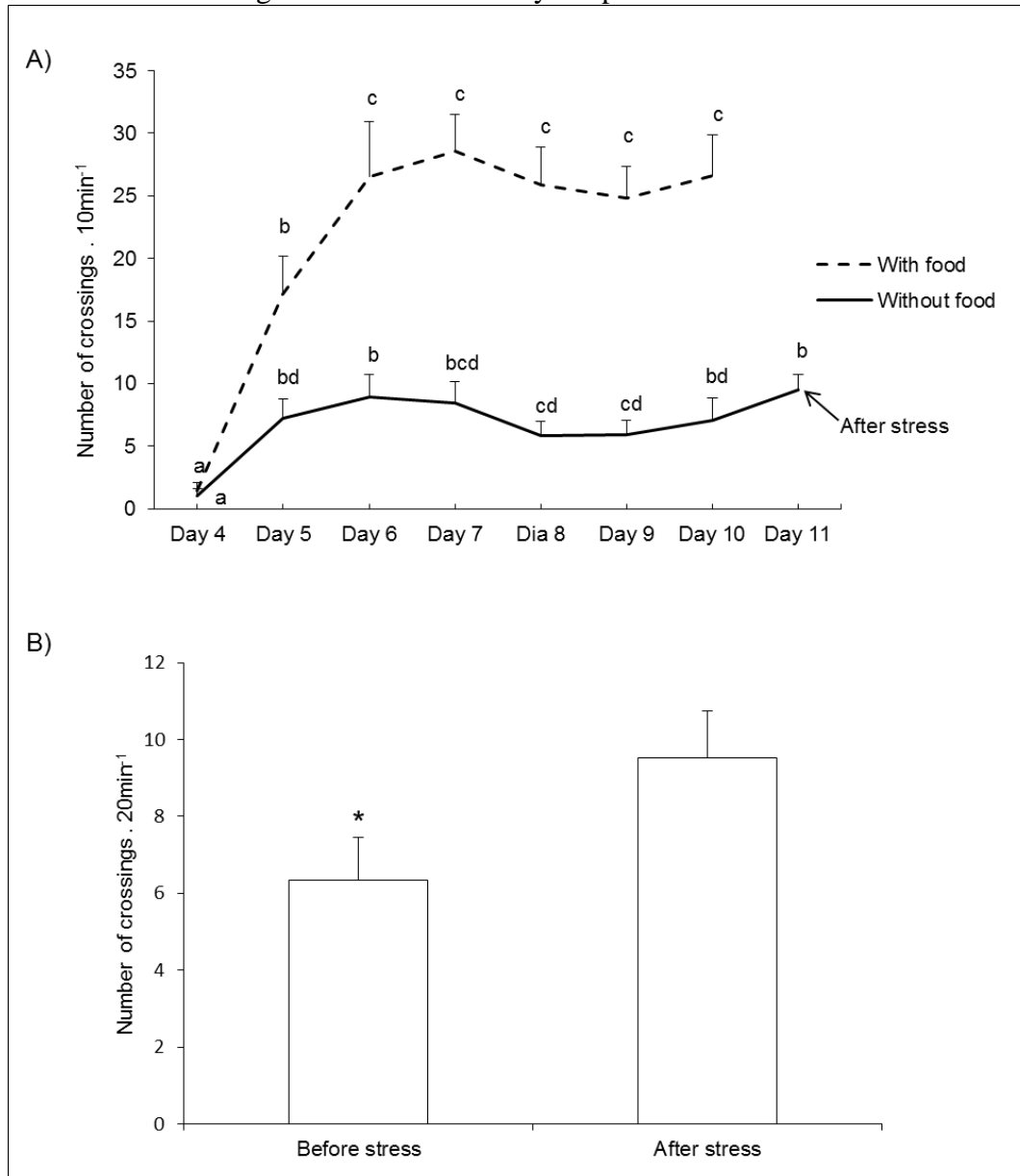
Table 1. Non-social stress experiment. Number of crossings per minute of the tactile stimulation treatment (15 replicates), in the three moments of records: before, during and after feed. Data from 7 days (from day 4 to 10) were summed and divided by 70 or 140, accordingly. Asterisk indicates significant differences. ANOVA for repeated measures, followed by Fisher-LSD post hoc test. Data are mean \pm SE.

