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Preference for reaching tactile stimulation in a territorial fish is not affected by social stress

São José do Rio Preto, SP, Brasil.

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de São José do Rio Preto.

Orientadora: Prof^a. Dr^a. Eliane Gonçalves de Freitas

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RESUMO

O bem-estar animal é avaliado por indicadores fisiológicos e comportamentais, como nível de estresse, taxa de crescimento, comportamentos estereotipados e desempenho cognitivo. Uma abordagem recente utiliza a percepção do próprio animal como uma forma de saber se o mesmo se encontra em bom estado de bem-estar, por meio de testes de preferência e motivação, considerando que o animal está bem saúde quando está em condições por ele preferidas. Neste estudo, usamos essa abordagem para testar se peixes territoriais percebem a estimulação táctil corporal como um recurso positivo, uma vez que essa condição tem sido considerada uma maneira de melhorar o bem-estar em peixes. Assim, testamos se a tilápia-do-nilo prefere e está motivada a acessar a estimulação tátil; e se a preferência aumenta após um estímulo negativo, como o estresse oriundo de interações agressivas (estresse social). Machos adultos foram isolados por oito dias em aquários contendo um estimulador táctil central formado por hastes plásticas verticais com cerdas de silicone, pelas quais os peixes passam recebendo estimulação táctil corporal. Posteriormente, os animais foram submetidos a um teste de preferência no qual o peixe tinha a opção de atravessar uma área com estimulação táctil ou uma área sem estimulação. Em seguida, os peixes passaram por um teste de motivação, no qual deviam superar estímulos aversivos (alta iluminação) para acessar a estimulação táctil. Depois disso, os animais foram pareados para estabelecer a hierarquia de social por meio de interações agressivas, e novamente submetidos a testes de preferência e motivação. Um grupo controle foi submetido aos mesmos procedimentos, mas sem o estressor social. Observamos que o número de atravessamentos foi maior pela área sem estimulação táctil, tanto nos testes de preferência quanto nos de motivação, embora algumas diferenças individuais tenham sido detectadas. No entanto, os peixes não evitaram a área com estimulação, mesmo com a opção de permanecer em áreas neutras; os animais também superaram a rota aversiva de alta iluminação para obter o acesso ao estimulador táctil. O estresse social não afetou o comportamento de preferência e motivação. Além disso, observamos que os peixes que atravessaram mais o estimulador se tornaram submissos, de modo que a estimulação táctil atuou modulando a hierarquia social reduzindo a agressividade. No geral, concluímos que os peixes não percebem a estimulação tátil como um alívio de estresse social. No entanto, como os animais não evitaram a estimulação e enfrentaram um estímulo aversivo para obter acesso a ela, sugerimos que a estimulação táctil pode representar uma condição positiva para peixes territoriais.

Palavras-chave: bem-estar de peixes, escolha, motivação, comportamento social.

ABSTRACT

Animal welfare is evaluated by several physiological and behavioral indicators such as stress level, growth rate, stereotyped behavior and cognitive performance. A recent approach uses the perception of the animal itself as a way to evaluate if this animal is in a good welfare state, by preference and motivation tests, considering that the animal is in good welfare when it is in conditions that it chooses itself. Here, we used this approach to test whether territorial fish perceive body tactile stimulation as a positive resource, since this condition has been considered a way to improve fish welfare. Thus, we tested whether the fish Nile tilapia prefers and are motivated to access tactile stimulation; and if the preference increases after a negative stimulus, such as the stress from aggressive interaction (social stress). Adult males were isolated for eight days in aquaria containing a central tactile stimulator device formed by vertical plastic sticks with silicone bristles, whose fish pass throughout, receiving body tactile stimulation. Afterwards, they were assigned to a preference test in which the fish had the option of crossing between an area with tactile stimulation and an area without stimulation. Then, fish went to a motivation test, in which it must overcome aversive stimuli (bright light) to access the tactile stimulation. Thereafter, they were paired to establish social rank by aggressive interactions, and subjected again to preference and motivation tests. A control group underwent the same procedures but without the social stressor. We observed that the number of crossings was higher in the area without tactile stimulation either in preference or motivation tests, although some individual differences were detected. Nevertheless, the fish did not avoid the area with stimulation, even with the option of remaining in neutral areas; and also overcame the aversive route to obtain the access to the tactile stimulator. Social stress did not affect the preference and motivation behavior. Additionally, we observed that fish that crossed more through the apparatus became subordinate, thus that tactile stimulation modulates social rank hierarchy by reducing aggressiveness. Overall, we conclude that fish does not perceive tactile stimulation as a social stressor relieve. However, as fish did not avoid the stimulation and faced an aversive stimulus to gain access to it, we suggest that tactile stimulation may represent a positive condition for territorial fish.

Keywords: fish welfare, choice, motivation, social behavior.

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1. INTRODUCTION

Attention to animal welfare has been a frequent concern of society in recent years (BROOM, 2011). Studies on fish welfare and aquaculture conditions have been increasing considerably (ASHLEY, 2007) as long as fish are considered sentient beings (VOLPATO *et al.*, 2007), capable of presenting consciousness of feelings and sensations (GALHARDO; OLIVEIRA, 2006). The most traditional animal welfare research relies on indicators of animal state as physiological responses, such as catecholamine and corticosteroid levels in blood plasma, which suggest stress levels variation (HUNTINGFORD, 2006); behavioral responses such as the presence of abnormal behaviors (HUNTINGFROD, 2006), stereotyped behaviors (ALMAZÁN-RUEDA *et al.*, 2004), changes in aggressive behavior (BOSCOLO *et al.*, 2011) and cognitive performance (BRANDÃO *et al.*, 2015). A more recent approach considers that a welfare condition can be inferred by the perception of animal needs and from the own animal perspective (DUNCAN, 2006; KIRKDEN; PAJOR, 2006; NICOL *et al.*, 2009; GALHARDO; OLIVEIRA, 2011; FRANKS, 2019).

An efficient way to investigate whether a particular condition confers welfare by animal perception is to test the choice and preference of these animals for such condition (DUNCAN, 1992; VOLPATO *et al.*, 2007; MAIA; VOLPATO, 2016). These tests are designed to analyze how the animal feels by functional and behavioral traits (DUNCAN, 2006) and the animal's ability to choose the absence of negative states (DAWKINS, 2006; BOISSY *et al.*, 2007) and the presence of positive ones (DUNCAN, 2002). Thus, if the animal presents this positive perception regarding some environment's condition, it is expected that it will choose to access or remain in that condition (VOLPATO *et al.*, 2007; DUNCAN, 2002), for example the preference for structured environment in zebrafish (*Danio rerio*) and checker barbs (*Puntius oligolepis*) (KISTLER *et al.*, 2011) and the preference for substrate in Mozambique tilapia (GALHARDO; OLIVEIRA, 2011). It is important to emphasize that choice and preference may represent different concepts depending on the context (MAIA; VOLPATO, 2016). The preference-based approach requires consistency between the choices over time (VOLPATO *et al.*, 2007; MAIA; VOLPATO, 2016).

As a complement to the preference tests, it is important to understand how animals are motivated to achieve a particular condition (DUNCAN, 1992; DAWKINS, 2006; MILLOT *et al.*, 2014; MAIA *et al.*, 2017; FRANKS, 2019) according to the idea that the more important a condition is to the animal, the higher is the "price it is willing to pay" to reach it (KIRKDEN;

PAJOR, 2006; GALHARDO; OLIVEIRA, 2011). The study of motivation is crucial to understand how animals make their decisions (BERRIDGE, 2004; JENSEN; PEDERSEN, 2008). It corresponds to the balance between the external stimuli and the internal state of the animal in the previous moment to a decision making (KIRKDEN; PAJOR, 2006), for example, the choice to access a particular resource. Motivation tests can, then, indicate how valuable a resource is to the animals (MAIA; VOLPATO, 2016), establishing a connection across perception, needing and preference in the analysis of welfare (JENSEN; PEDERSEN, 2008). In this context, one of the most suitable experimental designs to study motivation is the use of aversive obstacles or stimuli, which the animal must overcome in order to reach a particular resource (DUNCAN, 1992; MAIA *et al.*, 2017). The way an individual cope with these obstacles and stimuli indicates the importance of the condition or resource to be achieved by the animal (GALHARDO; OLIVEIRA, 2011). Physical obstacles like push-door operant devices are commonly used (OLSSON *et al.*, 2002). In this model, increased costs are imposed by adding weights to a door and the animal has to push these heavy doors to access a certain resource. Psychological effort tests are also efficient in demonstrating animal motivation (MAIA *et al.*, 2017), for example pathways with aversive stimulus that animals have to cross to reach the resource. These stimuli can be a high light route (MAIA *et al.*, 2017) or the presence of artificial water currents for fish (SULLIVAN *et al.*, 2016). Thus, different resources or attributes in the environment can be tested by preference and motivation.

A recent way thought to improve welfare and increase positive state perception in vertebrates is the body tactile stimulation (GONÇALVES-DE-FREITAS *et al.*, 2019). In mammals, for example, physical interactions as body massage or touches are able to reduce stress (FIELD *et al.*, 2005. HERNANDEZ-REIF *et al.*, 2007; PROBST *et al.*, 2012); to relieve pain (FIELD, 1998; JANE *et al.*, 2009); to elevate serotonin levels (FIELD *et al.*, 2005) to minimize behavioral traits of anxiety and depression (FREITAS *et al.*, 2015; ANTONIAZZI *et al.*, 2017) and to prevent learning deficit (DE LOS ANGELES *et al.*, 2016). Furthermore, teleost fishes have been tested for this effect as well. Soares *et al.* (2009) were the first to show that tactile stimulation reduces stress in a coral reef fish which perform a client-cleaner interaction (*Ctenochaetus striatus* and *Labroides dimidiatus*, respectively). These authors observed that the clients seek for tactile stimulus promoted by the cleaners, and that such stimulation reduces cortisol levels of the client fish after confinement stress. Another study demonstrated that tactile stimulation reduces aggressive interaction in pairs of male Nile tilapia, a territorial species (BOLOGNESI *et al.*, 2019), although did not reduce

cortisol levels. In Soares *et al.* (2009) study, tactile stimulation is part of the species' natural behavior. However, Nile tilapia is a territorial fish; therefore it is not clear yet if tactile stimulation is perceived as a positive condition, in order to improve their welfare. In this context, a preference-based approach is adequate to answer this question.

