UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CENTRO DE AQUICULTURA DA UNESP

TACTILE STIMULATION AND WELFARE IN NILE TILAPIA: EFFECTS ON STRESS, AGGRESSIVENESS AND COGNITIVE ABILITY

Marcela Cesar Bolognesi

Jaboticabal, SP
2022

UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

TACTILE STIMULATION AND WELFARE IN NILE TILAPIA: EFFECTS ON STRESS, AGGRESSIVENESS AND COGNITIVE ABILITY

Marcela Cesar Bolognesi

Orientadora: Profa. Dra. Eliane Gonçalves de Freitas

Tese apresentada ao Programa de Pós - Graduação em Aquicultura do Centro de Aquicultura da UNESP - CAUNESP, como parte dos requisitos para obtenção do título de doutora.

Jaboticabal, SP

2022

Bolognesi, Marcela Cesar
B693t Tactile stimulation and welfare in Nile tilapia : effects on stress, aggressiveness and cognitive ability / Marcela Cesar Bolognesi. – – Jaboticabal, 2022
ix, 73 p. : il. ; 29 cm

Tese (doutorado) - Universidade Estadual Paulista, Centro de Aquicultura, 2022
Orientadora: Eliane Gonçalves de Freitas
Banca examinadora: Aline Cristina Sant'anna, Angélica da Silva Vasconcellos, Elisabeth Criscuolo Urbinati, Thais Billalba Carvalho
Bibliografia

1. Aggressive behavior in animals. 2. Welfare. 3. Tilápia-do-Nilo. 4. Stress. 5. Cognition. I. Título. II. Jaboticabal-Centro de Aquicultura.

CDU 639.3.05

Ficha Catalográfica elaborada pela STATI - Biblioteca da UNESP
Campus de Jaboticabal/SP - Karina Gimenes Fernandes - CRB 8/7418

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Centro de Aqüicultura da Unesp - CAUNESP

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Tactile stimulation and welfare in Nile tilapia: effects on stress, aggressiveness, and cognitive ability

AUTORA: MARCELA CESAR BOLOGNESI
ORIENTADORA: ELIANE GONÇALVES DE FREITAS

Aprovada como parte das exigências para obtenção do Título de Doutora em AQUICULTURA, pela Comissão Examinadora:

Profa. Dra. ELIANE GONÇALVES DE FREITAS (Participação Virtual)
Departamento de Ciências Biológicas / UNESP Campus de São José do Rio Preto, SP

Profa. Dra. ALINE CRISTINA SANT'ANNA (Participação Virtual)
Departamento de Zoologia / ICB/UFJF, Juiz de Fora-MG

Profa. Dra. ANGÉLICA DA SILVA VASCONCELLOS (Participação Virtual)
PPG Biologia de Vertebrados / PUC Minas, Belo Horizonte-MG

Profa. Dra. ELISABETH CRISCUOLO URBINATI (Participação Virtual)
Departamento de Morfologia e Fisiologia, Faculdade de Ciências Agrárias e Veterinárias - UNESP/Jaboticabal, SP

Profa. Dra. THAIS BILLALBA CARVALHO (Participação Virtual)
Departamento de Ciências Fisiológicas / Universidade Federal do Amazonas, Manaus-AM

Jaboticabal, 17 de fevereiro de 2022

Centro de Aqüicultura da Unesp
Coordenação do Programa de Pós-Graduação
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 Jaboticabal SP Brasil
Tel 55 16 32032110 ramal 214 fax 55 16 32032260
pgaqui@caunesp.unesp.br www.caunesp.unesp.br

SUMMARY

DEDICATION

I dedicate this work to the Brazilian scientists and researchers who have been facing a dark, unscientific and denialist government since 2019. To researchers who have been suffering financial cuts from government, whose work, research and post graduate students training have been hampered. This is a huge loss to our country, which has been mourning the covid-19 pandemic and lack of governmental responsibility.

In this public document, I leave my intention for a country more considerate to science and education. I also emphasize the great importance of feminist women in science, who fight for space, respect and recognition.

ACKNOWLEDGEMENTS

I’m grateful to my advisor, Profa. Dra. Eliane Gonçalves-de-Freitas for these 13 years of many teachings, partnership and friendship. This thanks cannot be put into words. I leave a special thanks for the support this past year, without your help I couldn't make it this far. Thank you for the words of kind and hope, again. You are an example that I was lucky to have in my academic and personal life.

I thank the CAUNESP coordination, which understood the difficulties faced by students during the covid-19 pandemic and allowed a time extension for the thesis to be fully completed.

I am grateful to the Animal Behavior Society for the ABS Latin American Mentoring Program. The goal of the mentorship program is to enhance opportunities for professional development and career advancement of Latin American students of animal behavior who are currently studying in Latin American institutions. We have been assigned with professor Jeff Podos from University of Massachusetts, whom I thank for the activities during the past year, the mentorship, advices, academic tips, and review of this thesis. We had several very pleasant meetings, where I could virtually meet his laboratory and the lab students, could know about their research and also share and discuss this thesis.

I thank Carlos Eduardo Sousa and Roselene Costa Ferreira for their technical support at the Animal Behavior Laboratory at UNESP - Ibilce.

I thank all my colleagues at the Animal Behavior Laboratory at UNESP – Ibilce, who have been with me all these years for their friendship, support and help with all the experiments. Each research is the result of a lot of collaborative work, without you this thesis would not exist, so thank you very much.

FINANCIAL SUPPORT

This thesis had the following financial support:

Marcela Cesar Bolognesi: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. Phd scholarship.

Eliane Gonçalves de Freitas: Research Assistance (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq - Universal Notice - process 428296/2016-5).

ABSTRACT

Tactile stimulation (TS) is a mechanical stimulus applied to an animal's body, such as massages and manual touches. It can decrease stress and stimulate positive effects that increase welfare in humans and other animals. The application of body TS reduces stress in mammals and fish, being an appropriate form of enrichment for these animals. We know that fish farming conditions can promote stress, particularly due to high stocking density, netting and social stress. If stress lasts for a long time, it becomes chronic (distress), as it results in the constant release of glucocorticoids, which will cause damage to growth and reproduction, in addition to a decrease in neurogenesis, with consequent effects on cognitive ability. In this context, TS is efficient in reducing stress in a marine fish. Furthermore, in a previous study, we observed that TS reduces aggression in male Nile tilapia, but without reducing cortisol, indicating that more studies are needed to better understand or affirm whether there is a positive effect of TS on stress in territorial fish. Recently, we have demonstrated that TS for Nile tilapia is non-aversive and that fish search for TS even though they need to cross an aversive route to reach it. Thus, some hypotheses were raised: 1. ET reduces the stress caused by confinement in Nile tilapia more quickly than fish without ET; 2. ET reduces social stress in Nile tilapia groups compared to groups without ET; 3. ET improves cognitive ability of Nile tilapia compared to fish without ET. We designed three studies to test these hypotheses in Nile tilapia, *Oreochromis niloticus*, a territorial fish widely used in fish farming worldwide. We used a TS device that contains silicone bristles to promote body TS for fish. In the first study, we tested whether TS reduces more rapidly the stress caused by confinement in Nile tilapia. In the second, we tested the effect of TS on aggression, stress and growth in Nile tilapia groups. And in the third, we tested whether TS improves the cognitive ability of Nile tilapia. We conclude that TS reduces aggressiveness and promotes higher growth in Nile tilapia groups. It also improves learning in isolated fish. However, TS did not decrease stress in either isolated or grouped Nile tilapia. These positive effects on aggression, growth and learning allow us to infer that TS promotes positive states and increases welfare in this species. Thus, TS can be a strategy to improve the welfare of fish in aquaculture conditions.

Key-words: learning, confinement, cortisol, social stress, aquaculture, growth.

RESUMO

A estimulação táctil (ET) é um estímulo mecânico aplicado no corpo de um animal, como, por exemplo, massagens e toques manuais. Ela pode diminuir o estresse e estimular efeitos positivos que aumentam o bem-estar em humanos e outros animais. A aplicação de ET corporal reduz o estresse em mamíferos e peixes, sendo uma forma adequada de enriquecimento para esses animais. Sabemos que a condição de criação de peixes pode promover estresse, particularmente devido à alta densidade de lotação, captura com rede e estresse social. Se o estresse permanece por um tempo prolongado ele se torna crônico (distresse), pois tem como consequência a liberação constante de glicocorticoides, que vai gerar prejuízos no crescimento, na reprodução, além da diminuição da neurogênese, com consequentes efeitos sobre a habilidade cognitiva. Nesse contexto, a ET é eficiente em reduzir o estresse em um peixe marinho. Além disso, em um estudo anterior, observamos que a ET reduz a agressividade em machos de tilápia-do-nilo, porém sem reduzir o cortisol, indicando que mais estudos são necessários para melhor compreender ou afirmar se há um efeito positivo da ET sobre o estresse em peixes territoriais. Recentemente também demonstramos que a ET para tilápia-do-nilo não é aversiva e que os peixes buscam por ela mesmo precisando atravessar uma rota aversiva. Dessa forma, algumas hipóteses foram levantadas: 1. ET diminui mais rapidamente o estresse causado pelo confinamento em tilápia-do-nilo do que peixes sem ET; 2. ET reduz o estresse social em grupos de tilápia-do-nilo comparado com grupos sem ET; 3. ET melhora a capacidade cognitiva de tilápia-do-nilo comparado a peixes sem ET. Assim, delineamos três estudos para testar essas hipóteses na tilápia-do-nilo, *Oreochromis niloticus*, um peixe territorial amplamente utilizado na piscicultura mundial. Utilizamos um estimulador táctil que contém cerdas de silicone, que promovem a ET corporal nos peixes. No primeiro estudo, testamos se a ET diminui mais rapidamente o estresse provocado pelo confinamento em tilápia-do-nilo; no segundo, testamos o efeito da ET sobre a agressividade, o estresse e o crescimento em grupos de tilápia-do-nilo; e no terceiro testamos se a ET melhora a habilidade cognitiva de tilápia-do-nilo. Concluímos que a ET reduz a agressividade e promove maior crescimento em grupos de tilápia-do-nilo. Além disso, melhora a aprendizagem em tilápias-do-nilo isoladas. Entretanto, a TS não diminui o estresse tanto em tilápias-do-nilo isoladas ou agrupadas. Esses efeitos positivos na agressividade, crescimento e aprendizagem nos permitem inferir que a ET corporal promove estados positivos e aumenta o bem-estar nessa espécie. Assim, a estimulação táctil pode ser uma estratégia para melhorar o bem-estar de peixes em condição de aquicultura.