Periods of video-recording		
Before feeder introduction	During feed	After feeder being removed
3.34 \pm 0.08	16.09 \pm 0.38 *	6.18 \pm 0.15

The number of crossings through the tactile stimulator was quantified both before (from day 4 to 10) and after the confinement stress (day 11). Data from both daily-performed observations were summed, followed by the split of total time in two distinct periods: with (20 min / day) and without feed (20 min / day). Regarding the period with feed, a difference was observed among days (ANOVA for repeated measures, $F_{(6,84)} = 14.12$, $p < 0.0001$, Fig. 3A). Fisher-LSD test showed that on day 4, when the tactile stimulator was introduced, the number of crossings was lower, compared to the following days ($p < 0.0001$). On day 5, the number of crossings increased ($p = 0.000036$). From day 6, there was an increase in crossings' number, compared to the previous days ($p < 0.01$), which remained constant until day 10 ($p > 0.29$). In the period without feed, a difference among days was observed (ANOVA for repeated measures, $F_{(7,98)} = 6.37$, $p < 0.0001$, Fig. 3A). The Fisher-LSD test showed that on the day 4, when the stimulator was introduced, the number of crossings was low, in relation to the other days ($p < 0.0001$). Regarding the day 5, the number of crossings increased, comparing to day 4 ($p = 0.000076$). This raise remained until day 11 ($p = 0.12$). In this day, there was not a period with feed, we only performed the stressor application. An increase was observed in the number of crossings through the stimulator after stress (day 11)

when compared to the mean of the previous days (days 4 to 10) (paired t-test, $p = 0.04$, Fig. 3B).

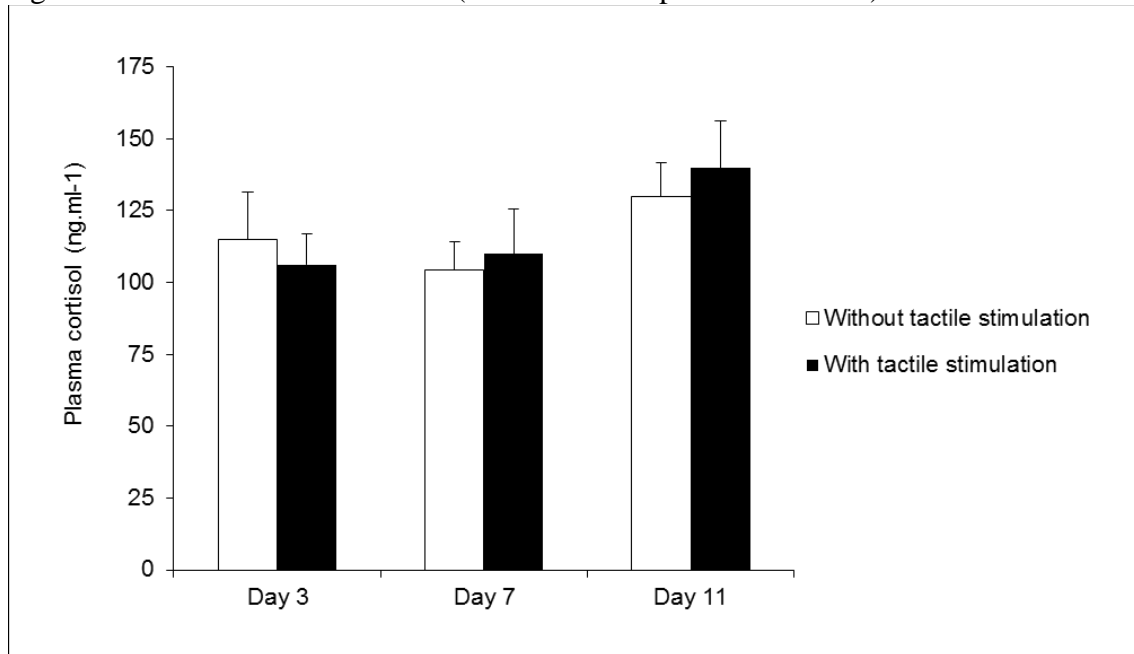
Figure 3. A) Mean \pm SE of the number of crossings through the stimulator during period with feed and period without feed. It is noteworthy that on day 11, fish were not fed during throughout the experiment. ANOVA for repeated measures, followed the Fisher-LSD test. Letters compare values within periods. Values followed by at least one equal letter did not present significant difference. B) Mean \pm SE of the number of crossings before stress (average of 7 days before the stressor application) and on day 11, after the application of the stressor. Asterisk indicates significant differences by the paired t-test.