Thus, in this study we tested whether Nile tilapia freely choose tactile stimulation and also their motivation to access this resource. We also tested if such preference and motivation increase after a negative stimulus, such as the stress from aggressive interactions (social stress). If the perception of tactile stimulation is generalized and positive in vertebrates, we expected that territorial fish such as Nile tilapia would freely choose to access this resource and that they would be more motivated to access tactile stimulation after experiencing a stressful situation. Nile tilapia, *Oreochromis niloticus* (L.) is a very important species in aquaculture (BARCELLOS *et al.*, 1999. GONÇALVES-DE-FREITAS *et al.*, 2019) and the knowledge of tools that promote welfare of these animals is highly relevant (VOLPATO *et al.*, 2009; GONÇALVES-DE-FREITAS *et al.*, 2019). In social system of the Nile tilapia, the males establish a dominance hierarchy by aggressive confrontations (ALVARENGA; VOLPATO, 1995, CARVALHO *et al.*, 2008; BARRETO *et al.*, 2011). The occurrence of these confrontations between conspecifics causes social stress, one of the main causes of chronic stress in Nile tilapia rearing (BARCELLOS *et al.*, 1999; BOSCOLO *et al.*, 2011). The social stress arising from the establishment of the dominance hierarchy can generate serious damages to these animals, thus becoming an important factor of poor welfare (GONÇALVES-DE-FREITAS *et al.*, 2019). Therefore, it is important to investigate conditions that reduce detrimental effects from social interaction in Nile tilapia, such as tactile stimulation.

2. METHODS

2.1. Fish housing

Adult males of GIFT Nile tilapia from a commercial supplier (Ribeirão Preto, SP, Brazil), were kept in outdoor ponds at the IBILCE, UNESP, São José do Rio Preto, SP, Brazil. The fish were collected for the study and taken to the laboratory where they all were acclimated together for 15 days in a polyethylene water tank (ca. 500 L, 1 fish/10 L) with water at 28°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) once a day (10:00 a.m.). The water quality was maintained by biological filters with filtration of 400 L/h, and constant aeration.

2.2. Strategy of study

Nile tilapia males were initially isolated for 8 days in an aquarium with the tactile stimulator apparatus on its center to allow them experience tactile stimulation. On the 9th day they underwent a preference test with four sessions of choice for tactile stimulation and on the 10th day, a motivation test in which the fish had to cross an aversive area to achieve the stimulation. Then the fish were paired with another male of the experiment to set a social rank (dominant and subordinate) and create social stress (16 replicas). After that, the preference and motivation tests were repeated. A control group was subjected to the same procedure, but without social stress (16 replicas).

Before isolation the animals were anesthetized by immersion in Benzocaine (0.03 g.L⁻¹), weighted, sized and individually identified by green elastomer (VIE tags), inserted under 2 or 3 scales on each side of the body. The mean (\pm S.E.) standard length and weight of fish were respectively: Treatment with social stress: 10.76 cm \pm 0.61 cm; 41.74 g \pm 7.86 g; Control group: 10.42 cm \pm 0.71 cm; 39.02 g \pm 9.55 g. The animals were observed in glass aquaria (80 x 30 x 40 cm) coated with blue plastic to avoid visual contact between 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, and cameras placed on tripods in front of the aquaria. The photoperiod was set to 12L:12D (from 7:00 a.m. to 7:00 p.m.) and temperature to 28° C. The water quality was monitored by commercial kits and electronic devices: Ammonia (0.025 ppm) and Nitrite (0.125 ppm); pH (8.36 \pm 0.25).

2.3. Experiencing tactile stimulation

We used an apparatus developed by Bolognesi *et al.* (2019) to provide tactile stimulation in fish, which is made of a rectangular PVC (polyvinyl chloride) structure, filled with vertical plastic sticks with silicone bristles each side (Figure 1A). Silicone bristles were chosen because they are soft, thus avoiding mucus removing from the skin, scale loss and other possible 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 receive tactile stimulation (Figure 1B). To stimulate fish to cross through the apparatus, we offered food (dry shrimps) on the aquarium side, opposite to the side where the fish was, using a handling feeder (Figure 1 C).

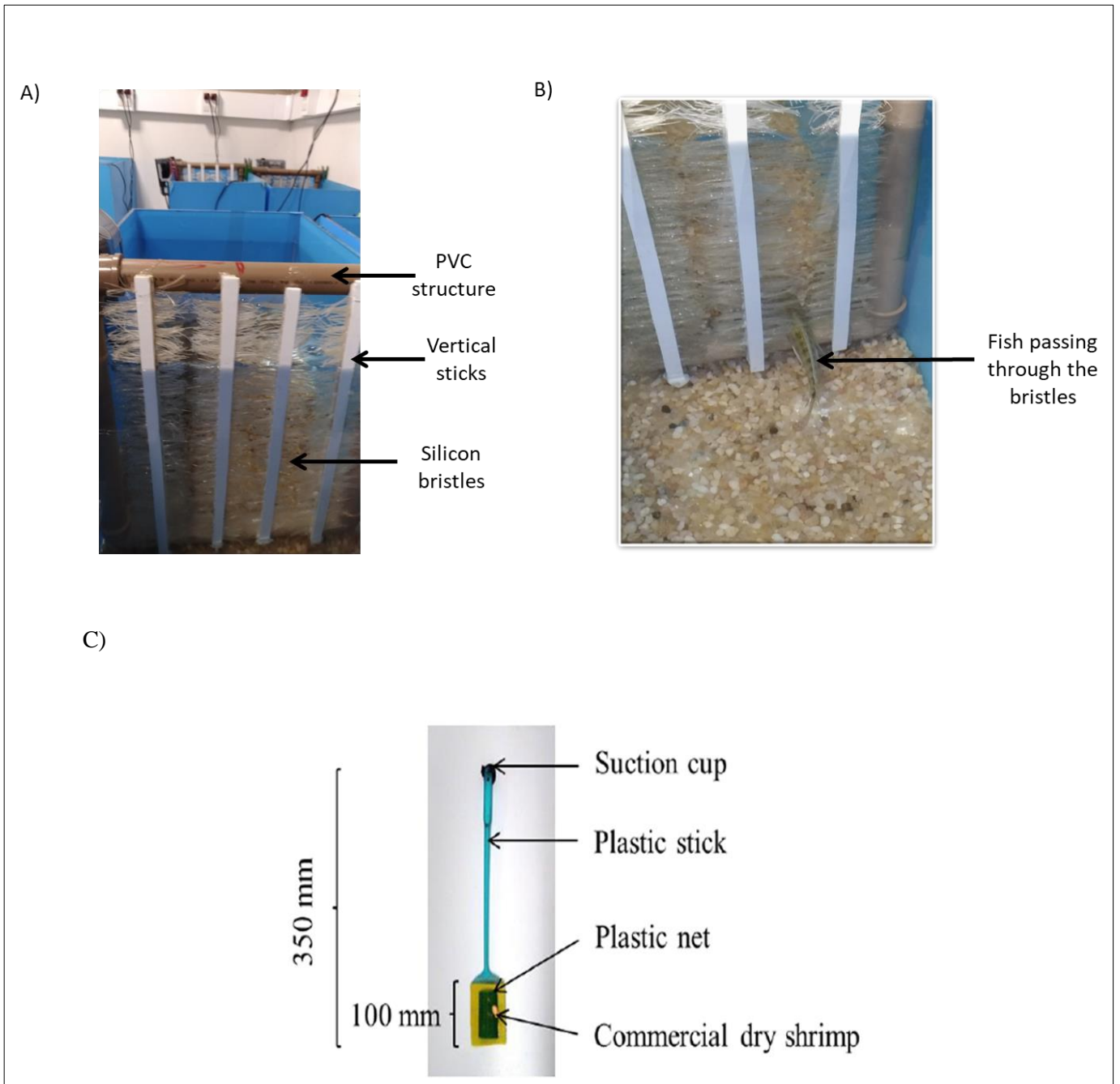


Figure 1. A) Photo of the apparatus for tactile stimulation positioned in the aquarium's center. B) Example of a fish passing through the silicon bristles receiving the body tactile stimulation. C) Feeder.

Before the beginning of the experiment, the fish underwent a period of habituation to this feeder. Here, the term "habituation" does not refer to the extinction or decrease of a behavior, but to the fact that the fish becomes accustomed to a new experimental condition. The food was the stimulus for the fish to experience initial contact with the tactile stimulator. The food used was dried shrimp, attached to the feeder. The fish were removed from the water tank and placed together, randomly in an aquarium (60x60x40 cm, ca. 140L, four fish

per aquarium), where they remained for 3 days under the same conditions as those used in acclimation, except for feeding. During this period the animals were fed with the feeders six times a day being three times in the morning (8:00 a.m., 9:30 a.m. and 11:00 a.m.) and three times in the afternoon (2:00 p.m., 3:30 p.m. and 5:00 p.m.).

2.4. Experimental Procedures

On the 1st day of the experiment the animals were removed from the habituation aquaria and isolated in the experimental aquarium with the tactile apparatus. Two feeding sessions were held in the morning (9:00 a.m. and 11:00 a.m.) and two in the afternoon (2:00 p.m. and 4:00 p.m.) for 8 days. All feeding sessions were video-recorded 5 minutes before, 5 minutes during and 5 minutes after feeding. For data analysis, we considered the number of crossings before and after feeding. This protocol had been previously tested and validated by Bolognesi *et al.* (2019). Afterwards, fish underwent preference and motivation tests, followed by the effect of social stress.

On the 9th day the animals were assigned to preference test with 4 sessions of choice (20 min each) at 9:00 a.m., 11:00 a.m., 2:00 p.m. and 4:00 p.m. On the 10th day, the animals were assigned to 2 sessions of the motivation test (20 min each) at 9:00 a.m. and 2:00 p.m. On the 11th day, the fish were removed from their original aquarium and paired with another one, also from the experiment, in a neutral aquarium for aggressive interactions (20 min; 8:30 a.m.), thus generating social stress. After that, the animals returned to their original aquarium and were assigned again to the preference test (9:00 a.m. and 11:00 a.m.). The pairing was repeated at 1:30 pm, followed by the preference test (2:00 p.m. and 4:00 p.m.). On the 12th day the fish were removed again from their original aquarium and paired with another one in neutral aquarium for aggressive interactions (20 minutes; 9:30 am). After that, the animals returned to their original aquarium and were assigned to the motivation test (10:00 am). The pairing was repeated at 2:30 p.m., followed by the motivation test (3:00 p.m.).

For the control group, the procedures and schedules were identical, except for the agonistic interaction. At this time, instead of pairing with another male, the fish were only placed in a new aquarium for 20 min so they had the same manipulation as the social stress treatment. Then, the animals returned to the aquariums for the tests of preference and motivation.

2.4.1. Preference Test

In this test, the tactile apparatus had half of its area filled with silicone bristles, and the other half with the sticks but without the silicone bristles so that fish could choose one of these areas (with or without tactile stimulation) (Figure 2). During the test, the animals were filmed for 20 minutes and the frequency of crossings through the two areas was recorded. In this test we evaluated the spontaneous choice of the fish and, therefore, we did not use the feeder, avoiding the effect of conditioning on the preference. The test was repeated in the afternoon to evaluate the consistency and performed in a way that did not present any clue that could bias the animal's preference. In addition, the positions of the regions of the apparatus (with the bristles and without the bristles) were alternated between the replicates.