Palavras-chave: aprendizagem, confinamento, cortisol, estresse social, aquicultura, crescimento.

General Introduction

Animal welfare is a scientific term that is under constant construction. For example, Broom (1986) defined welfare as an individual's state in relation to their attempts to deal with their environment. Dawkins (1990) highlights concepts of animal suffering and looking at what the animal wants and how it feels. Volpato, Gonçalves-de-Freitas and Fernandes-de-Castilho (2007) define welfare as an internal state of an animal when it is in conditions that it freely chose. According to Webster (2013), welfare is determined by the individual's perception of the animal's emotional and physical state. Thus, the concept of animal welfare is related to the animal's physical and mental health, and involves different and complementary definitions. The common thread is that welfare is a scientific concept that can be measured and varies on a scale from very good to very poor.

Global aquaculture production has been increasing for many years (FAO, 2017), being recognized as a major food producer (Conte, 2004). Thus, the questioning of the impact of this practice on the welfare of fish also grew (Ashley, 2007). The idea that fish are sentient animals (Volpato, Gonçalves-de-Freitas, Fernandes-de-Castilho, 2007), prone to feeling pain, fear and stress, has deepened the interest in improving the welfare of these animals under captivity (Chandroo, Duncan, Moccia, 2004). For this, physiological indicators are widely used to infer the level of welfare, such as plasma cortisol levels, since the higher the levels of this hormone, the greater the state of stress can be and, therefore, the poorer the welfare (Huntingford et al., 2006). However, the assessment of welfare must go beyond physiology and adequately extend to the cognitive abilities of fish (Chandroo, Duncan, Moccia, 2004) and to behavioral indicators, such as changes in cognitive performance (Braithwaite, 2006; Brandão, Braithwaite, Gonçalves-de-Freitas, 2015), the animal's ability to make choices in the environment (Freitas, Volpato, 2013; Gauy et al., 2021) and changes in aggressive behavior (Boscolo, Morais, Gonçalves- de-Freitas, 2011). According to Fraser et al (1997) and Huntingford et al (2014), the welfare in fish, as in other animals, is considered under three main approaches: 1. Function-based approach considers that animals are doing well if their biological functions are adequately expressed, that is, fish are in good physical health and are able to adjust to the artificial environment. 2. Feeling-based approach assumes that fish are sentient beings and therefore are doing well when they are free from suffering or other negative experiences such as pain, fear, and hunger. 3. Nature-based approach considers fish to be doing well if they can express their natural behavioral repertoire. Thus, several indicators are used to infer fish welfare,

although most can be classified into two main sets: physiological and behavioral indicators (Martins et al., 2012; Brandão et al., 2021).

Stress is a fundamental and adaptive aspect of animal biology that initiates physiological and behavioral responses that have been selected to ameliorate the effects of poor quality environments (Sneddon, Wolfenden, Thomson, 2016). Initial responses to stress, such as fight-or-flight response, are followed by longer-term HPI (hypothalamus, pituitary, interrenal) activity, which returns the organism to homeostasis (Pankhurst, 2011). The magnitude of stress can be measured through change in HPI axis activity as measured through, for example, plasma cortisol (Sneddon, Wolfenden, Thomson, 2016). In artificial environments, fish are subject to several stressors that can harm their welfare due to constant manipulation resulting from management, such as confinement (Pottinger, Prunet, Pickering, 1992), capture with nets (Ellis et al., 2004) , overpopulation (Schram et al., 2006), exacerbated aggressive interactions (Boscolo, Morais, Gonçalves-de-Freitas, 2011) among others. In many cases, aggressive interactions can increase excessively, being harmful to social species that compete for territory, such as cichlids (Barlow, 2002). This increase can lead to social stress (Boscolo, Morais, Gonçalves-de-Freitas, 2011). Thus, it is important to study ways to reduce the impacts of these stressors on fish welfare. Furthermore, the constant release of glucocorticoids (e.g. cortisol) can generate negative effects for the animal, such as unbalanced changes in metabolism (Dibattista et al., 2006), high mortality rate (Johnsson, Winberg, Sloman, 2006), reduced growth rate, depressed immune response (Pottinger, 2008), and decreased neurogenesis (Sørensen et al., 2012). Therefore, it is important to investigate ways to reduce or prevent this stress and consequently increase the animals' welfare.

Tactile stimulation (TS) can be understood as a mechanical stimulus produced on the body or part of the body of an animal, which perceives this stimulus by the sensory nervous system related to touch. It can generate beneficial (positive) effects, for example, massage in humans, performed by pressing the hands over the body, promotes relaxation (by elevation of serotonin level) and decrease in stress (showed by lower cortisol level) (Field et al., 2005). Massage also decreases stress-related behavior in human newborns (Hernandez-Reif, Diego, Field, 2007). Some positive effects have also been seen in other mammals such as stress (cortisol) reduction in cattle (Probst et al., 2012), heart rate reduction in piglets (Tallet et al., 2014), lambs (Coulon et al., 2015) and dairy cows (Schmied et al., 2010), with TS made through manual touches promoted by humans. TS

also improves memory in adult rats after manual stimulation made by humans (Antoniazzi et al., 2017).

Artificial stimulation devices can also be used to promote TS and, consequently, their positive effects. It is known that cows raised in intensive systems have a high motivation to access automatic mechanical brushes that promote TS throughout their body (McConnachie et al., 2018) and that TS is an important feature, as cows have a natural grooming behavior (McConnachie et al., 2018). In addition, Soares et al (2011) demonstrated the decrease in cortisol levels in a fish that received artificial TS. The fish is the surgeon fish *Ctenochaetus striatus*, a species of client fish that maintains a cooperative relationship with another species, *Labroides dimidiatus*, a cleaner fish. In this marine fish interaction, the cleaner fish feed on the ectoparasites found in the body of the client fish, maintaining a cooperative relationship between species (Grutter, 1995). However, in addition to feeding on the parasites, cleaner fish also cheat by eating mucus from the client fish's body (Bshary, Grutter 2002). Bshary and Wurth (2001) observed that cleaner fish also promoted TS in the client fish body by using their pectoral fins, and that this stimulation offered benefits to the cleaner fish, such as being able to eat more mucus suffering fewer attacks or predation by the client fish. Then, Soares et al (2011) investigated what would be the benefit for the client fish in receiving TS and, for that, they used artificial models of cleaner fish that promoted TS by small brushes. They observed that client fish that received TS had lower levels of cortisol after being exposed to a confinement stressor (Soares et al., 2011).

In 2019, Bolognesi, Gauy and Gonçalves-de-Freitas tested the effect of TS on the aggressive behavior and stress in Nile tilapia, *Oreochromis niloticus*. Nile tilapia is of great importance to world aquaculture and is a territorial species that does not naturally present tactile interaction like the client and cleaner fishes. For Nile tilapia, the tactile interaction occurs by fights, where there is physical contact between the fish and also occurs in the reproductive context (Carvalho, Gonçalves-de-Freitas, 2008). Bolognesi, Gauy and Gonçalves-de-Freitas (2019) developed a TS device formed by a PVC structure with plastic sticks that contain silicone bristles, responsible for promoting TS in fish. The authors observed that TS reduces aggressive interactions in pairs of fish, but does not reduce stress, measured by cortisol, immediately after exposure to social or confinement stressors. In another study, we found that male Nile tilapia do not avoid this type of artificial TS, the fish could choose to pass through a device half with, half without the TS.

Although they preferred the area without TS they also passed spontaneously through the TS area (Gauy et al., 2021). Besides, they overcame an aversive high-intensity lighted route to reach the TS device, showing motivation to access it (Gauy et al., 2021).

Starting from the possible promotion of positive states from TS, we sought to study the effect of TS in other conditions, such as: stress recovery, aggressive behavior of social groups, and cognitive ability of fish. The thesis is that TS has a positive effect on welfare, reducing levels of cortisol, reducing aggressiveness and improving cognitive ability in fish. The hypotheses and predictions are as follows:

Study 1) TS decreases more rapidly the stress caused by confinement in territorial fish. We expect fish with TS to reduce more quickly the cortisol levels after exposure to a stressor than fish without TS.