No significant differences were observed on plasmatic levels of cortisol between treatments with and without tactile stimulation (control), as well as among sampling days (ANOVA for repeated measures, $F_{(2,36)} = 0.24$, $p = 0.78$, Fig. 4). Regarding the tactile

stimulation treatment, no correlation was found between crossings' number and cortisol levels on day 7 ($r = -0.38$, $p = 0.27$), as well as the period after stress on day 11 ($r = -0.35$, $p = 0.31$).

Figure 4. Mean \pm SE of the concentration of plasmatic cortisol after confinement stress. No significant differences were found (ANOVA for repeated measures).



4.2 Social Stress experiment

A different number of crossings per minute through the tactile stimulator was found before stress (from day 4 to 10) (ANOVA for repeated measures, $F_{(2,12)} = 22.69$, $p < 0.0001$, Table 2). Fisher-LSD test showed an increased number of crossings during the presence of feed when compared to periods before and after feed ($p < 0.0001$).

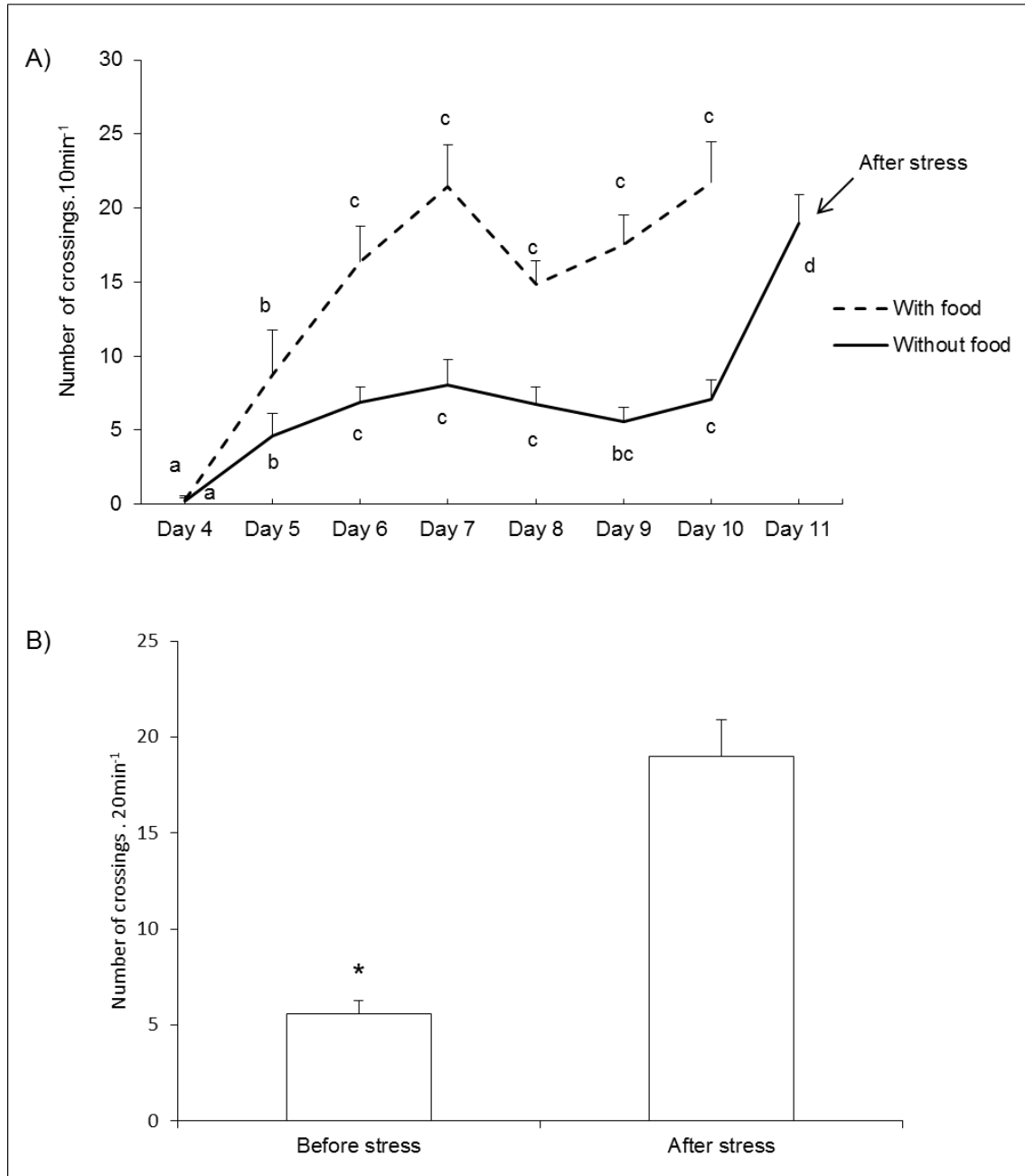
Table 2. Social stress experiment. Number of crossings per minute of the tactile stimulation treatment (14 replicates), in the three moments of records: before, during and after feed. Data from 7 days (from day 4 to 10) were summed and divided by 70 or 140 min, accordingly. Asterisk indicates significant difference. ANOVA for repeated measures, followed by Fisher-LSD post hoc test. Data are mean \pm SE.

Periods of video-recording		
Before feeder introduction	During feed	After feeder being removed
4.00 \pm 0.04	10.09 \pm 0.11 *	3.71 \pm 0.04