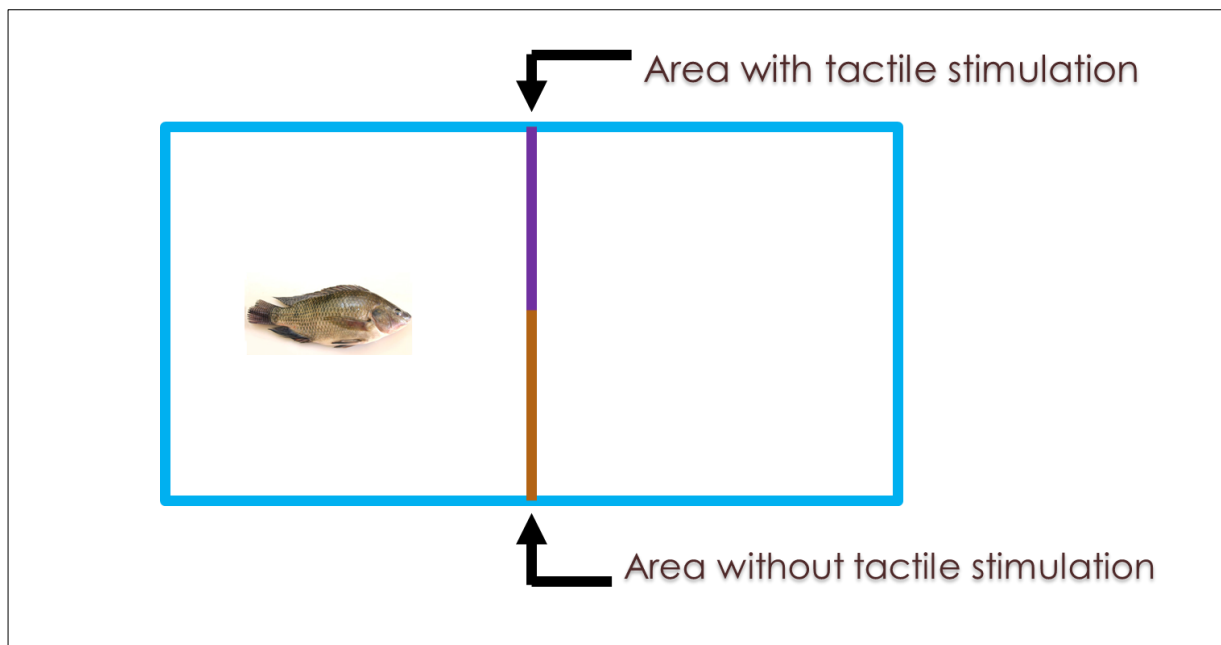


Figure 2. Design of the preference test.

2.4.2. Motivation Test

This test was based on the study of Maia *et al.* (2017), aiming to test the fish's propensity to surpass an aversive obstacle to reach a resource. For this, a small area in one aquarium's end was covered by a black fabric, creating a dark area (refugee). The fish were initially confined in this region through the use of transparent plastic plates. On the opposite side to the dark area the tactile stimulator was fixed, corresponding to the model with an area with the silicone bristles and another without the silicone bristles, like the preference tests. Between the dark area and the tactile stimulator, we made a route lightened by a LED lamp (900 lumens), positioned above the aquarium, thus creating an aversive obstacle for the animal, similar to the protocol from Maia *et al.* (2017) for rainbow trout. Light was used

because it is a negative stimulus for several fish species (MARCHESAN *et al.*, 2005. MESQUITA *et al.*, 2007), including Nile tilapia (MAXIMINO *et al.*, 2007).

The "price to be paid" by the fish (motivation) was inferred from the latency for leaving the dark area and crossing the aversive route to access the stimulator (device). The animals were released from the dark area after 5 minutes and then filmed for 20 minutes. The latency to access the apparatus and the number of crossings in both areas of the apparatus were recorded.

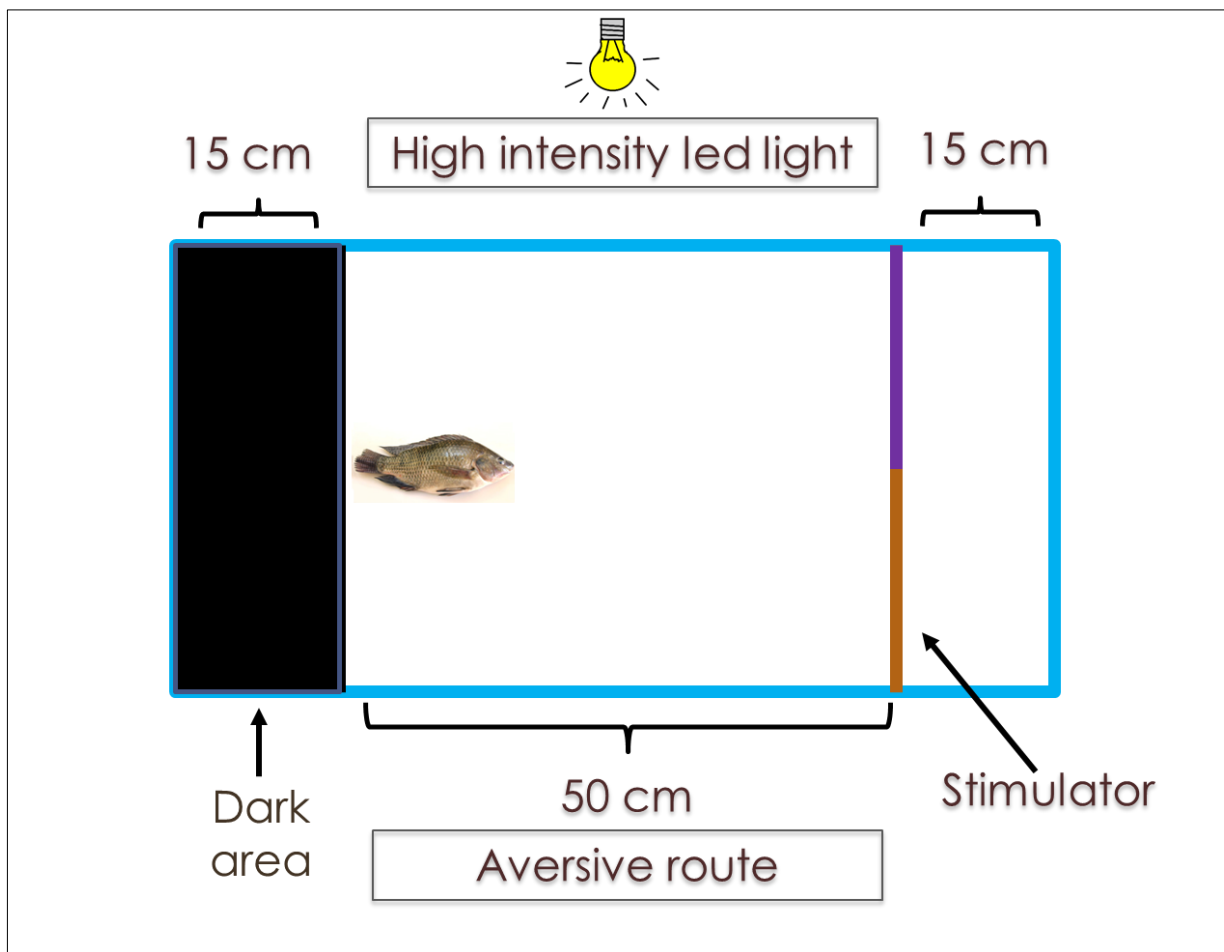


Figure 3. Design of the motivation test.

2.4.3. Social stress

We analyzed the aggressive interactions performed by each fish during the pairing (treatment with social stress), according to the previously described ethogram for Nile tilapia (GONÇALVES-DE-FREITAS *et al.*, 2008). Aggressive behavior was divided as attacks and displays. Attacks are the interactions with physical contact and higher energy expenditure (as nipping, mouth fight and undulation), and the displays are interactions without physical contact and lower energy expenditure (ROS *et al.*, 2006) (as lateral threat and circling). Aggressive interactions were quantified to determine the social rank of the paired fish in the

dominance hierarchy (dominants and subordinates). We used the dominance index to determine the social rank. The dominance index was calculated for each individual as the number of interactions emitted divided by the number of interactions emitted and received (GONÇALVES-DE-FREITAS *et al.*, 2008). This value varies from 0 to 1, and the animal with the index closer to 1 is considered the dominant and closer to 0 the subordinate (GONÇALVES-DE-FREITAS *et al.*, 2008).