Study 2) TS reduces social stress in groups of fish. We expect groups of fish with TS to show lower levels of aggression and social stress than fish without TS.

Study 3) TS improves the cognitive ability of fish. We expect fish with TS to perform better in a cognitive test than fish without TS.

Here are the three studies designed to test the above hypotheses. Each chapter, therefore, corresponds to an independent study, that will be submitted to the following journals:

Chapter I - *Tactile stimulation has no effect on stress recovery in Nile tilapia (Oreochromis niloticus)*, will be submitted as a brief communication to Journal of Fish Biology.

Chapter II - T*actile stimulation reduces aggressive interaction and increased specific growth rate in Nile tilapia groups*, will be submitted to Animal Behavior.

Chapter III - *Tactile stimulation improves learning ability in cichlid fish*, will be submitted to Animal Cognition.

CHAPTER I

Tactile stimulation has no effect on stress recovery in Nile tilapia (*Oreochromis niloticus*)

Marcela Cesar Bolognesi[1,2], Ana Carolina dos Santos Gauy[1,2], Eliane Gonçalves-de-Freitas[1,2]

[1] Centro de Aquicultura da Unesp - UNESP. Universidade Estadual Paulista. Via de Acesso Prof. Paulo Donato Castellane s/n, 14884-900, Jaboticabal/SP, Brasil.

[2] Instituto de Biociências, Letras e Ciências Exatas – UNESP. Universidade Estadual Paulista. Rua Cristóvão Colombo 2265, 15055-000, São José do Rio Preto/SP, Brasil.

Abstract

Body tactile stimulation (TS) was tested as a stimulus to alleviate stress in Nile tilapia, *Oreochromis niloticus*, as has been found for humans and other mammals. Isolated males were kept in tanks with or without TS for eight days and then assigned to a confinement
16 stressor. Plasma cortisol was measured in independent groups at 0, 30, 60, 90 or 120 min after stressor. We found no significant differences in cortisol between treatments with and without TS. Therefore, TS does not affect recovering from confinement stress in this territorial species.

Key-words: cortisol, confinement, animal welfare, aquaculture.

Introduction

Tactile stimulation (TS) is a mechanical stimulus made on an animal's body, which can generate a positive effect for the animal. For example, massage in humans lowers cortisol and elevates serotonin (Field et al., 2005), reducing stress and anxiety behaviors. TS made by hand touch also reduce cortisol and, consequently, stress in beef cattle (Probst et al., 2012). Besides mammals, TS artificially provided by a bristled mobile model reduces stress after confinement in a marine teleost fish, *Ctenochaetus striatus*, (Soares et al., 2011). In 2019, Bolognesi, Gauy and Gonçalves-de-Freitas showed that artificial TS provided by silicone bristles does not decrease cortisol levels in Nile tilapia after confinement, although reduces fish aggressiveness. Whereas the TS is not aversive to Nile

tilapia (Gauy et al., 2021), it raised the question of a possible later effect of TS over cortisol levels, i.e., during stress recovering.

According to Schreck and Tort (2016), stress is the body's physiological response and stressor is the agent that causes this response. In vertebrates there are three stages of the stress response when a stressor is detected by the central nervous system: alarm, resistance and exhaustion (Schreck, 2000). In fish, in the alarm phase, the sympathetic autonomic nervous system is activated and the chromaffin cells produces catecholamines (adrenaline and noradrenaline), the HPI (hypothalamus, pituitary, interrenal) axis is also activated (Ellis et al., 2012). The stressor is perceived by the nervous system and the hypothalamus releases CRH (corticotrophin releasing hormone), which stimulates the release of ACTH (adrenocorticotropic hormone) by the anterior pituitary into the bloodstream (Ellis et al., 2012). ACTH will act on the target tissue of the interrenal, which will produce glucocorticoids, such as cortisol (Ellis et al., 2012). In the resistance phase, physiological changes occur, such as, increased red cells in the blood, increased glicose in the blood. Behavioral changes such as reduced feeding, also occur. This happens to enable quick reactions as the fight-or-flight response. Cortisol will act by providing the body with necessary energy to deal with stress. If the stressor is removed or ended, the organism tends to return to homeostasis. But when stress is intense, prolonged, or occurs repeatedly, the exhaustion phase occurs. In this phase, the constant release and accumulation of cortisol in the body happens (Wendelaar Bonga, 1997). In this case, the animal is unable to regain homeostasis and the negative consequences of the accumulation of cortisol in the body occur, such as damage to growth, reproduction and the immune system, reflecting the body's difficulty in coping with stress (Portz, Woodley, Cech, 2006). Fish also present all three phases of stress response and may experience chronic stress if the third phase persists.

When the stressor is removed or the animal is able to cope with the stress, the organism tends to return to homeostasis (Schreck, Tort, 2016). Thus, with the application and subsequent removal of a stressor, the cortisol level is expected to increase and then, progressively, decrease. Foo and Lam (1993), for instance, in a study with Mozambique tilapia, *O. mossambicus*, observed that plasma cortisol level increases approximately 4 min after application of the stressor (net capture followed by confinement and exposure to air for 5 min), peaks within 30 min after application of the stressor, and returns to baseline

within near 60 min. This process of returning to baseline levels is considered the stress recovery.

Although there are studies showing the effect of TS in reducing stress (e.g. Field et al., 2005; Probst et al., 2012; Soares et al., 2011), the mechanism by which it acts is still uncertain. One possibility is that cortisol reduction occurs because of serotonin, which hampers the production of cortisol since serotonin production is stimulated by TS (Field et al., 2005). TS may also have a more direct effect on the HPA / HPI axis, inhibiting the synthesis of cortisol. These mechanisms remain to be tested.

Fish are subject to many stressors in rearing environments, such as overpopulation (Schram et al., 2006), net capture (Ellis et al., 2007) and social stress resulting from aggressive interactions (Boscolo; Morais and Gonçalves-de- Freitas, 2011). The consequence of this intense and / or prolonged stress is the constant release and accumulation of cortisol in the body (Wendelaar Bonga, 1997). Excess cortisol promote negative consequences for fish, such as reduced growth rate (Sadoul, Vijayan, 2016), immune response depression (Yada, Tort, 2016), reduced reproductive capacity (Pankhurst, 2016), neuronal destruction (Mcewen, Sapolsky, 1995), decreased neurogenesis (Sørensen et al., 2012) and death.

One of the alternatives used in fishkeeping and more recently in aquaculture to lower stress and raise welfare is the environmental enrichment (Arechavala-Lopez et al., 2021; Brandão et al., 2021). Some of the aims of environmental enrichment are increase the animal's positive utilization of the environment and increase its ability to cope with challenges in a more natural way (Young, 2003). In this case, TS could be configured as a sensory enrichment for fish (Gonçalves-de-Freitas et al., 2019). Thus, we are looking for alternatives to reduce stress and, consequently, increase the welfare of fish, especially in species of world importance, such as Nile tilapia, *O. niloticus*, the third most produced species in the world (FAO, 2017). Improving stress recovery in breeding environments can help reduce stress and consequently increase the welfare of the species produced. Thus, we tested if fish with TS decreases more quickly the stress caused by confinement in Nile tilapia than fish without TS , improving stress recovery.

Methods

Subjects and housing

We used 150 adult males sex reverted Nile tilapia, *Oreochromis niloticus* (GIFT – Genetically Improved Farmed Tilapia variety) from a commercial supplier, and that were kept in artificial ponds at the University facility. In the lab, the fish were acclimated for 15 days in polypropylene water tanks (capacity 500 L, 1 fish / 10 L) under the following conditions: temperature maintained at 27 ° C using heaters and thermostats, photoperiod of 12 h of light (7:00 a.m. – 19:00 p.m.) timer controlled and tropical fish food (28 % crude protein; at apparent satiety). The water quality was maintained by Canister biological filters (400 L / h filtration) and the water tanks were regularly siphoned to remove wastes. The length and weight of the fish used are described in Appendix 1.

Tactile stimulation (TS) device

The artificial TS device was a rectangular structure of PVC tubes (40 x 30 cm) and plastic sticks containing silicone bristles, which promote TS in fish (Figure 1A). A similar device was used by Bolognesi, Gauy and Gonçalves-de-Freitas (2019) and was efficient in promoting TS for Nile tilapia. We used a similar device as control, without the silicone bristles, not promoting TS (Figure 1B). The device was positioned in the center of the tank and the fish passed through it, receiving body TS (Figure 1C).