The number of crossing through the tactile stimulator was quantified both before (from day 4 to 10) and after social stress (day 11). Data from both daily-performed observations were summed, followed by the split of total time in two distinct periods: with (20 min / day)

and without feed (20 min / day). Regarding the period with feed, differences were observed among days (ANOVA for repeated measures, $F_{(6,78)} = 32.03$, $p < 0.0001$, Fig. 5A). Fisher-LSD test showed that on day 4, when the tactile stimulator was introduced, the number of crossings was also low, regarding other days ($p > 0.0001$). As for day 5, the number of crossings increased, when compared to day 4 ($p = 0.000002$). From day 6, an increase in crossings' number was observed, comparing to the previous days ($p < 0.0001$), which remained until day 10 ($p > 0.15$). In the period without feed, we observed a difference among days (ANOVA for repeated measures, $F_{(7,91)} = 16.23$, $p < 0.0001$, Fig. 5A). Fisher-LSD test showed that on day 4, the number of crossings was low, regarding other days ($p < 0.0004$). As for day 5, crossings' number increased, comparing to day 4 ($p = 0.0004$). As for day 6, the number of crossing has also increased concerning the previous days ($p < 0.01$), remaining until day 10 ($p > 0.34$). On day 11, an increase in crossings number was observed regarding the previous days ($p < 0.0005$). On this day, there was not a period with feed, only the stressor application was performed. Increased crossings through the stimulator were observed after stress (day 11) when compared to the average of the days before stress (days 4 to 10) (paired t-test, $p = 0.00002$, Fig. 5B).