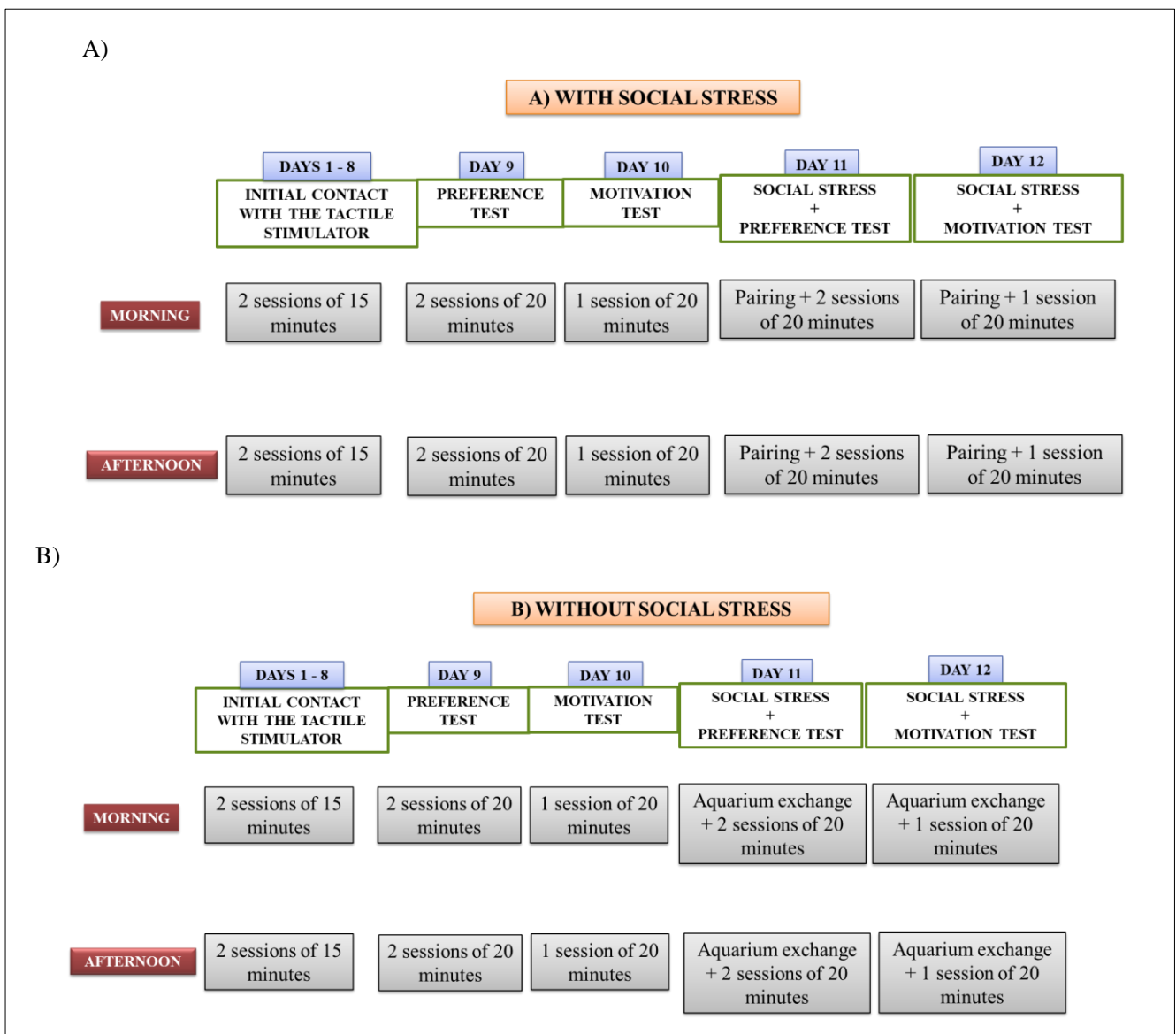


Figure 4. Sequence of events during the experiment in: A) Treatment group B) Control group.

2.5. Data analysis

The data were tested for normality by Kolmogorov-Smirnov test and homoscedasticity by Fmax (LEHNER, 1996). Mixed model ANOVA was used to compare between the

treatments (with social stress and without social stress) and inside each treatment: the number of crossings in the initial 8 days of contact with the tactile stimulator and in the preference and motivation tests; the latency of the access to the tactile stimulator in motivation tests. Data were also contrasted by Planned Comparisons.

In the first 8 days of the experiment we quantified the number of crossings through the tactile stimulator by the fish 5 minutes before and 5 minutes after feeding to test the efficiency of using the apparatus over time. On the days of the preference test we quantified the number of crossings through the tactile stimulator in the area with and without the bristles, between treatments. In motivation tests we quantified the latency to access the tactile stimulator, in addition to the number of crossings in each areas (with and without the bristles) and also the frequency of aggressive interactions emitted by each fish during the pairing (treatment with social stress). The association between aggressive interactions and elevated plasma cortisol levels is already well established for Nile tilapia (CORREA *et al.*, 2003; BARRETO *et al.*, 2015; BOLOGNESI *et al.*, 2019) and therefore we did not measure the cortisol levels of treatments.

2.5.1. Preference Index

We used the preference index (PI) based on the study of Maia & Volpato (2016) to complement the analysis of the number of crossings in the preference test of each fish, individually. This analyze allows us to check over preferred and non-preferred items in a preference test, in our case, with two options of choice. The index calculation follows some steps that are represented in an example in Table 1. First, the frequency of crossings in each area (with or without tactile stimulation) was summed. Then, we calculated the areas above the line of cumulative-frequency so that these calculated areas increase as the preference test trials progress. The most recent choices may better represent what the animal really prefers considering its choices over time (MAIA; VOLPATO, 2016; MAIA *et al.*, 2017). Then, all the calculated areas are cumulative. The positive PIs represent the preferred option, meanwhile the negative PI values indicate the non-preferred option, and the values of PI represent the intensity of preference/dispreference responses (MAIA; VOLPATO, 2016; MAIA *et al.*, 2017). We calculated for each fish, separated PI values that include four test trials before manipulation and four test trials after manipulation (manipulation was: male pairing in a new aquarium in the treatment group or isolated in a new aquarium in the control group).

Table 1. Preference Index steps. Example of treatment's group fish 1 PI values for the area with tactile stimulation before manipulation.

Test period	Frequency	Cumulative frequency	Area	Cumulative area	Expected area	Area variation	Preference Index (PI)
1	2	2	1	1	5,25	-4,25	-4,25
2	6	8	9	10	30,75	-20,75	-25
3	8	16	20	30	50,75	-20,75	-45,75
4	5	21	17,5	47,5	80,5	-33	-78,75

3. ETHICAL NOTE

This study is in accordance with the Ethical Principles 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 #171/2017.

4. RESULTS

4.1. Number of crossings from day 1 to 8

A different number of crossings through the tactile stimulator was found in the 8 days of initial contact with the apparatus ($F_{(7,105)} = 20.57$ $p < 0.0001$, Figure 5). The number of crossings in day 2 increased from day 1 ($p = 0.00015$) and was lower than in the remaining days ($p < 0.0008$). The number of crossings became stable from day 3 to 8 ($p > 0.664$).

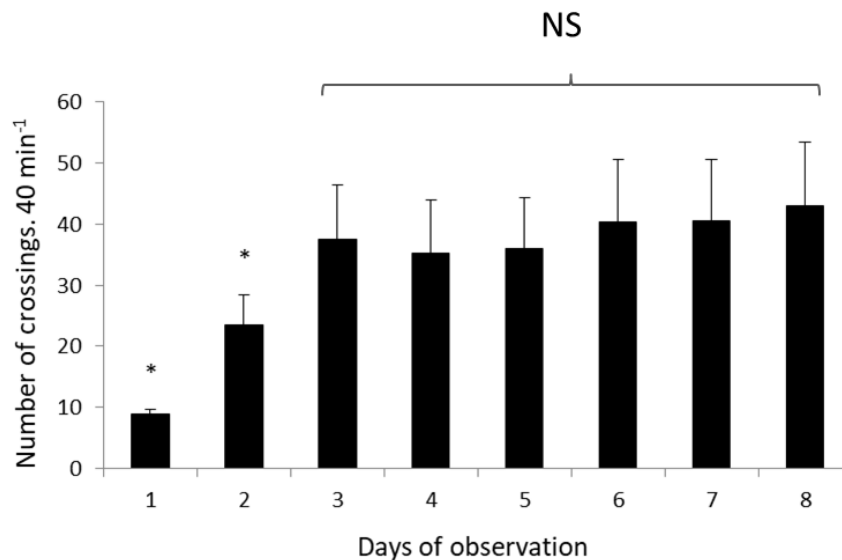


Figure 5. Number of crossings through the tactile stimulator during the eight days of initial contact with the apparatus (32 replicas). ANOVA for repeated measures followed by Fisher-LSD test. Asterisk indicates significant differences between the days. Data are mean \pm SE.

4.2. Preference test

There was no significant statistical interaction for the number of crossings through tactile device between treatments in the preference test (with vs. without social stress; $F_{(3,90)} = 0.71$, $p = 0.54$, Figure 6). However, we observed differences in the number of crossings between the areas with and without tactile stimulation before and after manipulation ($F_{(3,90)} = 19.44$, $p = 0.0001$). Planned comparisons showed that fish crossed more times through the area without tactile stimulation than through the area with tactile stimulation in the control, before ($F_{(1,30)} = 7.03$, $p = 0.012$) and after ($F_{(1,30)} = 12.17$, $p = 0.015$) manipulation and in the social stress treatment before ($F_{(1,30)} = 7.53$, $p = 0.010$) and after ($F_{(1,30)} = 12.17$, $p = 0.001$) manipulation. We also observed by planned comparisons that after fighting, the number of crossings through the area with tactile stimulation decreased ($F_{(1,30)} = 13.34$, $p = 0.009$) such as the number of crossings through the area without tactile stimulation ($F_{(1,30)} = 13.09$, $p = 0.008$), but was similar before ($F_{(1,30)} = 2.05$, $p = 0.122$) and after ($F_{(1,30)} = 2.77$, $p = 0.106$) manipulation in the control group.