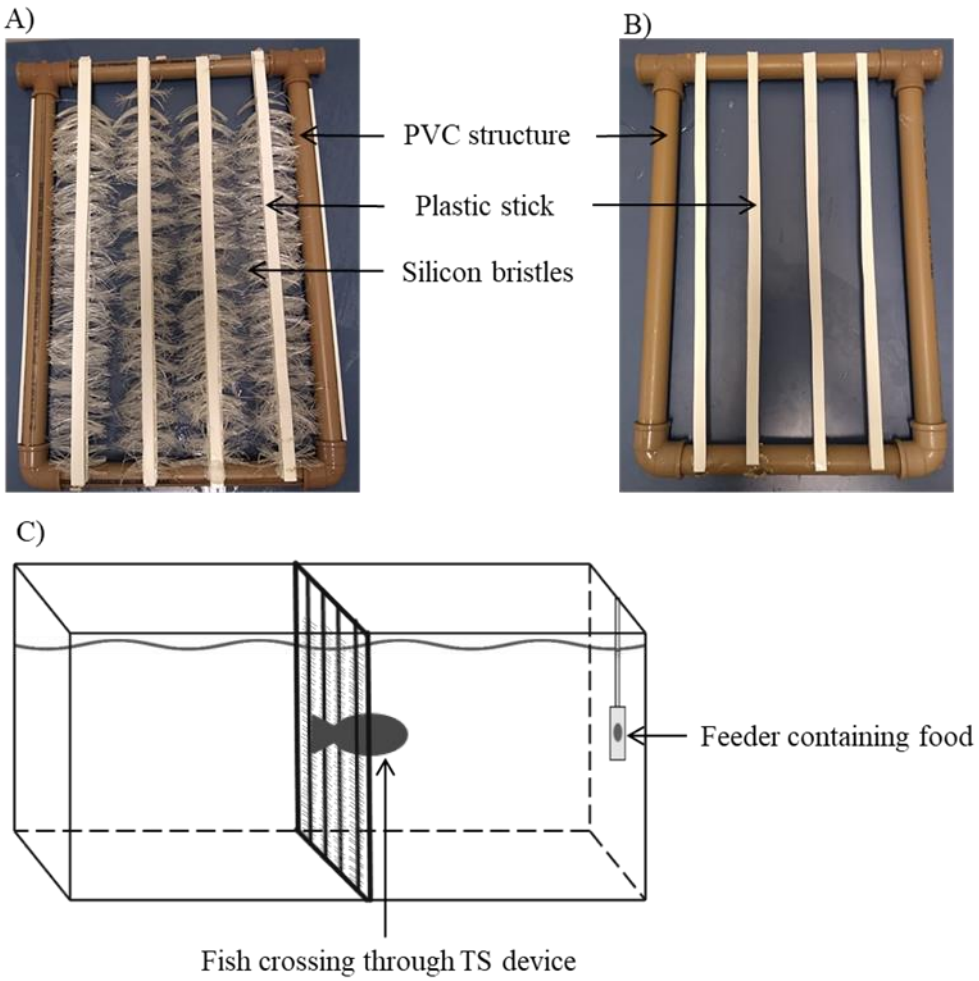

Figure 1. A) Photo of the TS device made by a PVC structure and plastic sticks. A) With silicone bristles. B) Without silicone bristles. C) Scheme of a tank with the TS device with silicon bristles in the center, a fish is crossing through the device and the feeder is placed in one tank wall.

Experimental design

We made 5 independent treatments with TS (with 15 replicates each) that were analyzed in different stress recovery times: 0, 30, 60, 90 and 120 min. The fish were individually anesthetized (immersion in 0.03 g / L benzocaine), measured and weighed, and then isolated for eight days in glass tanks (40 x 30 x 40 cm, capacity 48 L) with the TS device or control device. Once a day the fish received food by a feeder positioned at the opposite side of the fish in relation to the TS device. On the ninth day, fish were submitted to confinement stressor for 30 min. This type of stressor is known to increase the plasma cortisol concentration (Barreto et al., 2009). For this, we used an opaque plastic plate to keep the fish confined in a small space between the tank wall and the plate (less than 10 % of the available water volume). After the end of the confinement, a blood sample was made

to measure the plasmatic cortisol: in the 0 min treatment the collection was done immediately after the end of the confinement, in the 30 min treatment it was done 30 min after the end of the confinement, in the 60 min treatment it was done 60 min after the end of the confinement, in the 90 min treatment it was done 90 min after the end of the confinement, and, in the 120 min treatment, it was done 120 min after the end of the confinement. In the meantime fish remain in their tanks with or without TS. These times were chosen to assess the fish's progressive physiological response in relation to recovery from confinement stress.

Feed habituation

To make the fish try the TS device we used food (a small piece of sausage) as a stimulus. Before the experiment we grouped 10 fish per tank in glass tanks (60 x 60 x 60 cm, capacity 216 L) for 2 days and offered food in a feeder attached to one tank wall. During this period, they were fed eight times a day. This is enough time for the fish to habituate to the feeder (Bolognesi, Gauy, Gonçalves-de-Freitas, 2019).

Hormonal analysis

The physiological stress indicator used was plasma cortisol. For blood sampling, the animals were individually anesthetized (immersion in 0.09 g / L benzocaine) and collection was performed by puncture of the tail vein using hypodermic needles and heparinized syringes. The management of capture and anesthesia of the fish was performed in less than 2 min to avoid interfering with the results (e.g. Pottinger, 2008). Blood was centrifuged at 3,000 rpm for 10 min and plasma separated and frozen at – 20 º C until analysis. Cortisol was measured using the ELISA method - Enzyme Linked Immunosorbent Assay, using commercial kits (Cortisol: IBL - Immuno Biological Laboratories, Hamburg, Germany), validated for Nile tilapia (e.g. Bolognesi, Gauy, Gonçalves-de -Freitas 2019; Boscolo, Morais, Gonçalves-de-Freitas, 2011). The intra-assay coefficients of variation of plates were: 8.09 %, 4.52 %, 6.12 % and 6.17 %, and the inter-assay coefficient of variation of plates was 6.22 %.

Data analysis

All data were analyzed using Statistica Software (StatSoft). Data were evaluated for normality by Kolmogorov-Smirnov and for homoscedasticity by the Fmax test. An outlier was identified by the Grubbs test in the treatment 90 min without TS and was replaced by the mean value of the cortisol concentration of the replicas in this group. Plasma cortisol

concentration data were analyzed by General Linear Models (GLM), Mann-Whitney and post hoc Fisher LSD. We considered $p < 0.05$ for statistical significance.

Ethics approval

All procedures in this work followed the ethical principles of the National Council for the Control of Animal Experiments (CONCEA). The present study was approved by the Ethics Committee in the Use of Animals of the Instituto de Biociências, Letras e Ciências Exatas, UNESP, São José do Rio Preto (CEUA-IBILCE/UNESP), protocol nº 201/2019.

Results

Plasma cortisol concentration between treatments with and without TS

We did not observe any difference in plasma cortisol level between treatment with and without TS (GLM, $F_{1,140} = 2.14$, $p = 0.14$, Figure 2A) nor between recovery times (GLM, $F_{4.140} = 1.02$, $p = 0.39$, Figure 2A). We also found no significant difference in plasma cortisol level when comparing treatments and each recovery time (GLM, $F_{4.140} = 0.34$, $p = 0.84$, Figure 2A). Because of the high variation found in cortisol concentration we divided the replicates in two profiles according to cortisol measures: low or high responders.

Comparison between Low e High responders

For both treatments we divided the 15 replicates of each recovery time (0, 30, 60, 90 and 120 min) according to their cortisol level profile, using the median. In other words, we formed two groups: the high responders (7 fish), who had cortisol levels above the median value found for that group; and the low responders (7 fish), who had cortisol levels below the median value found for that group. A similar approach was used by Pottinger, Pickering, and Hurley (1992); Pottinger and Carrick (1999), due to the individual differences that occur within the population regarding the stress response.

We analyzed the data by GLM and Fisher LSD post hoc and observed that the cortisol level of the low responders is lower than the high responders, both for treatment with TS and without TS (GLM, $F_{1,120} = 169.2$, $p < 0.0001$, Figure 2B). We also observed a difference between treatments with and without TS (GLM, $F_{1,120} = 4.17$, $p = 0.043$, Figure 2B). The only difference we observed between treatments for the same recovery

time was in the low responders at 90 min, where cortisol concentration was lower in the treatment with TS than in the treatment without TS (Mann-Whitney, p = 0.035, Figure 2B).

Profile Low Responders

For the profile low responders, we did not observe any difference between treatment with and without TS (GLM, $F_{1,60} = 1.26$, p = 0.26, Figure 2B), nor in the comparison between treatments and recovery time (GLM, $F_{4,60} = 0.81$, p = 0.52, Figure 2B). However, we observed a difference between recovery times (GLM, $F_{4,60} = 4.14$, p = 0.004, Figure 2B) and in the interaction between treatments and recovery times (GLM, F 1,60 = 329.4, p < 0.0001, Figure 2B). For the low responder profile without TS, we observed that the cortisol level of the low responders in the treatment without TS was higher at time 0 min than at 30 min, 90 min and 120 min (p > 0.012). For the low responder profile with TS, we observed that the cortisol level of the low responders in the treatment with TS was lower for 90 min than 0 min and 120 min (p < 0.005).

Profile High Responders

For the profile high responder, we did not observe any difference between treatment with and without TS (GLM, $F_{1,60} = 3.39$, p = 0.07, Figure 2B), nor between recovery times (GLM, $F_{4,60} = 1.88$, p = 0.12, Figure 2B) and neither when comparing treatments with recovery time (GLM, $F_{4,60} = 0.89$, p = 0.47, Figure 2B). However, we found a difference in the interaction between treatments and recovery times (GLM, $F_{1,60} = 303.6$, p < 0.0001, Figure 2B). For the high responders profile without TS we observed that the cortisol level of the high responders in the treatment without TS was lower in the 90 min time than in the 30 min and 120 min (p > 0.039). For the high responder profile with TS, we did not observe any difference in the cortisol level of the high responders in the treatment with TS (p > 0.07).