Figure 5. A) Mean \pm SE of the number of crossings through the stimulator during period with feed and period without feed. It is noteworthy that on day 11, fish were not fed during the experiment. ANOVA for repeated measures, followed by the Fisher-LSD test. Letters compare values within periods. Values followed by at least one equal letter, did not present significant difference. B) Mean \pm SE of the number of crossings before stress (average of 7 days before the stressor application) and on day 11, after its application. An asterisk indicates significant differences after the paired t-test.

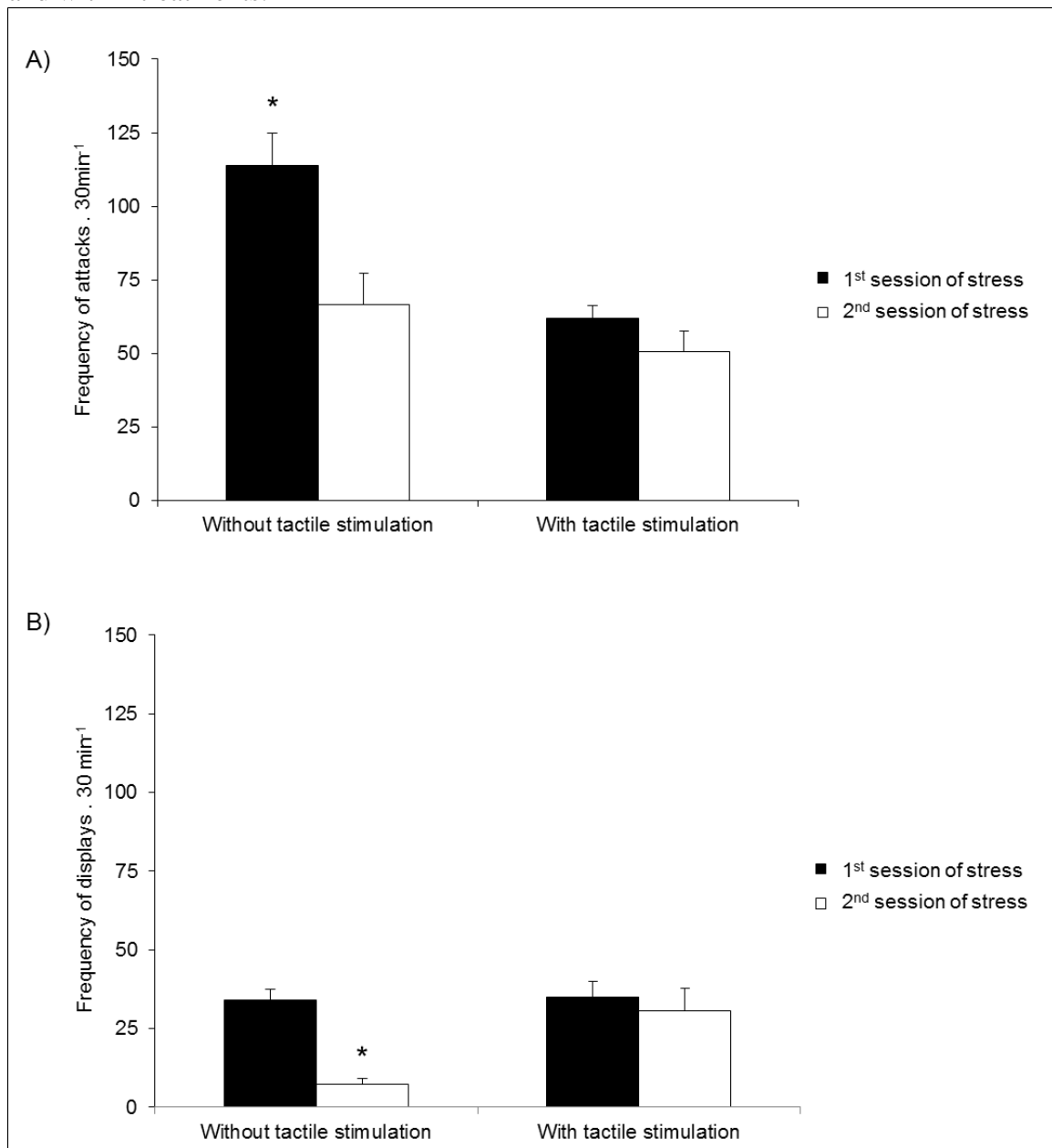


There was significant statistical interaction between treatments of the frequency of attacks (ANOVA for repeated measures, $F_{(1,26)} = 10.61$, $p = 0.003$, Fig. 6A) and between the stressor applications ($F_{(1,26)} = 9.45$, $p = 0.004$, Fig. 6A). However, no interaction was observed (for treatments and stressor applications) on the frequency of attacks ($F_{(1,26)} = 1.19$,