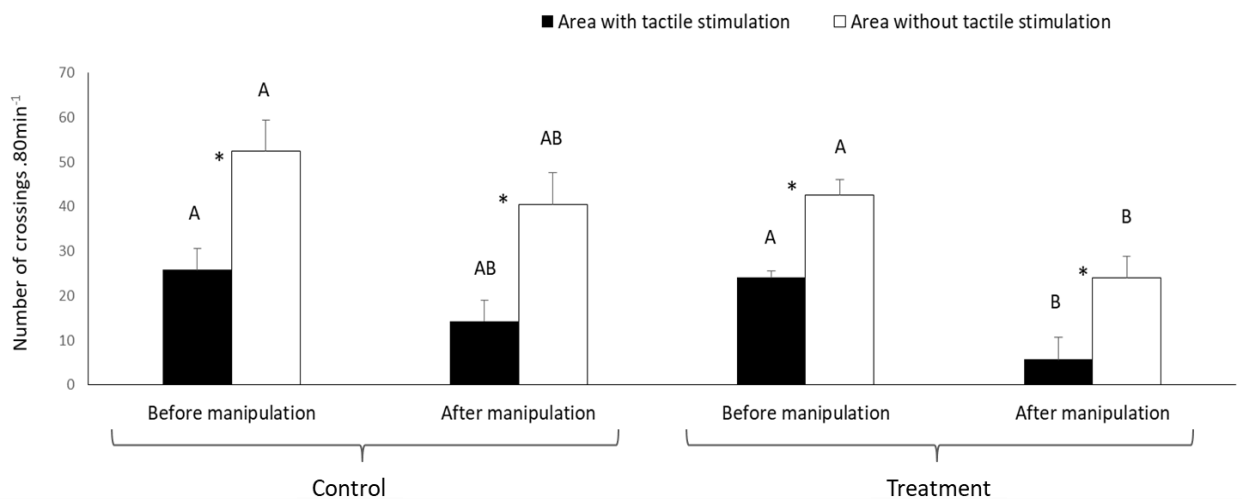


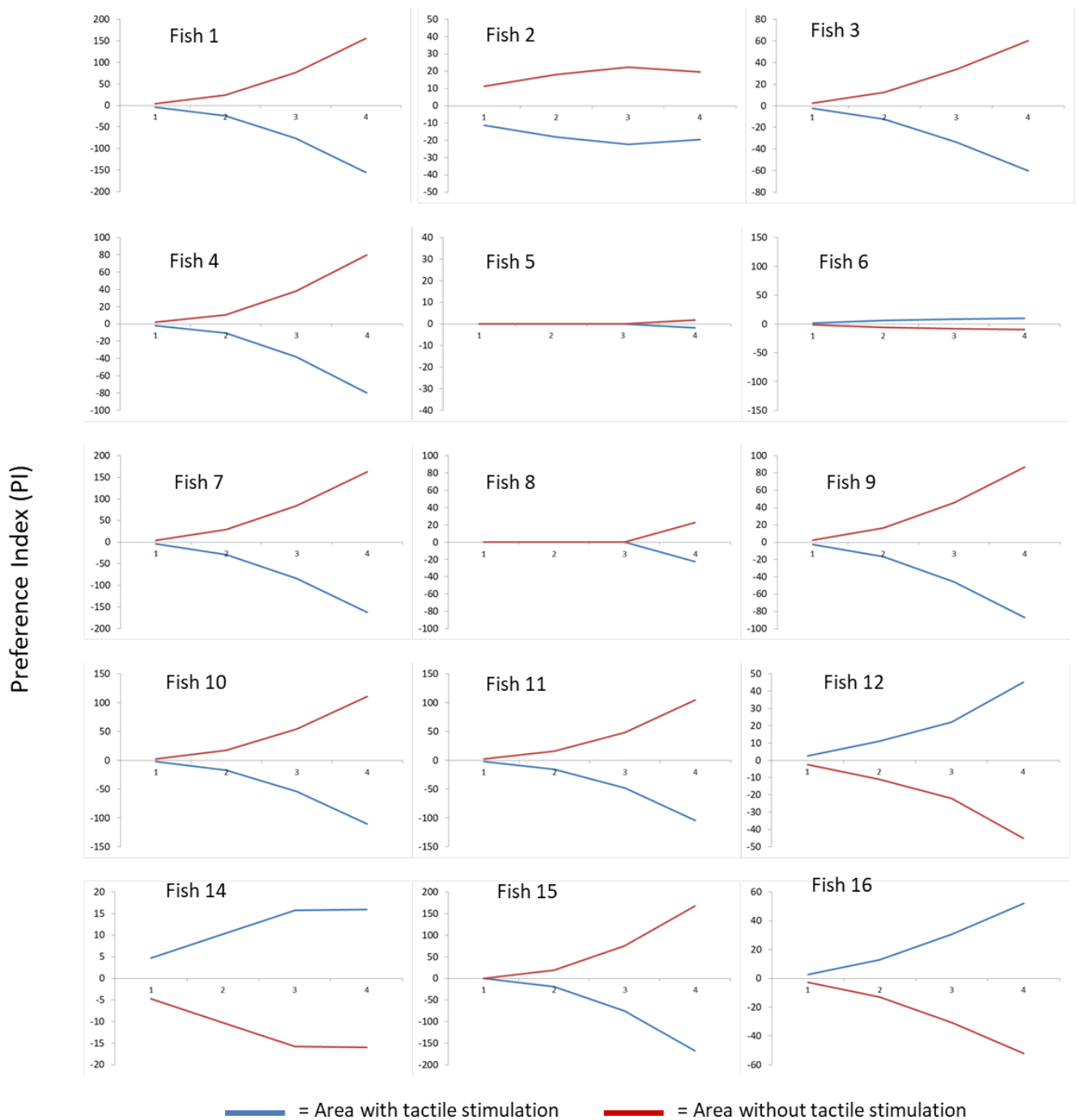
Figure 6. Number of crossings in preference test through the area with or without stimulation over the periods before and after manipulation (male pairing in a new aquarium in the treatment group or isolated in a new aquarium in the control group). ANOVA for repeated measures and planned comparisons. Asterisk indicates significant differences within treatments in the periods. Letters compares before and after manipulation for control and treatment groups. Similar letters indicate no significant differences. Data are mean \pm SE.

4.3. Preference Index

In the control group (16 replicas) only individuals 6, 12, 14 and 16 preferred to access the area with tactile stimulation before manipulation (Figure 7A), and individuals 2, 4, 12 and

16 preferred tactile stimulation after manipulation (Figure 7B). In the treatment group (16 replicas) only individuals 4, 6, 8, 12, and 14 preferred to access the area with tactile stimulation, meanwhile the rest of the fish preferred to access the area without tactile stimulation (Figure 8A). In the period after manipulation only fish 8 preferred to access the area with tactile stimulation, meanwhile the rest of the fish preferred to access the area without tactile stimulation (Figure 8B).

A)



B)

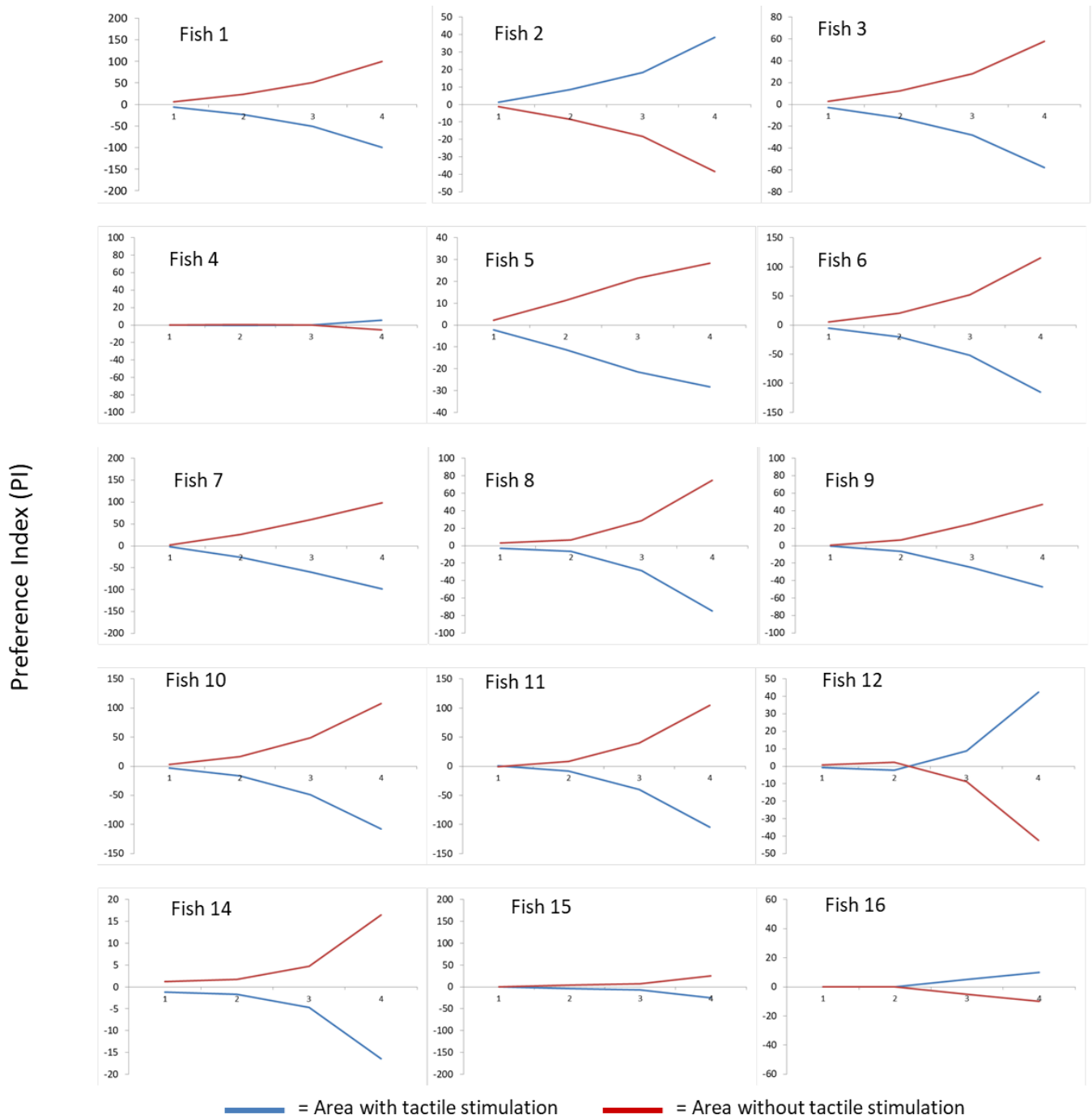
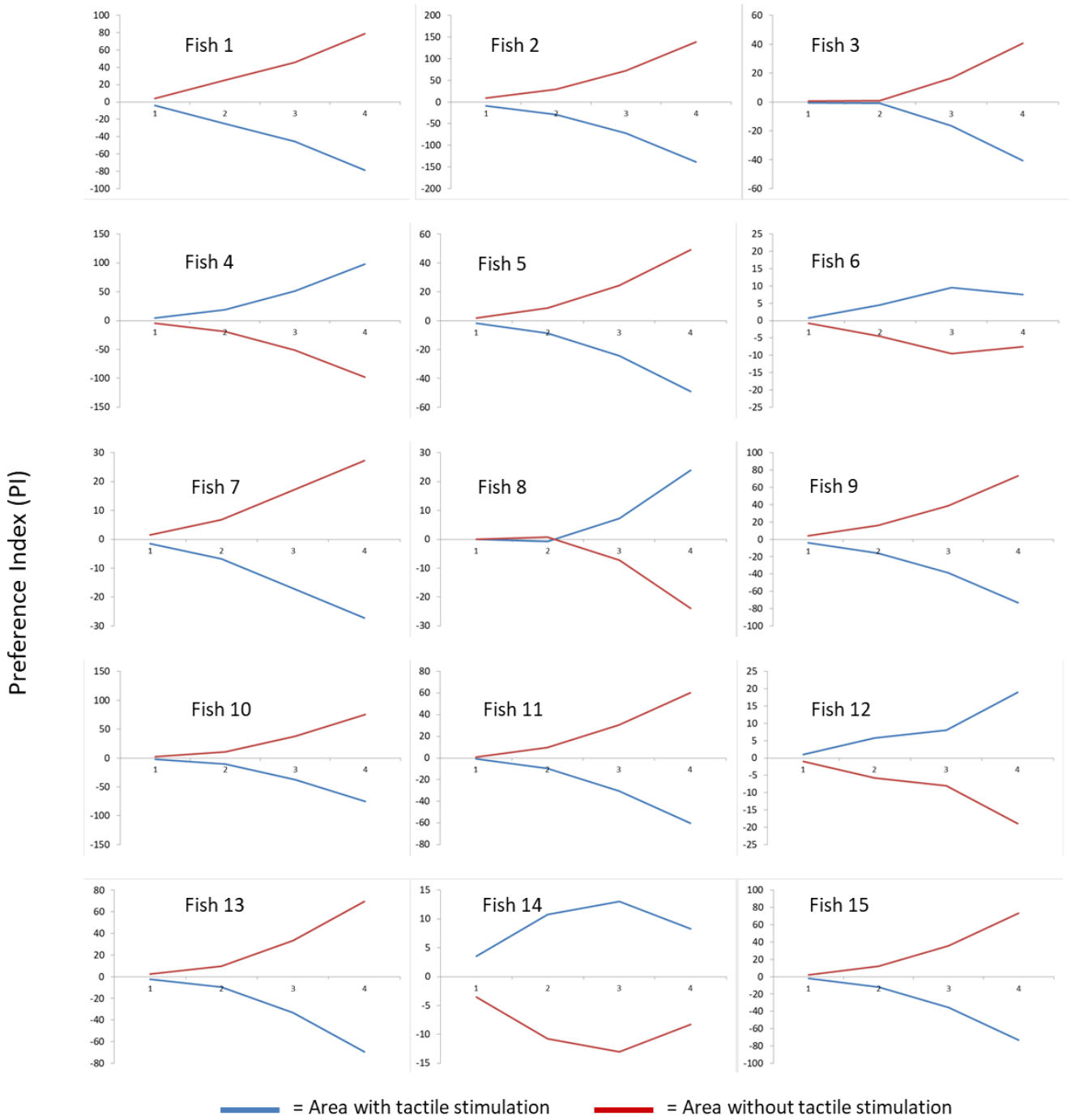


Figure 7. Preference Index of control group in the period before (A) and after (B) manipulation (isolation in a new aquarium). We did not count fish 13 in these calculations because it did not cross through the tactile stimulator in one of the periods.