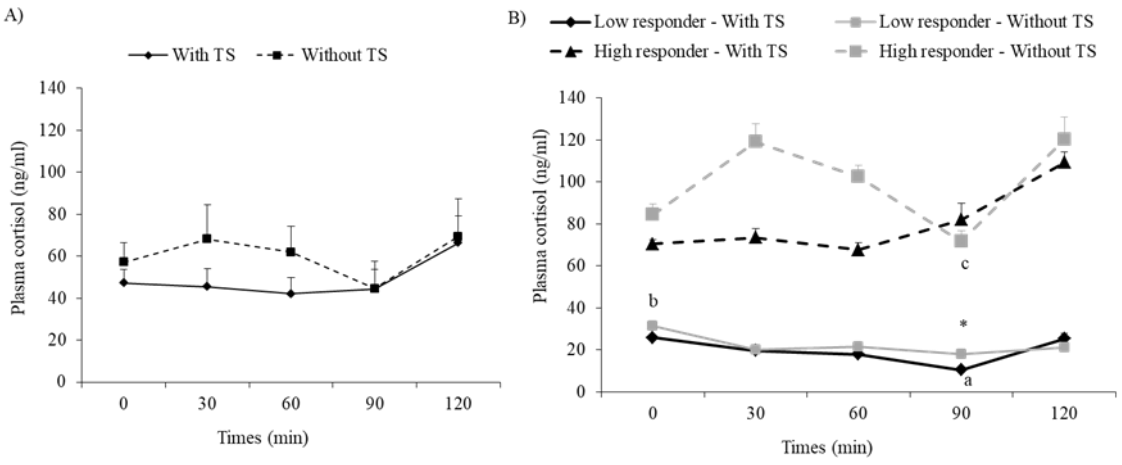

Figure 2. Mean ± Standard Error of plasma cortisol concentration (ng / ml) at different recovery times after confinement stress. A) Between treatments with TS (solid line) and without TS (dashed line). B) According to the Low and High responder profile. Solid black line corresponds to the low responder profile with TS (letter a represents difference between 90 min with 0 min and 120 min); solid gray line corresponds to the low responder profile without TS (letter b represents difference between 0 min with 30 min, 90min and 120 min); black dashed line corresponds to the high responder profile with TS; and gray dashed line corresponds to the high responder profile without TS (letter c represents difference between 90 min with 30 min and 120 min), GLM followed by Fisher LSD. Asterisk represents the difference between Low responder with TS and Low responder without TS, Mann-Whitney.

Discussion

This work traced the profile of the progressive physiological cortisol response of Nile tilapia (*O. niloticus*, GIFT variety) in relation to stress recovery. Groups of fish that had and had not have access to TS were studied at different times after application of a confinement stressor. We expected to find a cortisol concentration curve where it would rise after the stressor and, progressively, decline. It is interesting to note that we observed this curve in the low responder profile without TS, where cortisol was higher at 0 min; in the low responder profile with TS, where cortisol was higher at 0 min and in the high responder profile without TS, where cortisol was higher at 30 min compared to 90 min; but we did not observe in the high responder profile with TS, where cortisol remained constant. We also expected that TS would more quickly reduce cortisol levels, caused by confinement, when compared to fish without TS (control), improving stress recovery. But this was not observed and we conclude that TS has no effect on stress recovery in this species.

Confinement is a common stressor in the rearing process of fish such as Nile tilapia (e.g. net capture: Ellis et al., 2007). Therefore, it is widely used in studies to induce stress in this species (e.g. Pottinger, Pickering, Hurley, 1992; Volpato, Barreto, 2001; Barreto, Volpato 2004; Barreto et al., 2009; Backstrom et al., 2011; Bolognesi, Gauy , Gonçalves-de-Freitas, 2019). After a stressor is applied, the stress is expected to be high, which we notice by the increase and subsequent decrease in plasma cortisol levels. This happens due to the rapid activation of the HPI axis in fish after contact with a stressor, the result is a

hormonal cascade that culminates in the production of cortisol by the interrenal cells (Wendelaar Bonga, 1997). After a rise in cortisol, a fall is expected to occur, if the stressor is removed (Foo, Lam, 1993), the rise and fall profile of cortisol shows stress recovery. Considering the positive effect of TS previously demonstrated for other vertebrates, we expected that TS would more rapidly decrease the elevated cortisol level caused by confinement, as it could act by reducing cortisol levels over time. However, we did not see a clear answer in our data.

A possible explanation for the absence of differences between treatments is that cortisol varied significantly between individuals in each treatment and, with high variation, the differences are not evident. Thus, due to plasticity in the stress response, we divided the observed groups into low responders and high responders according to the level of cortisol presented. According to Pottinger and Carrick (1999); Øverli, Winberg and Pottinger (2005); Øverli et al (2007) and Winberg, Höglund and Øverli (2016), individuals of the same species can vary their response to stress and environmental disturbances, culminating in different changes in the level of cortisol and catecholamines in individuals subject to the same stressor. An individual's stress response is associated with factors such as health, nutrition, general physiological state, previous experience, age, social status and sex (Winberg, Höglund, Øverli, 2016). Furthermore, in coping style, we can identify proactive and reactive individuals, according to a set of physiological and behavioral characteristics (Winberg, Höglund, Øverli, 2016). The proactive are those who have a higher level of activity and aggressiveness, take more risks and are bolder, are more inclined to show a small range of behaviors and little behavioral flexibility, using more previous experiences than current information from the environment (Benus et al., 1990; Ruiz-Gomez et al., 2011). The reactive, on the other hand, are those who present more evasion, immobility, low levels of aggression, shyness, greater behavioral flexibility, and are more perceptive to changes in the environment (Benus et al., 1990; Ruiz-Gomez et al., 2011). These behavioral differences are also associated with physiological differences. The proactive ones show a lower post-stress cortisol level, while the reactive ones show a higher post-stress cortisol level (Koolhaas et al., 1999; Øverli, Winberg, Pottinger, 2005). Despite separating individuals into low and high responders, we still observed that there was a great variation in cortisol levels in fish, indicating that the response is not only associated with the way they cope with the environment, but with other individual characteristics.

In the profile low responder without TS, the cortisol level was higher at 0 min, indicating the effect of confinement. The cortisol level drops in 30 min and stays lower in 90 min and 120 min. The highest cortisol immediately after confinement was expected, followed by a decrease over time. In the low responder profile with TS, the cortisol level decreased from 0 min to 90 min and then increased at 120 min. A decrease in cortisol from 0 min was expected, as the stressor had been removed and the fish had time to recover. In the high responders profile without TS, we observed a peak at 30 min and a decrease only in 90 min, showing stress recovery. In the high responder profile with TS, we did not observe the effect of TS, but we did observe a constant cortisol level. Thus, the expected curve was observed in the profile low responder without TS, low responder with TS and high responder without TS, but we did not observe in the high responder with TS. Comparing between treatments we observed that at the 90 min time for low responders, the cortisol level was lower in the treatment with TS than in the treatment without TS, which may indicate a punctual effect of the TS, which can act by intensifying the decrease in the levels observed in 90 min. This result is in agreement with that found by Barcellos et al (1999) for Nile tilapia. The author applied a confinement stressor, in which the fish remained 60 s out of the water in nets. Barcellos et al (1999) observed a peak of cortisol concentration 60 min after application of the stressor and, after this period, the concentration began to decrease, which was observed after 4 h. We did not see a peak in cortisol at 60 min, but we did observe a fall at 90 min.

Bolognesi, Gauy and Gonçalves-de-Freitas (2019) kept Nile tilapia isolated for seven days with a TS device, and then the fish were submitted to a confinement stressor, which consisted of a 90 % reduction in the space of the fish tank with the use of an opaque plate, confining the fish to one end of the fish tank for 30 min. In this situation, the fish still had space to move. In the present study, we kept Nile tilapia isolated for eight days with TS device, and then the fish were subjected to the same confinement stressor, but with more than 90 % of the fish tank space reduced. The fish was confined between the plate and the tank wall having no space to move, which could characterize a more intense stressor than the one used by Bolognesi, Gauy and Gonçalves-de-Freitas (2019). The plasma cortisol values obtained immediately after the application of the stressor in the present study were lower than those obtained by Bolognesi, Gauy and Gonçalves-de-Freitas (2019) (Table 1).

Table 1. Demonstration of means (± standard error) of plasma cortisol levels (ng / ml) immediately after confinement.

	Without TS	With TS
Bolognesi, Gauy and Gonçalves-de-Freitas (2019)	129,8±11,8	139,7±16,6
This study (low + high responders)	57,0±9,2	47,2±6,3
This study (low responders)	31,5±1,5	25,8±0,7
This study (high responders)	84,4±4,9	70,55±1,9

Barreto et al (2009) confined Nile tilapia for 30 min to the bottom of the fish tank using a metal grid, and found mean ± standard error of 35.1 ± 6.6 ng / ml of post-confinement plasma cortisol, however fish were not separated into high and low responders. The value observed by Barreto et al (2009) is similar to the value found in our study for the low responders, 31.5 ± 1.5 ng / ml (without TS 0 min). This shows a variation between individuals of the same species subject to a similar stressor, as observed by Pottinger and Carrick (1999); Øverli, Winberg and Pottinger (2005); Øverli et al., 2007 and Winberg, Höglund and Øverli (2016).