$p = 0.28$, Fig. 6A). Fisher-LSD test showed that the frequency of attacks was higher regarding the treatment without tactile stimulation, on the first application of the social stressor, when compared to the second one ($p = 0.006$) and when compared to both applications of the tactile stimulation treatment ($p < 0.006$).

Another significant interaction was observed (treatment *vs.* stressor application) on the frequency of displays (ANOVA for repeated measures, $F_{(1,26)} = 7.99$, $p = 0.008$, Fig. 6B). Additionally, the Fisher-LSD test showed that the frequency of displays was lower on the treatment without tactile stimulation regarding the second stressor application, when compared to the first one ($p < 0.0001$) and when compared to both applications on the treatment with tactile stimulation ($p < 0.0003$).

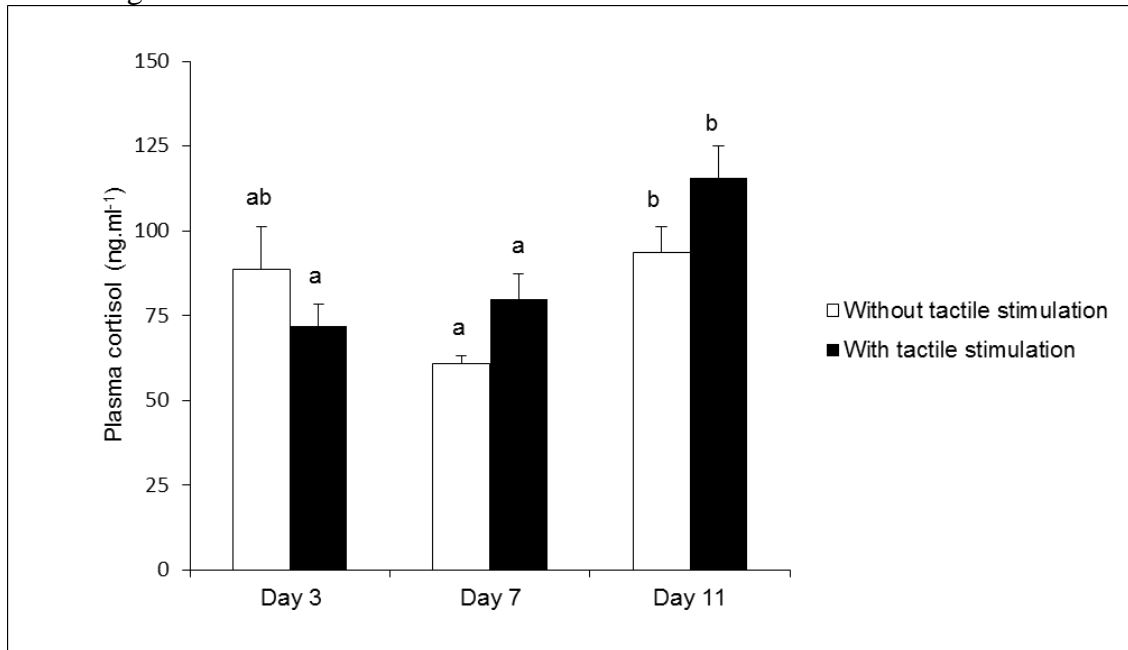
Figure 6. Mean \pm SE of the frequency of attacks (A) and displays (B). ANOVA for repeated measures, followed by the Fisher-LSD test. Asterisk indicates significant differences between and within treatments.