A)



B)

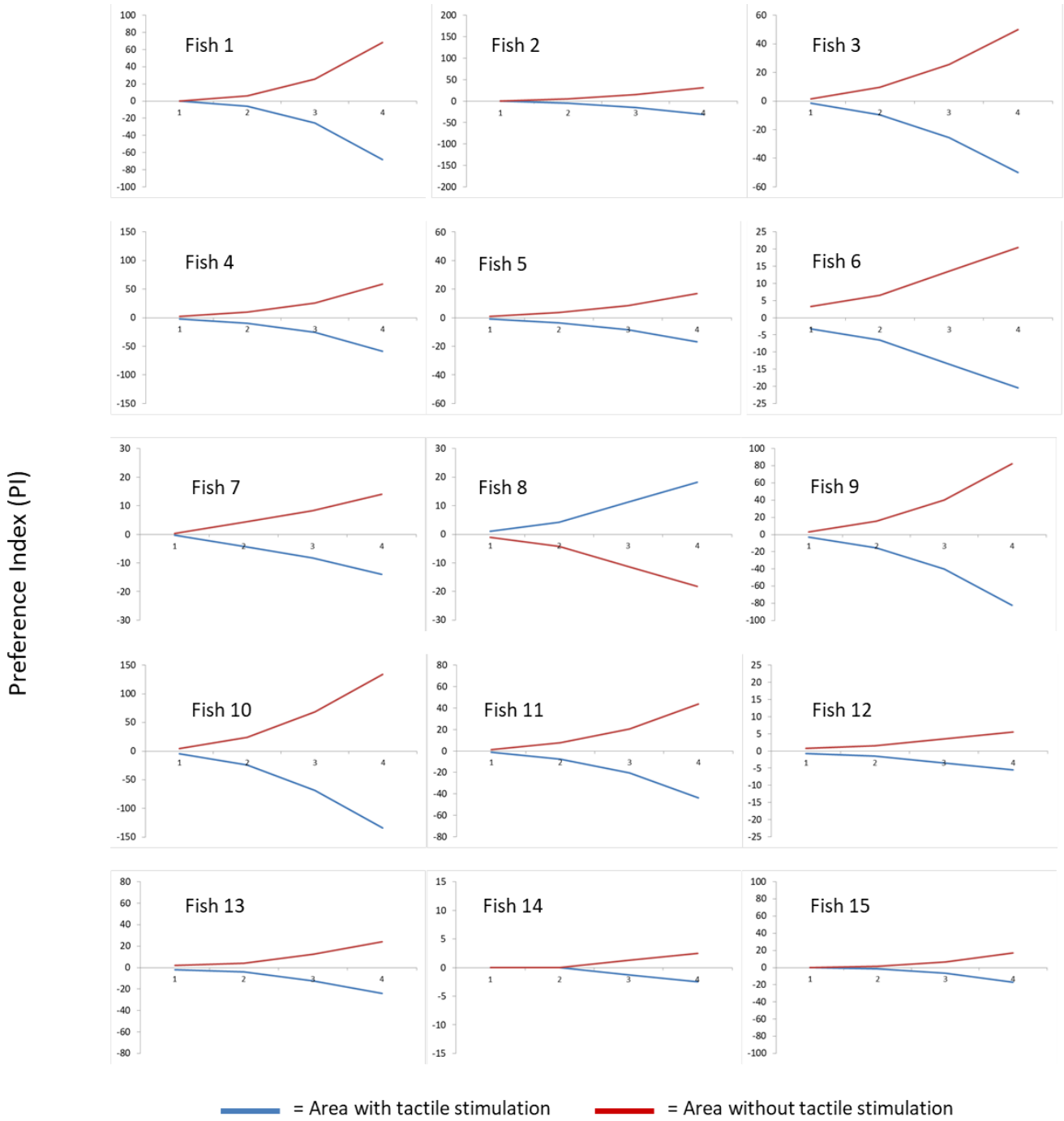


Figure 8. Preference Index of treatment group in the period before (A) and after (B) manipulation (pairing for fighting). We did not count fish 16 in these calculations because it did not cross through the tactile stimulator in one of the periods.

4.4. Motivation test

No difference was observed for the latency to reach the apparatus between the treatments (with or without fights) and over the periods before and after manipulation ($F_{(1,26)} = 0.34$, $p = 0.56$, Figure 9A). As the latency varied for each fish, we analyzed the number of crossing per minute available to the animal to access the tactile device (20 min – latency to fish leave the dark area). We did not find difference between the number of crossings of control and treatment groups ($F_{(1,30)} = 0.14$, $p = 0.703$, Figure 9B). Again, planned comparisons showed that fish crossed more times through the area without tactile stimulation ($F_{(3,90)} = 20.61$, $p = 0.0001$). In the control group, the number of crossings through the area without tactile stimulation was higher than the area with tactile stimulation before manipulation ($F_{(1,30)} = 15.85$, $p = 0.0004$). After manipulation there was no difference between the crossings in both areas ($F_{(1,30)} = 3.19$, $p = 0.083$). In the treatment group the number of crossings through the area without tactile stimulation was higher than the area with tactile stimulation before manipulation ($F_{(1,30)} = 32.15$, $p = 0.00004$) and after manipulation ($F_{(1,30)} = 14.70$, $p = 0.0006$).

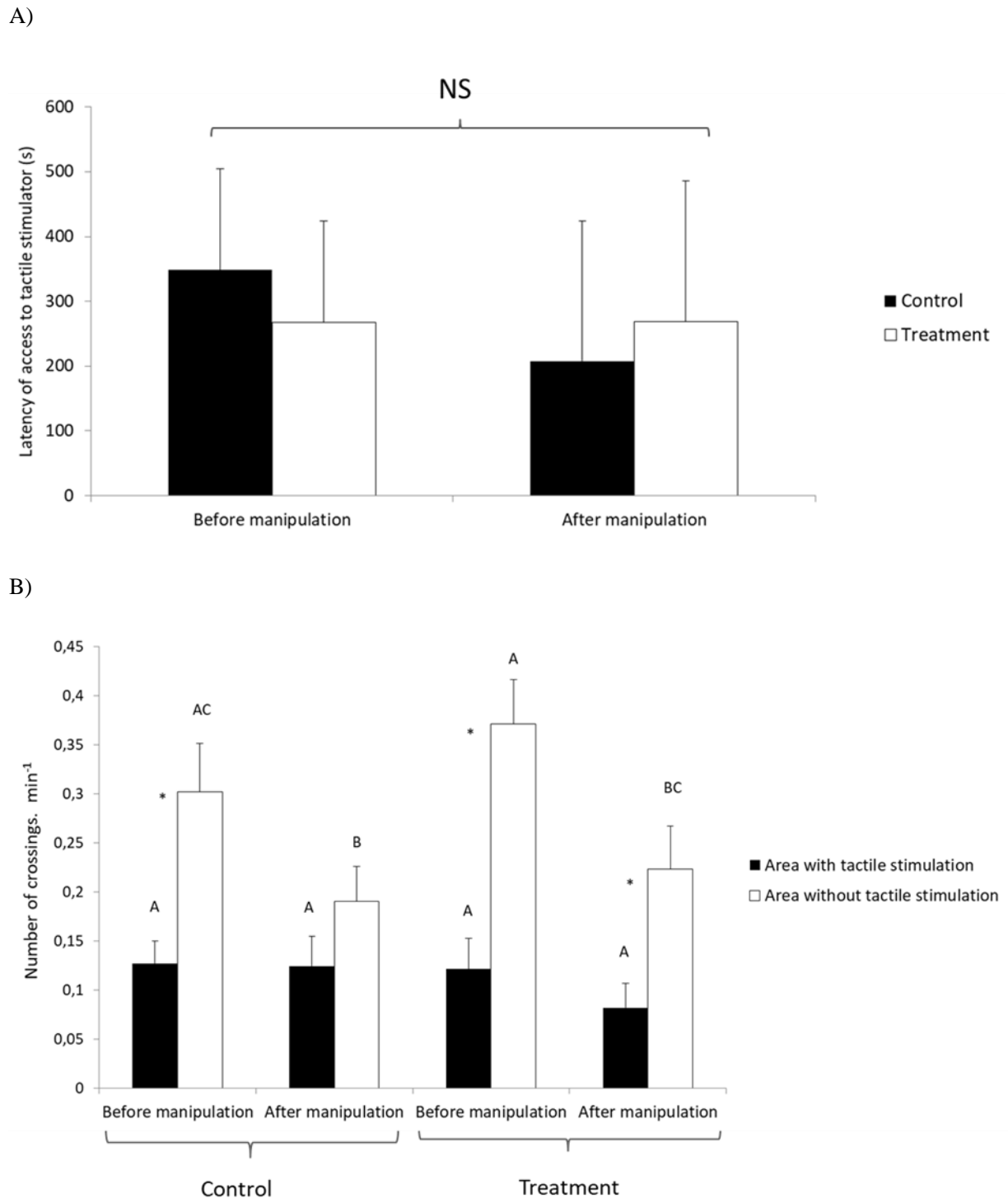


Figure 9. A) Latency to access the tactile stimulator in the periods before and after manipulation (male pairing in a new aquarium in the treatment group or isolated in a new aquarium in the control group) for control and treatment group in the motivation test. ANOVA for repeated measures and planned comparisons. B) Number of crossings through the two areas of tactile stimulator (with and without stimulation) during the motivation test over the periods before and after manipulation. ANOVA for repeated measures and planned comparisons. Asterisk indicates significant differences between the areas with and without tactile stimulation. Letters compares before and after manipulation

for control and treatment groups. Similar letters indicate no significant differences. Data are mean \pm SE.