Comparison between different studies using *O. niloticus* is desirable, but for confinement stress, we found many variations in the experimental protocol. For example, confinement can be done by keeping the fish in a net and taking it out of the water for a few seconds (e.g. Barcellos, 1999), restricting the area available to the fish, leaving 25 % of free area (e.g. Templonuevo, Vera-Cruz, 2016), 10 % free area (e.g. Bolognesi, Gauy, Gonçalves-de-Freitas, 2019), or less than 10 % free area, as in the present study. Space restriction can be done in several ways, for example, with a grid at the bottom of the fish tank (e.g. Barreto et al., 2009), or with plates, which reduce the size of the available area (e.g. Barreto, Volpato 2004; Bolognesi, Gauy, Gonçalves-de-Freitas, 2019). The confinement can also be done for different periods of time, such as 30 s, 5 min, 15 min and 30 min (e.g. Barreto, Volpato, 2004), 60 min and 90 min (e.g. Templonuevo, Vera-Cruz, 2016), 30 min (e.g. Bolognesi, Gauy, Gonçalves-de-Freitas, 2019; Barreto et al., 2009), 60 min (e.g. Volpato, Barreto, 2001), 30 min, 60 min, 120 min and 24 h (e.g. Auperin et al.,

1997). Furthermore, fish can be isolated or grouped (e.g. Auprin et al., 1997) before blood collection, which is an important variable for a social species such as Nile tilapia, and can interfere with the level of cortisol. Therefore, both for baseline levels and for post-stress levels of confinement the values may differ between studies. Therefore, it is interesting to interpret the context in which the fish was inserted before and during the application of the stressor.

TS had a punctual effect, decreasing the cortisol level 90 min after the stressor in the low responder profile, which may indicate a positive effect of TS for this profile in Nile tilapia. At 120 min, we have an increase in cortisol level, however, we did not observe any significant difference from this time to the others. Thus, in general, we did not observe other relevant effects on cortisol levels and concluded that TS has no effect on stress recovery in this species, unlike what occurs in the teleost fish, *C. striatus* (Soares et al., 2011) and in cattle (Probst et al., 2012). A possible explanation for this difference may be the fact that these species present natural behaviors related to TS. Mammals, such as cattle, lick their offspring at birth and exhibit licking and scratching behaviors on objects or barriers (Jensen, 2011), which promotes body TS. Many mammals exhibit grooming behavior (Dunbar, 2010), which also promotes TS. Client fish *C. striatus* receives TS from cleaner fish naturally during the cooperative relationship (Bshary, Wurth, 2001). Nile tilapia present physical contact in aggressive confrontations associated with the dominance hierarchy. Fights can have a negative meaning for Nile tilapia, as exaggerated fights can cause damage such as injuries and pain (Boscolo, Morais, Gonçalves-de-Freitas, 2011). But Nile tilapia also presents physical interactions during courtship (Gonçalves-de-Freitas, Nishida, 1998; Carvalho, Gonçalves-de-Freitas, 2008) and, in this case, they are expected to be positive interactions for males and females. Additionally, the TS device is designed to provide gentle stimulation through the silicone bristles and act as a sensory environmental enrichment for fish (Gonçalves-de-Freitas et al., 2019). In fact, this device is not aversive to Nile tilapia, as they choose to pass through the bristles spontaneously, even when they have the possibility to dodge them and also they demonstrate motivation to access the device (Gauy et al., 2021). Thus, we rule out the possibility that the TS device is aversive.

Since we found high variation in cortisol levels, we still need more studies to better understand the significance of TS on stress in this species.

References

ARECHAVALA-LOPEZ, P et al. Environmental enrichment in fish aquaculture: A review of fundamental and practical aspects. **Reviews in Aquaculture**, p. 1-25, 2021. DOI: https://doi.org/10.1111/raq.12620

AUPERIN, B et al. Effect of confinement stress on circulating levels of growth hormone and two prolactins in freshwater-adapted tilapia (Oreochromis niloticus). **General and Comparative Endocrinology**, v. 108, n. 1, p. 35-44, 1997. DOI:

https://doi.org/10.1006/gcen.1997.6938

BACKSTRÖM, T et al. Stress effects on AVT and CRF systems in two strains of rainbow trout (Oncorhynchus mykiss) divergent in stress responsiveness. **Hormones and Behavior**, v. 59, n. 1, p. 180-186, 2011. DOI: https://doi.org/10.1016/j.yhbeh.2010.11.008

BARCELLOS, LJG et al. Plasmatic levels of cortisol in the response to acute stress in Nile tilapia, Oreochromis niloticus (L.), previously exposed to chronic stress. **Aquaculture Research**, v. 30, n. 6, p. 437-444, 1999. DOI: https://doi.org/10.1046/j.1365-2109.1999.00348.x

BARRETO, RE et al. Aggressive behaviour traits predict physiological stress responses in Nile tilapia (Oreochromis niloticus). **Marine and Freshwater Behaviour and Physiology**, v. 42, n. 2, p. 109-118, 2009. DOI: https://doi.org/10.1080/10236240902850392

BARRETO, RE; VOLPATO, GL. Caution for using ventilatory frequency as an indicator of stress in fish. **Behavioural Processes**, v. 66, n. 1, p. 43-51, 2004. DOI: https://doi.org/10.1016/j.beproc.2004.01.001

BENUS, RF et al. Heritable variation for aggression as a reflection of individual coping strategies. **Experientia**, v. 47, n. 10, p. 1008-1019, 1991. DOI: https://doi.org/10.1007/BF01923336

BOLOGNESI, MC; GAUY, ACS; GONÇALVES-DE-FREITAS, E. Tactile stimulation reduces aggressiveness but does not lower stress in a territorial fish. **Scientific Reports**, v. 9, n. 1, p. 1-10, 2019. DOI: https://doi.org/10.1038/s41598-018-36876-1

BOSCOLO, CNP; MORAIS, RN; GONCALVES-DE-FREITAS, E. Same-sized fish groups increase aggressive interaction of sex-reversed males Nile tilapia GIFT strain.

Applied Animal Behaviour Science, v. 135, n. 1-2, p. 154-159, 2011. DOI: https://doi.org/10.1016/j.applanim.2011.10.003

BRANDÃO, M et al. Understanding behaviour to improve the welfare of an ornamental fish. **Journal of Fish Biology**, n. 99, p. 726– 739, 2021. DOI: https://doi.org/10.1111/jfb.14802

BSHARY, R; WÜRTH, M. Cleaner fish Labroides dimidiatus manipulate client reef fish by providing tactile stimulation. **Proceedings of the Royal Society of London. Series B: Biological Sciences**, v. 268, n. 1475, p. 1495-1501, 2001. DOI: https://doi.org/10.1098/rspb.2001.1495

CARVALHO, TB; GONÇALVES-DE-FREITAS, E. Sex group composition, social interaction, and metabolism in the fish Nile tilapia. **Brazilian Journal of Biology**, v. 68, n. 4, p. 807-812, 2008. DOI: https://doi.org/10.1590/S1519-69842008000400015

DUNBAR, RIM. The social role of touch in humans and primates: behavioural function and neurobiological mechanisms. **Neuroscience & Biobehavioral Reviews**, v. 34, n. 2, p. 260-268, 2010. DOI: https://doi.org/10.1016/j.neubiorev.2008.07.001

ELLIS, T et al. Cortisol and finfish welfare. Fish Physiology and Biochemistry, v. 38, n. 1, p. 163-188, 2012. DOI: https://doi.org/10.1007/s10695-011-9568-y

ELLIS, T et al. Non-invasive measurement of cortisol and melatonin in tanks stocked with seawater Atlantic salmon. **Aquaculture**, v. 272, n. 1-4, p. 698-706, 2007. DOI: https://doi.org/10.1016/j.aquaculture.2007.07.219

FAO. World Aquaculture 2015: A Brief Overview; **FAO Fisheries and Aquaculture Report**, n. 1140. Rome, Italy, 2017.

FIELD, T et al. Cortisol decreases and serotonin and dopamine increase following massage therapy. **International Journal of neuroscience**, v. 115, n. 10, p. 1397-1413, 2005. DOI: https://doi.org/10.1080/00207450590956459

FOO, JTW; LAM, TJ. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, Oreochromis mossambicus. **Aquaculture**, v. 115, n. 1-2, p. 145-158, 1993. DOI: https://doi.org/10.1016/0044-8486(93)90365-6

GAUY, ACS; BOLOGNESI, MC; MARTINS, GD; GONÇALVES-DE-FREITAS, E. Preference and motivation tests for body tactile stimulation in fish. **Animals**, 11, 2021. DOI: https://doi.org/10.3390/ani11072042

GONÇALVES-DE-FREITAS, E et al. Social behavior and welfare in Nile tilapia. **Fishes**, v. 4, n. 2, p. 23, 2019. DOI: https://doi.org/10.3390/fishes4020023

GONÇALVES-DE-FREITAS, E.; NISHIDA, SM. Sneaking behaviour of the Nile tilapia. **Boletim Técnico do CEPTA**, v. 11, p. 71-79, 1998.