A significant interaction (treatment *vs.* sampling days) on the level of plasmatic cortisol was observed (ANOVA for repeated measures, $F_{(2,32)} = 4.02$, $p = 0.02$, Fig. 7). Tukey-HSD test showed that on the tactile stimulation treatment there was an increase following the second stressor application (day 11), when compared to the period before the stress (days 3 and 7) ($p < 0.03$). Furthermore, we observed that on the treatment without tactile stimulation, cortisol levels also raised after stress (day 11), comparing to a period before stress (day 7) ($p = 0.05$). However, there was no difference on cortisol levels after stress (day 11) with the period before stress (day 3) ($p = 0.99$). Concerning the tactile

stimulation treatment, no correlation was observed between the numbers of crossings through the stimulator on day 7 and the level of cortisol on the same day ($r = 0.24$, $p = 0.52$), as well as for day 11 ($r = 0.13$, $p = 0.72$).

Figure 7. Mean \pm SE of plasma cortisol in the three baseline days (day 3 and 7) and after social stress (day 11). ANOVA for repeated measures, followed by Tukey-HSD test. Letters compare within treatment. Bars followed by at least one same letter are not different. There was no significant difference between treatments.



5 DISCUSSION

In the present study, we found an efficient protocol for generating tactile stimulation in fish. However, such kind of stimulation does not immediately reduces stress on Nile-tilapia, but it acts by decreasing aggressiveness, thus probably generate positive effects on the fish's welfare.

The apparatus developed for tactile stimulating fish was efficient because fish increased the crosses in between bristles after being trained with food. In fact, crosses increased in both experiments, and increased even in periods without feed. This type of training is common for conditioning fish to achieve some goal (WRIGHT; EASTCOTT, 1982; YUE; DUNCAN; MOCCIA, 2008; HÄDERER; MICHIELS, 2016) and worked in this study. Moreover, the frequency of crosses before and after feed introduction show that fish uses the stimulator irrespective of feed presence. As expected, the animals increased crossed through the stimulator after the stressors application, being this fact more evident after social stress. However, an immediate stress reduction was not demonstrated.

We expected that after confinement stress, fish would seek stimulation more often, if it relieved stress. Although, we observed that despite this seeking starts right after confinement liberation, the number of crossings was similar to the previous stress condition. On the other hand, by comparing the average number of crossings of all days before stress with the period after stress, we observe that the animals spontaneously sought more for stimulation after the being stressed. The spontaneous seek for tactile stimulation, however, was clearly increased after fighting (social stress). This can indicates that tactile stimulation should relieve the effects from aggressive interaction. But another interpretation is that, although cortisol levels are similar, the social stress is more intense than non-social one, thus fish will seek for ways to alleviate more intense stress.

Regarding aggressive interactions, we observed that the number of attacks, a higher energy expenditure interaction with more physical conflicts (ROS, BECKER, OLIVEIRA, 2006; COPELAND et al., 2011) was lower on the group with access to tactile stimulation. Thus, a tactile stimulation effect on fish's aggressiveness existed. In humans, for example, body massages increases serotonin levels (FIELD et al., 2005) and in contrast, reduced serotonin increases aggressive behavior in fish (MUNRO, 1986; LEPAGE et al., 2005). Then, tactile simulation could be a mechanism for reducing aggressiveness by increasing serotonin in fish. Also, other studies showed that fights are more intense when fish are socially isolated (CORRÊA et al., 2003; EARLEY et al., 2006) as we did in this study. In this sense, reduced

aggressiveness shows an important effect of tactile stimulation. However, reduced aggressiveness was not followed by reduced cortisol.