4.5. Social rank

We analyzed the number of crossings through the tactile apparatus in the initial eight days according to the social rank (dominant and subordinate) that the fish acquired after agonistic interactions in the treatment group. There was a significant interaction between the social rank and the days of observation ($F_{(7,98)} = 4.49$, $p = 0.0002$, Figure 10A). Planned comparisons showed that the number of crossings through the stimulator of dominant fish was higher than subordinate one on day 2 ($F_{(1,14)} = 6.25$, $p = 0.05$). On day 8 we observed that the number of crossings of subordinate fish was higher than of dominant fish ($F_{(1,14)} = 8.22$, $p = 0.012$).

In the preference test there were differences in the number of crossings between the areas with and without tactile stimulation ($F_{(1,14)} = 8.50$, $p = 0.011$, Figure 10B) and between dominant and subordinate individuals ($F_{(3,42)} = 9.70$, $p = 0.0005$) but no significant interaction between preference and social rank ($F_{(3,42)} = 0.53$, $p = 0.66$). Planned comparisons showed that the number of crossings for dominant individuals was higher in the area without tactile stimulation before ($F_{(1,14)} = 14.64$, $p = 0.001$) and after aggressive interaction ($F_{(1,14)} = 5.52$, $p = 0.04$). There were no differences between the crossings in the different areas for subordinate individuals, either before ($F_{(1,14)} = 1.05$, $p = 0.321$) or after aggressive interaction ($F_{(1,14)} = 3.98$, $p = 0.065$).

In the area with tactile stimulation, planned comparisons showed that the number of crossings by subordinate individuals before manipulation was higher than the crossings by dominant individuals ($F_{(1,14)} = 4.97$, $p = 0.049$). We observed that for dominant individuals the number of crossings through the area without tactile stimulation after manipulation decreased in comparison with the period before manipulation ($F_{(1,14)} = 5.69$, $p = 0.032$). There was not difference between the crossings through the area with tactile stimulation in the periods before and after manipulation ($F_{(1,14)} = 1.84$, $p = 0.196$). Analyzing the subordinate fish, we observed that the crossings through the area with tactile stimulation were decreased after manipulation in comparison with before manipulation ($F_{(1,14)} = 7.54$, $p = 0.015$) such as the crossings through the area without tactile stimulation ($F_{(1,14)} = 8.51$, $p = 0.011$).

We did not observe difference between the latency of dominant and subordinate fish both, before and after manipulation ($F_{(1,14)} = 0.59$, $p = 0.456$, Figure 11A). Regarding the number of crossings, we observed difference between the crossings in the area with tactile

stimulation and in the area without tactile stimulation ($F_{(1,14)} = 9.29, p = 0.008$, Figure 11B). There was no difference between the number of crossings of subordinate and dominant individuals ($F_{(3,42)} = 1.79, p = 0.163$). The number of crossings in the area without tactile stimulation was higher than in the area with tactile stimulation for dominant individuals before the manipulation ($F_{(1,14)} = 12.60, p = 0.003$) and after the manipulation ($F_{(1,14)} = 9.11, p = 0.009$).

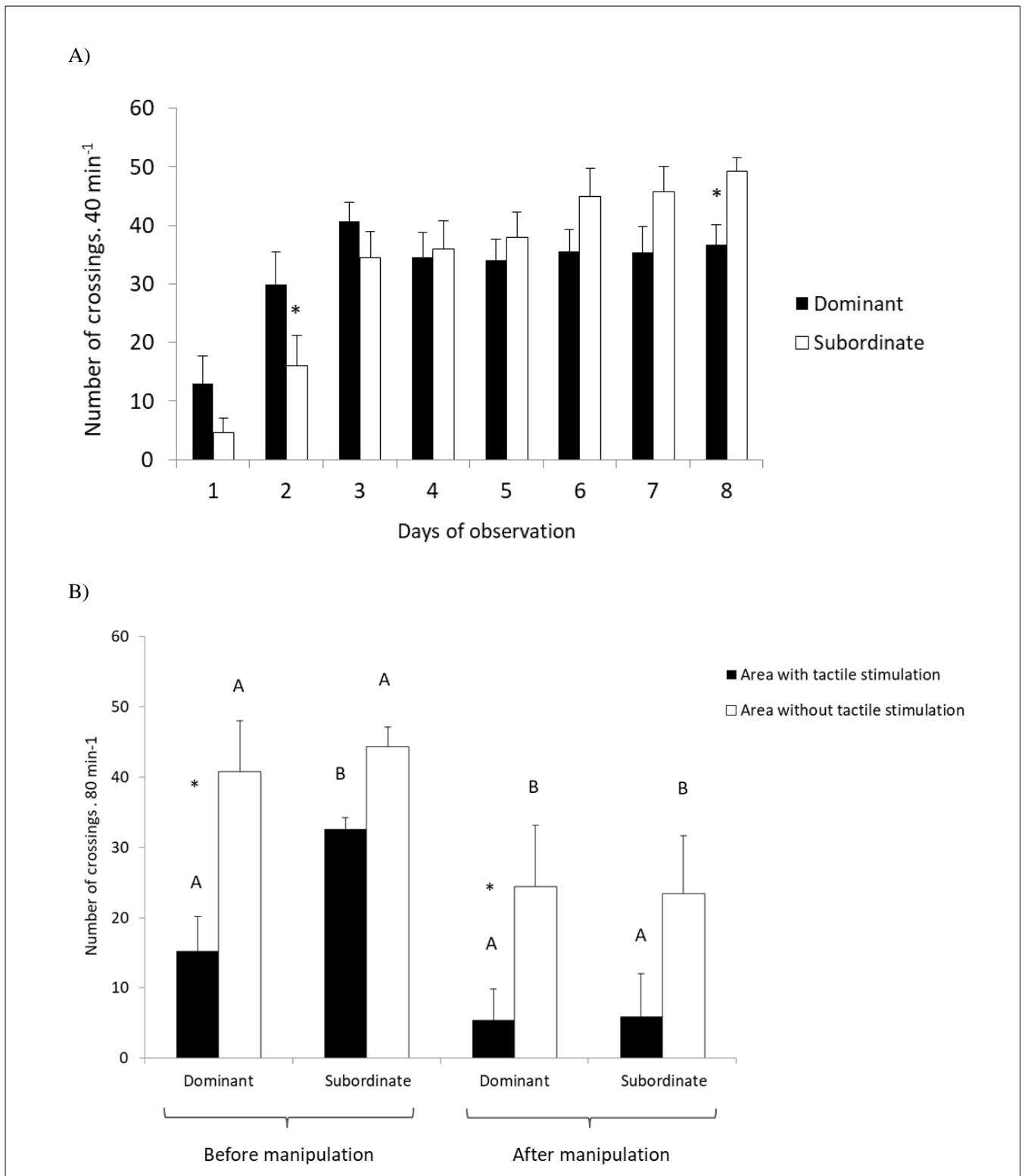


Figure 10. A) Number of crossings through the tactile stimulator during the first eight days of contact with the apparatus for dominant and subordinate individuals. ANOVA for repeated measures and planned comparisons. Asterisk indicates significant differences between the number of crossings of dominant and subordinate individuals. B) Number of crossings through the two areas of tactile stimulator (with and without stimulation) during the preference test over the periods before and after manipulation (male pairing in a new aquarium in the treatment group or isolated in a new aquarium in the control group) dominant and subordinate individuals. ANOVA for repeated measures and planned

comparisons. Asterisk indicates significant differences between the areas with and without tactile stimulation. Letters compares before and after manipulation for dominant and subordinates. Similar letters indicate no significant differences. Data are mean \pm SE.

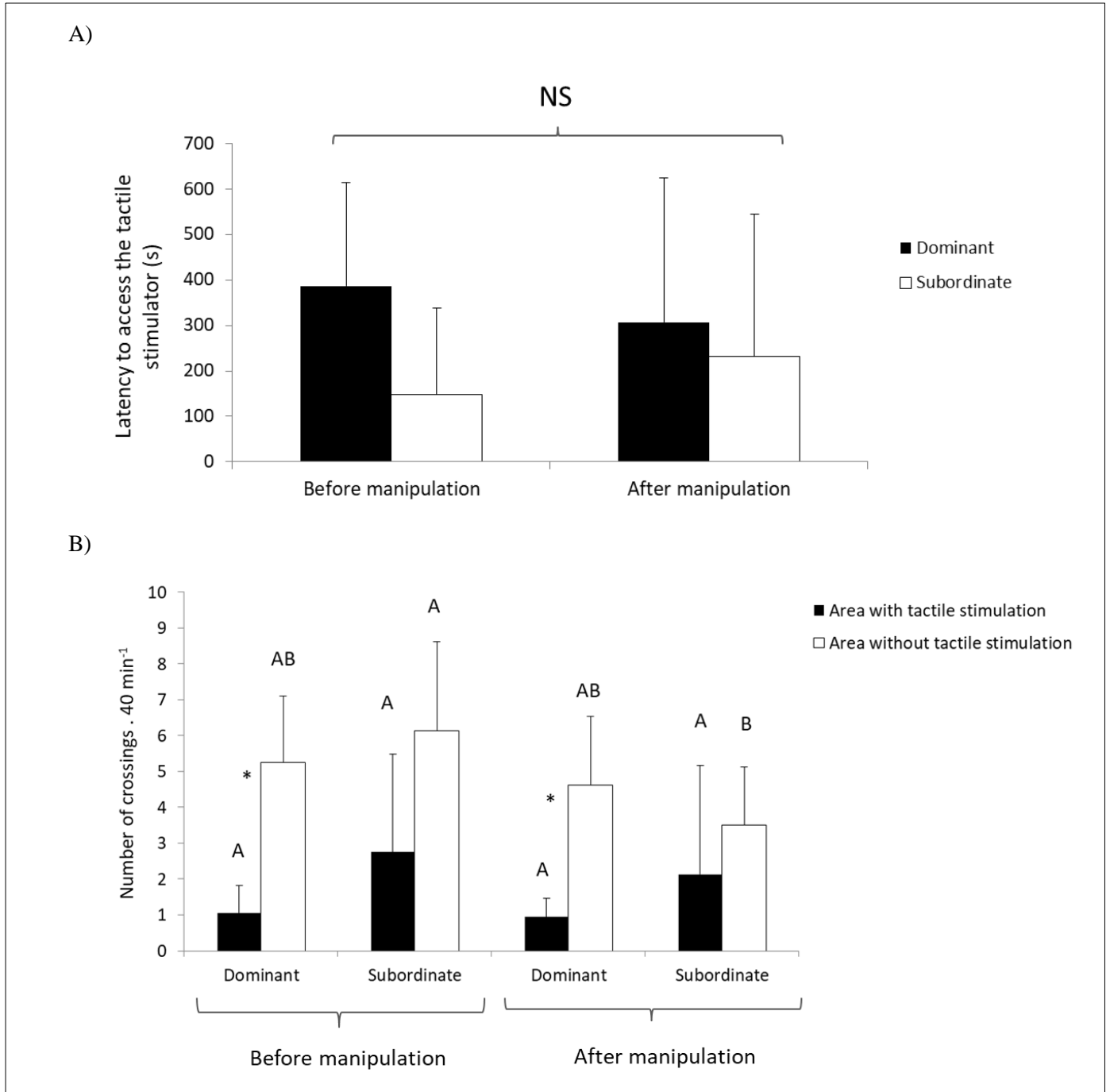


Figure 11. A) Latency of access to tactile stimulator in the periods before and after manipulation for subordinate and dominant individuals. ANOVA for repeated measures and planned comparisons. B) Number of crossings through the two areas of tactile stimulator (with and without stimulation) during the motivation test over the periods before and after manipulation (male pairing in a new aquarium in the treatment group or isolated in a new aquarium in the control group) for subordinate and dominant individuals. ANOVA for repeated measures followed by and planned comparisons. Asterisk indicates

significant differences between the areas with and without tactile stimulation. Letters indicates significant differences over the periods (before and after manipulation) for subordinate and dominant individuals. Data are mean \pm SE.