JENSEN, MB. The early behaviour of cow and calf in an individual calving pen. **Applied Animal Behaviour Science**, v. 134, n. 3-4, p. 92-99, 2011. DOI: https://doi.org/10.1016/j.applanim.2011.06.017

KOOLHAAS, JM et al. Coping styles in animals: current status in behavior and stress-physiology. **Neuroscience & Biobehavioral Reviews**, v. 23, n. 7, p. 925-935, 1999. DOI: https://doi.org/10.1016/S0149-7634(99)00026-3

MCEWEN, BS; SAPOLSKY, RM. Stress and cognitive function. **Current Opinion in Neurobiology**, v. 5, n. 2, p. 205-216, 1995. DOI: https://doi.org/10.1016/0959-4388(95)80028-X

ØVERLI, Ø et al. Evolutionary background for stress-coping styles: relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. **Neuroscience & Biobehavioral Reviews**, v. 31, n. 3, p. 396-412, 2007. DOI: https://doi.org/10.1016/j.neubiorev.2006.10.006

ØVERLI, Ø; WINBERG, S; POTTINGER, TG. Behavioral and neuroendocrine correlates of selection for stress responsiveness in rainbow trout - a review. **Integrative and Comparative Biology**, v. 45, n. 3, p. 463-474, 2005. DOI: https://doi.org/10.1093/icb/45.3.463

PANKHURST NW. Reproduction and Development. In: Schreck, CB; Tort, L; Farrell, AP; Brauner, CJ (Eds). **Biology of Stress in Fish** – Fish Physiology. Academic Press. 2016. p. 296-321.

PORTZ, DE; WOODLEY, CM; CECH, JJ. Stress-associated impacts of short-term holding on fishes. **Reviews in Fish Biology and Fisheries**, v. 16, n. 2, p. 125-170, 2006. DOI: https://doi.org/10.1007/s11160-006-9012-z

POTTINGER, T. The Stress Response in Fish: Mechanisms, Effects and Measurement. In: Branson, EJ (Ed). **Fish Welfare**. Oxford, Blackwell Publishing Ltd, 2008. p. 32-48.

POTTINGER, TG; CARRICK, TR. Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. **General and Comparative Endocrinology**, v. 116, n. 1, p. 122-132, 1999. DOI: https://doi.org/10.1006/gcen.1999.7355

POTTINGER, TG; PICKERING, AD; HURLEY, MA. Consistency in the stress response of individuals of two strains of rainbow trout, Oncorhynchus mykiss. **Aquaculture**, v. 103, n. 3-4, p. 275-289, 1992. DOI: https://doi.org/10.1016/0044-8486(92)90172-H

PROBST, JK et al. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. **Applied Animal Behaviour Science**, v. 139, n. 1-2, p. 42-49, 2012. DOI: https://doi.org/10.1016/j.applanim.2012.03.002

RUIZ-GOMEZ, ML et al. Response to environmental change in rainbow trout selected for divergent stress coping styles. **Physiology & Behavior**, v. 102, n. 3-4, p. 317-322, 2011. DOI: https://doi.org/10.1016/j.physbeh.2010.11.023

SADOUL, B; VIJAYAN, MM. Stress and Growth. In: Schreck, CB; Tort, L; Farrell, AP; Brauner, CJ (Eds). **Biology of Stress in Fish** – Fish Physiology. Academic Press. 2016. p. 168-190.

SCHRAM, E et al. Stocking density-dependent growth of Dover sole (Solea solea). **Aquaculture**, v. 252, n. 2-4, p. 339-347, 2006. DOI: ttps://doi.org/10.1016/j.aquaculture.2005.07.011

SCHRECK, CB. Accumulation and long-term effects of stress in fish. In: Moberg GP; Mench JA (Eds). **The Biology of Animal Stress**: Basic Principles and Implications for Animal Welfare. Wallingford: CAB International, 2000. p. 147–158.

SCHRECK, CB; TORT, L. The concept of stress in fish. In: Schreck, CB; Tort, L; Farrell, AP; Brauner, CJ (Eds). **Biology of Stress in Fish** - Fish physiology. Academic Press, 2016. p. 1-34.

SOARES, MC et al. Tactile stimulation lowers stress in fish. **Nature Communications**, v. 2, n. 1, p. 1-5, 2011. DOI: https://doi.org/10.1038/ncomms1547

SØRENSEN, C et al. Social stress reduces forebrain cell proliferation in rainbow trout (Oncorhynchus mykiss). **Behavioural Brain Research**, v. 227, n. 2, p. 311-318, 2012. DOI: https://doi.org/10.1016/j.bbr.2011.01.041

TEMPLONUEVO, RMC; VERA-CRUZ, EM. Responses of red Nile tilapia (Oreochromis niloticus L.) subjected to social and confinement stresses. **CLSU International Journal of**

Science & Technology, v. 1, n. 2, p. 7-14, 2016. DOI: https://doi.org/10.22137/ijst.2016.v1n2.02

VOLPATO, GL; BARRETO, RE. Environmental blue light prevents stress in the fish Nile tilapia. **Brazilian Journal of Medical and Biological Research**, v. 34, n. 8, p. 1041-1045, 2001. DOI: https://doi.org/10.1590/S0100-879X2001000800011

WENDELAAR BONGA, SE. The stress response in fish. **Physiological Reviews**, v. 77, n. 3, p. 591-625, 1997. DOI: https://doi.org/10.1152/physrev.1997.77.3.591

WINBERG, S; HÖGLUND, E; ØVERLI, Ø. Variation in the neuroendocrine stress response. In: Schreck, CB; Tort, L; Farrell, AP; Brauner, CJ (Eds). **Biology of Stress in Fish** – Fish Physiology. Academic Press. 2016. p. 35-74.

YADA, T; TORT, L. Stress and Disease Resistance: Immune System and Immunoendocrine Interactions. In: Schreck, CB; Tort, L; Farrell, AP; Brauner, CJ (Eds). **Biology of Stress in Fish** – Fish Physiology. Academic Press. 2016. p. 366-389. DOI: https://doi.org/10.1016/B978-0-12-802728-8.00010-2

YOUNG, RJ. **Environmental enrichment for captive animals**. Oxford, UK: Blackwell Publishing. 2003.

Appendix 1

Mean ± standard error of fish length and weight. We did not observe significant difference in fish length and weight between treatments with and without TS (Factorial ANOVA, $F_{1,144} = 0,00$, $p = 1.0$ for length; $F_{1,144} = 0,82$, $p = 0.36$ for weight).

	Treatments	Length (cm)	Weight (g)
With TS	0 min (15 fish)	8,40 ± 0,15	23,85 ± 1,38
	30 min (15 fish)	8,71 ± 0,22	22,82 ± 1,91
	60 min (15 fish)	8,76 ± 0,15	24,13 ± 1,49
	90 min (15 fish)	8,50 ± 0,15	21,64 ± 1,24
	120 min (15 fish)	8,73 ± 0,16	23,0 ± 1,32
Without TS	0 min (15 fish)	8,66 ± 0,14	22,72 ± 1,18
	30 min (15 fish)	8,79 ± 0,18	23,82 ± 1,66
	60 min (15 fish)	8,36 ± 0,15	20,53 ± 1,24
	90 min (15 fish)	8,71 ± 0,19	25,58 ± 1,79
	120 min (15 fish)	8,76 ± 0,18	23,78 ± 1,80

CHAPTER II

Tactile stimulation reduces aggressive interaction and improves growth in Nile tilapia groups

Marcela Cesar Bolognesi[1,2], Ana Carolina dos Santos Gauy[1,2], Kawan Carvalho Martins[2], Eliane Gonçalves-de-Freitas[1,2]

[1] Centro de Aquicultura da Unesp - UNESP. Universidade Estadual Paulista. Via de Acesso Prof. Paulo Donato Castellane s/n, 14884-900, Jaboticabal/SP, Brasil.

[2] Instituto de Biociências, Letras e Ciências Exatas – UNESP. Universidade Estadual Paulista. Rua Cristóvão Colombo 2265, 15055-000, São José do Rio Preto/SP, Brasil.

Abstract

The artificially applied body tactile stimulation (TS) is known to decrease stress and increase positive states in vertebrates. In fish, this type of sensory stimulus decreases stress and aggressiveness. Therefore, it probably could counteract the social stress usually increased in artificial environment. Thus, we tested the effect of TS on the aggressiveness and stress of Nile tilapia, *Oreochromis niloticus*, males. We kept 4 fish grouped with or without TS (15 replicates per treatment) and quantified the aggressive behavior, the specific growth rate (SGR) and the plasma cortisol of each social rank. We observed that TS reduced aggressive behavior (with a clearer effect in the dominant fish), that fish with TS showed higher growth (higher SGR), and that TS had no effect on cortisol levels. Thus, despite no effect on the cortisol levels, the effects on aggressiveness and growth indicate that TS has a positive effect for Nile tilapia social groups and may contribute to increase the welfare of this species.

Key-words: animal welfare, social hierarchy, specific growth rate, aquaculture.

Introduction

TS promote positive states in vertebrates, especially in mammals. For example, hand touches, a kind of TS, reverses adverse conditions caused by neonatal isolation in rats (Imanaka et al., 2008) and reduces stress in adult humans (Field et al., 2005; Hernandez-Heif, Diego, Field, 2007), cattle (Probst et al., 2012) and dairy cows (Schmied et al., 2010).