We designed a study to compare with the one performed by SOARES et al. (2011), in which the previous effect of tactile stimulation was observed in stress reduction of *Ctenochaetus striatus*, a fish species that naturally receives tactile stimulation by other fish (GRUTTER, 2004). In our case, we also tested the previous effect of tactile stimulation on stress, but in a non-natural situation, where the stimulation was artificially provided to fishes that does not possesses this kind of positive interaction. Nevertheless, we expected that the plasmatic level of cortisol of the animals with tactile stimulation should be lower after non-social and social stress, as shown by SOARES et al. (2011) for non-social stress on the coral reef fish *Ctenochaetus striatus*. However, no differences were observed regarding plasmatic cortisol levels between treatments with and without tactile stimulation and social stress. We have also observed that the plasmatic level of cortisol increased after social stress. In fact, the animals presented high levels of cortisol all along the experiment, in contrast to basal levels of cortisol in GIFT Nile tilapia (e.g. BOSCOLO; MORAIS; GONÇALVES-DE-FREITAS, 2011), which could be explained by social isolation (EARLEY et al., 2006; GALHARDO; OLIVEIRA, 2014). In this way, fish could be already stressed so that we did not observe significant increment on plasma cortisol. We highlight that after non-social stress, even with no decrease, plasmatic cortisol levels did not increase with the presence of the stimulator, showing that the stimulator did not impaired the stress status of the animal. Another study performed in our laboratory also shown high levels of plasmatic cortisol in isolated GIFT Nile-tilapia (non-published data). Increased levels of cortisol after social stress occurred after aggressive confrontation, as demonstrated in fish by several authors (e.g. CORRÊA et al., 2003; GALHARDO; OLIVEIRA, 2014). However, this goes against what we expected and shows that there was no effect of tactile stimulation immediately after social stress. These results may be due to the blood sampling, which occurred during cortisol peak (BARCELLOS et al., 1999). In this sense, it is important to evaluate the previous effect of tactile stimulation in this hormonal profile during the stage of stress recovering and observe the effect of tactile stimulation after stress.

Studies regarding tactile stimulation in mammals usually count on human presence and stimulation is provided by touch (e.g. PROBST et al., 2012, PROBST et al., 2013), petting or stroking (e.g. COULON et al., 2015; SCHMIED et al., 2008; SCHMIED et al., 2010; TALLET et al., 2014). These studies were mainly performed with domestic (e.g. cattle, swine and lambs) mammals. These interactions show both positive and negative effects for

the animals. Certain studies focus on the affection and bonds that are created between humans and animals (e.g. TALLET; VEISSIER; BOIVIN, 2008; COULON et al., 2013; HOUSEY; MELFI, 2014), thus not eliminating the social context of human contact, that may influence the animal's perception. Regarding the studies with fish and this study, we isolated the tactile stimulation variable to understand if it could be responsible for the effect of this stimulation. Thus, we highlight that for non-social stress, seeking for tactile stimulation occurred after the stressor application on the similar intensity before the stress. For social stress, searching for tactile stimulation raised right after stress, but decreased stress was not observed in both cases. In addition, aggressiveness decreased on fish in contact with stimulation, effect evidenced on attacks, interactions that has physical contact and greater energy expenditure (ROS; BECKER; OLIVEIRA, 2006; COPELAND et al., 2011). Therefore, we demonstrated for the first time that tropical territorial fish have spontaneously searched for tactile stimulation, including after the application of two kinds of stressors. We highlight the potential of tactile stimulation to reduce aggressiveness in Nile-tilapia, which may indicate improved welfare. We suggest that more studies should be performed regarding tactile stimulation and stress reduction that does not verify effects only immediately after stress, but also in stress recovery.

6 CONCLUSION

We conclude that for non-social stress, fish seek for tactile stimulation after the stressor application on the similar intensity before the stress. For social stress, fish seek for tactile stimulation raise right after stress. Decrease in stress was not observed in both cases. In addition, aggressiveness decreases on fish in contact with stimulation.

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