5. DISCUSSION

In this study we showed that territorial fish use tactile stimulation spontaneously and the preference for the access varies individually. The animals went through an aversive route to obtain the access to tactile stimulation, and did not avoid the apparatus, thus suggesting that tactile stimulation is a resource of some importance. On the other hand, social stress did not influence the preference and motivation, showing that fish do not use tactile stimulation to relieve the effects of social aggressive interaction. However, we detected an effect of social rank, with subordinate fish accessing tactile stimulation more frequently than dominant ones, suggesting that tactile stimulation has a more effective influence in these animals.

The pattern of crossings in the initial eight days of the experiment indicates that the tactile stimulator was efficient for providing body tactile stimulation, as previously showed by Bolognesi *et al.* (2019). Furthermore, fish gradually increased the crossings through the apparatus, irrespective of food presence followed by a stabilization, which means that fish need some time to adjust to the presence of the apparatus, as showed by Bolognesi *et al.* (2019) as well. On the other hand, we can interpret that fish increase the access to the apparatus after the initial days because it is a positive condition for it (DAWKINS, 2006; BOLOGNESI *et al.*, 2019). Thus, the first part of this study provided an adequate experience of individual tactile stimulation.

When the access to a resource that is not part of the natural range of animal's life increases, probably that resource is interpreted as something good (KIRKDEN; PAJOR, 2006; GALHARDO; OLIVEIRA, 2011), therefore we expect that the individual chooses that resource. However, despite the fish spontaneously crosses through the tactile device in the first phase, they do not prefer this condition in the preference test. Then, the higher number of crossings through the area without tactile stimulation can be explained by some factors. Natural physical contacts in Nile tilapia are mainly related to aggressive interactions (DAMSGARD; HUNTINGFORD, 2012) than contacts such as the client-cleaner interaction in coral-reef species (GRUTTER, 1995) or any other non aggressive contact, for example in shoal species (HUTH; WISSEL, 1992). In this way, the artificial contact provided by the tactile stimulator could be interpreted by fish as a negative interaction, what can explain the higher number of crossings through the area without tactile stimulation. Another explanation

would be the fact that the stimulator apparatus could act as a visual barrier to the fish. For cichlids like Nile tilapia, vision plays a fundamental role in the species ecology (CARLETON, 2009) and is an important sensorial path of movement and space localization (DOUGLAS; HAWRYSHYN, 1990), so that fish would prefer to move by an area with no restriction to the movements. Besides, physical barriers could represent obstacles to swimming capacity (AMARAL *et al.*, 2017) what can increase the energy expenditure e oxygen consume (BOTHA *et al.*, 2018) allowing the occurrence of negative consequences for the animal's energetic balance. Yet, simply because it is easy to move by ways with no barriers.

The question that remains here is why, then, fish do not avoid the tactile stimulation area if it is easier to detour the "barrier"? Besides, if the fish had the option of remaining in neutral areas of the aquarium, why did they cross the stimulation area? If the stimulation was negative, we would expect fish to avoid it. In fact, they did not. On the contrary, they were motivated to cross an aversive route to access the tactile stimulator. It would be possible that the route of high illumination created might not represent a true aversive stimulus for Nile tilapia. However, the quantity of lumens used in this study has already been applied to other fish species in motivation tests (MAIA *et al.*, 2017), and Nile tilapia is sensible to increased light intensity as well (CARVALHO *et al.*, 2013). In this way, the fact that the animals left a shaded area and then crossed to the aquarium's extremity, where the apparatus was positioned, indicates that fish take some risk to reach the tactile stimulation.

The preference indexes obtained for the preference test reinforce the general idea that most of the fish does not prefer to move through tactile device. However, the PI also shows the access to tactile stimulation varies individually, as shown for other resource types, as environmental color and shelter (BROWNE *et al.*, 2010; MAIA; VOLPATO, 2019). Therefore, we must consider such an individuality to evaluate preferences instead of concludes so fast that territorial fish, such as Nile tilapia, does not prefer tactile stimulation. Thus, considering that Nile tilapia does not avoid the apparatus, and looking to individual variations, we conclude that tactile stimulation is a condition that fish spontaneously reach for, thus meaning a positive resource for territorial fishes.

Bolognesi *et al.* (2019) have already shown that tactile stimulation does not lowers cortisol after both social and non-social stress in Nile tilapia males. However, cortisol per se could not be the best indicator of positive effect of tactile stimulation. In this way, preference tests bring an advantage for evidencing the animal feelings and perceptions, irrespective of association with physiological indicators (VOLPATO *et al.*, 2007). Thus, considering the hypothesis that tactile stimulation would have a positive effect on relieving consequences

from negative stimulus, we predicted that a social stressor would cause an increase on the access of the animals to tactile stimulation, as previously observed by Bolognesi *et al.* (2019). They found an increase of Nile tilapia fish movement by the environment after social aggressive interaction, making fish pass through the tactile apparatus in the middle of the aquarium. However, we observed that in both the preference test and in the motivation test this type of stressful condition did not cause changes in the pattern of crossings through the apparatus. By comparing the periods before and after the manipulation, we detected a reduction in the number of crossings; however, this reduction occurs in the two options of choice, that is, areas with and without stimulation. This suggests that the social stressor in this study had an effect of reducing the activity of the animals in a general way, as showed by other authors (CONTE, 2004; SCHRECK, 2016). Differences of our study from Bolognesi *et al.* (2019)'s study would be the longer time of fighting and smaller size of aquaria, thus putting fish in a more stressful condition. In fact, higher stress level in fish can cause a decrease in movement (SCHRECK, 1990) swimming performance (KUTTY; SUKURMAN, 1975; LANKFORD *et al.*, 2005) and in the animal's motivation to perform some spatial task (GAIKWAD *et al.*, 2011; WOOD *et al.*, 2011).

According to the latency to reach the apparatus, animals that experienced or not social stressor seems to have the same motivation to access the tactile stimulator. The pattern of crosses in the motivation test is in line with the results from preference test, reinforcing the idea that motivation complements the analysis of what the animal actually prefers (DUNCAN, 2002; MAIA *et al.*, 2017; FRANKS, 2019). Again, by evaluating the PI, we observed a preference inversion after manipulation (aquarium exchange) in the control group, with fish 2 and fish 4 that previously preferred to access the area without tactile stimulation, preferring the area with stimulation and fish 6 and fish 14 that previously preferred to access the area with tactile stimulation, preferring the area without stimulation. These results, besides reinforcing the individual variability of preference responses, suggest that the simple manipulation of the animals is enough to change the pattern of crossings. Regarding the treatment group, we observed that after aggressive interaction (pairing) fish 2, fish 4, fish 6 and fish 14, which previously preferred to access the area with tactile stimulation, preferred the area without stimulation. This result reinforces the idea that the social stress seems to have acted in the decrease of the animal's activity, including the crossings through the area with tactile stimulation.

The association between the social rank and the number of crossings in the 8 days previous to preference and motivation tests demonstrates the tendency of the animals that

accessed more frequently the stimulation to become subordinate. In fact, tactile stimulation reduces the aggressiveness of Nile tilapia (BOLOGNESI *et al.*, 2019), therefore individuals who experienced more tactile stimulation should become less aggressive and lose the contest, as aggressive ability define the winner and the loser individual (BOSCOLO *et al.*, 2011). Another interesting fact regarding subordinate fish is that in the preference test, they equally accessed the areas with and without stimulation, unlike the dominant ones. This result is in agreement with the pattern of crossings in the initial 8 days of the fish that would become subordinate. However, after social stressor, subordinate and dominant showed the same number of crossings by the stimulator. It is known that the establishment social hierarchy in Nile tilapia could be stressful in a similar way either for subordinate or dominant animals (CORREA *et al.*, 2003). Therefore, after pairing, the stress levels of dominant and subordinate individuals could be equal, allowing a similar pattern of access to tactile stimulation for both. Despite this, some studies show that the effects of agonistic interactions can be higher in subordinate individuals in a longer period (EJICK; SCHRECK, 1980) what could explain the decrease in crossings through the area with tactile stimulation and through the area without tactile stimulation after the pairing for aggressive interactions in subordinate fish.

In this study, we could observe that the pattern of preference and motivation responses by tactile stimulation is very variable for Nile tilapia. We had the purpose of testing whether or not the fish prefers tactile stimulation and, therefore, it was limited to two options of choice. In conditions in which few choices are offered to animals, we cannot conclude assertively that the one chosen more frequently will always be preferred, especially if it is a variable environment (MAIA; VOLPATO, 2019) and where the options of choice can have gradual effects on the animal, as it is the case of tactile stimulation. In some studies, such as this one, preference and motivation responses are analyzed together (SULLIVAN *et al.*, 2016). The conclusions of these analyzes can sometimes extrapolate the individual views of each test, showing that even if an option of choice is not accessed more often, animals may rather be motivated to access this option and consequently, this condition may represent a positive aspect from the perspective of the animal itself.

6. CONCLUSION

We conclude that tactile stimulation is not aversive to Nile tilapia, since animals do not avoid contact with the stimulator; social stress has no effect on preference and motivation, since fish do not increase the search for tactile stimulation after this situation; tactile

stimulation can be determinant in the modulation of dominance hierarchy by reducing aggressiveness and making individuals become subordinate; animals are motivated to access tactile stimulation as they face an aversive stimulus to gain such access, suggesting that stimulation may represent a positive condition for these animals.

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