Dairy cows also decrease fear behaviors (Shahin, 2018) and are highly motivated to have access to a brush that promotes body TS (McConnachie et al., 2018). Fish are also able to sense external tactile stimuli (Kazumyan, 2011).

Fishes use tactile sensory information during behavior such as schooling, prey-predator interactions (Butler, Maruska, 2016a), orientation, foraging, social interactions, defense and reproduction (Kazumyan, 2011). They have tactile receptors such as Merkel cells, Rohon-Beard cells and free nerve endings throughout the body and also grouped in organs such as barbels, rostrum, fin-free rays etc., in addition to a rich innervation of these structures to the brain (Kazumyan, 2011), demonstrating their ability to feel touch, tension, pressure and vibration (Kazumyan, 2011). Fish also perceive mechanical stimuli through the lateral line mechanosensory system that detects water movements and vibrations (Coombs, 1994). This system is composed of neuromasts distributed throughout the body and head of fish (Dijkgraaf, 1963). Butler and Maruska (2016b) showed that mechanoception is a mode of social communication used in the expression of aggression, reproduction and parental care in the African cichlid *Astatotilapia burtoni*. In addition, lateral line mechanical stimulation activates brain regions where social decisions are also processed (telencephalon and hypothalamus) (Butler, Maruska, 2016b), indicating that the mechanosensory system is part of social interactions and mediates brain interactions used in social decision-making.

The lateral line and the free nerve endings are considered the main sensory channels in fish, but a recent study by Cohen et al (2020) showed the presence of a push-rod-like mechanoreceptor complex that respond to touch on the adhesive disc (modified dorsal fin) of eight species of remoras. They allow remoras to sense when the disc has made contact with a host and quickly initiate and modulate the attachment (Cohen et al., 2020). This type of mechanoreceptor was only found in monotremes so far, suggesting that mechanoreception in fishes is more complex than previously thought (Cohen et al., 2020).

Soares et al (2011) demonstrated the positive effect of body TS in reducing stress in the client fish *Ctenochaetus striatus*, which maintains a cooperative relationship with a cleaner fish, *Labroides dimidiatus*. In this relationship, the cleaner fish feeds on the ectoparasites present in the client fish and promotes TS in the client fish's body with the movement of their pectoral fins (Bshary, Wurth, 2001). Soares et al (2011) showed that artificial TS, per se, reduce stress on client fish, resembling a massage effect in humans. In addition, Bolognesi, Gauy and Gonçalves-de-Freitas (2019) showed that TS reduces

aggressiveness in pair staged fights of Nile tilapia, *Oreochromis niloticus*, although they did not observe a decrease in stress in this species. Furthermore, Gauy et al (2021) demonstrated that Nile tilapia spontaneously accesses artificial TS devices and that fish overcome an aversive lighted rout to achieve it, demonstrating strong motivation to guarantee TS access, and then showing the positive meaning of TS for this species. As the effects of TS seem to be positive, it is possible to correlate it with the degree of welfare of these animals.

Physiological and behavioral indicators are generally used to assess animal welfare (Huntingford, 2020). A physiological indicator such as low corticosteroids levels, suggests lower levels of stress (Huntingford, 2006) and can be correlated with better welfare. The specific growth rate (SGR) can also help indicate welfare, as it infers the fish's growth (Crane, Ogle, Shoup, 2019). A behavioral indicator such as reduced aggression is also correlated with better welfare (Boscolo, Morais, Gonçalves-de-Freitas, 2011; Gonçalves-de-Freitas et al., 2019). These indicators can be used to assess the welfare of fish in farming systems.

Several practices associated with aquaculture can be considered harmful to fish welfare, for example, high stocking density, transport, confinement in ponds, pre-slaughter food deprivation (Ashley, 2007) and exacerbated agonistic interactions (Boscolo, Morais, Gonçalves-de-Freitas, 2011). Nile tilapia is a species of great importance in aquaculture and industry worldwide, with a high capacity to adjust to different environments and varied forms of farming (Vera-Cruz, Brown, 2007).

Like other territorial cichlids, Nile tilapia presents a social system of dominance hierarchy (Gonçalves-de-Freitas et al., 2019), in which males dispute territories and the resources present in it (Carvalho, Gonçalves-de-Freitas, 2008). In the hierarchy, dominant fish (highest social position) have priority access to resources compared to submissive fish (lowest social position), particularly in the reproductive context (Gilmour, Dibattista, Thomas, 2005). The hierarchy is established by aggressive interactions, which can be separated into two main groups: attacks (overt fights) and threats (restrained fights) (e.g. Noleto-Filho et al., 2017; Gauy, Boscolo, Gonçalves-de-Freitas, 2017). Attacks are aggressive interactions that demand greater energy expenditure and generally present physical contact between individuals (Ros, Becker, Oliveira, 2006). Threats, on the other hand, are interactions that demand less energy expenditure and generally do not have physical contact between individuals (Ros, Becker, Oliveira, 2006). Aggressive

interactions are part of the natural behavior of the species, but if they occur exaggeratedly, they increase social stress (Boscolo, Morais, Gonçalves-de-Freitas, 2011) which is perceived by higher levels of cortisol (Sloman et al., 2001). If stress is prolonged or cannot be avoided, like in breeding systems, chronic stress occurs and the excess of cortisol produced will negatively affect the body. These detrimental effects can be: immune system depression (Pottinger, 2008), reduced growth rate (Barton, Morgan, Vijayan, 2002), impaired neurogenesis (Sørensen et al., 2012) and death.

Therefore, we must look for alternatives to reduce stress and aggressiveness and, consequently, increase the welfare of fish species in captivity. Here we tested the effect of TS on aggressiveness, growth and stress in Nile tilapia social groups.

Methods

Subjects and housing

We used 120 adult sex reverted males Nile tilapia, *Oreochromis niloticus*, from a commercial supplier. The fish were kept in artificial ponds at Instituto de Biociências, Letras e Ciências Exatas of São José do Rio Preto (IBILCE-UNESP). The fish were acclimated for 15 days in blue polypropylene water tanks (capacity 500 L, 1 fish / 10 L), in which the temperature was maintained at 27°C by heaters and thermostats. The tanks were blue because blue color prevents stress in Nile tilapia (Volpato, Barreto 2001). The photoperiod was 12h of light (7:00 a.m. – 7:00 p.m.) controlled by timer. Fish were fed with tropical fish food (28 % crude protein; ad libitum). Water quality was maintained using Canister biological filters (400 L / h filtration) and the water tanks were siphoned to remove waste. The standard length of the fish was measured with a manual caliper and the weight was measured with a precision scale on the first and last days of the experiment. Fish lengths were: mean ± SE 10.06 ± 0.11 cm for treatment with TS and mean ± SE 9.18 ± 0.11 cm for treatment without TS. Fish weights were: mean ± SE 34.37 ± 1.19 g, for treatment with TS and 32.67 ± 1.15 g, for treatment without TS. We did not observe significant difference on fish length and weight between treatments (Independent t test, $t_{(118)} = 1.55$, $p = 0.12$ for length; independent t test, $t_{(118)} = 1.02$, $p = 0.30$ for weight). After the experiment all fish were individually euthanized (immersion in benzocaine 0.18 g / L), the gonads and liver were inspected to certify age and health.

Tactile stimulation (TS) device

The artificial TS was provides by the TS device that was made by a rectangular structure of PVC tubes (60 x 40 cm) and vertical plastic sticks, in which silicone bristles are inserted (Figure 1A), they promote body TS. The control device is similar but without the silicone bristles, not promoting TS (Figure 1B). The fish received the stimulus when passing through the silicone bristles (Figure 1C). The TS device was positioned in the center of the tank during the experiment. This TS device was used by Bolognesi, Gauy and Gonçalves-de-Freitas (2019) and Gauy et al (2021) and proved to be efficient in promoting TS, as fish pass through it spontaneously and present motivation to access it (Gauy et al., 2021).

Experimental design

We use 120 fish from 8 to 12 cm standard length. Each replica was formed by a group of 4 fish with heterogeneous size. Groups of fish with heterogeneous size present less aggressiveness than groups of homogeneous size, this prevents injuries and death of grouped fish (Boscolo, Morais, Gonçalves-de-Freitas, 2011).

The fish were individually anesthetized (immersion in benzocaine 0.03 g / L), measured, weighed and identified (with Visible Implant Fluorescent Elastomer – VIE, which was applied under some scales on both sides of the fish's body). They were allocated to one of two treatments called: with TS (15 replicates with 4 fish each) or without TS (15 replicates with 4 fish each). We used 60 x 60 x 40 cm fish tanks, 140 L capacity, coated with blue plastic on its sides to avoid visual contact with the other tanks.

The group remained in the tank for 12 days. In the first three days, we made daily records of 15 min, at 2 pm to quantify aggressive behavior and identify the social rank of each fish in the social hierarchy. On the third day, we collected blood from each fish to measure plasma cortisol. On the fourth day we introduced the TS device or control device. From the fourth to the eleventh day, we made daily records (15 min) to quantify the number of crossings through the TS device or control device and the aggressive behavior. After the last record we did a second blood sample. For the filming we used hand cam cameras positioned in front of the tanks. The schematic design can be seen in Figure 1D.

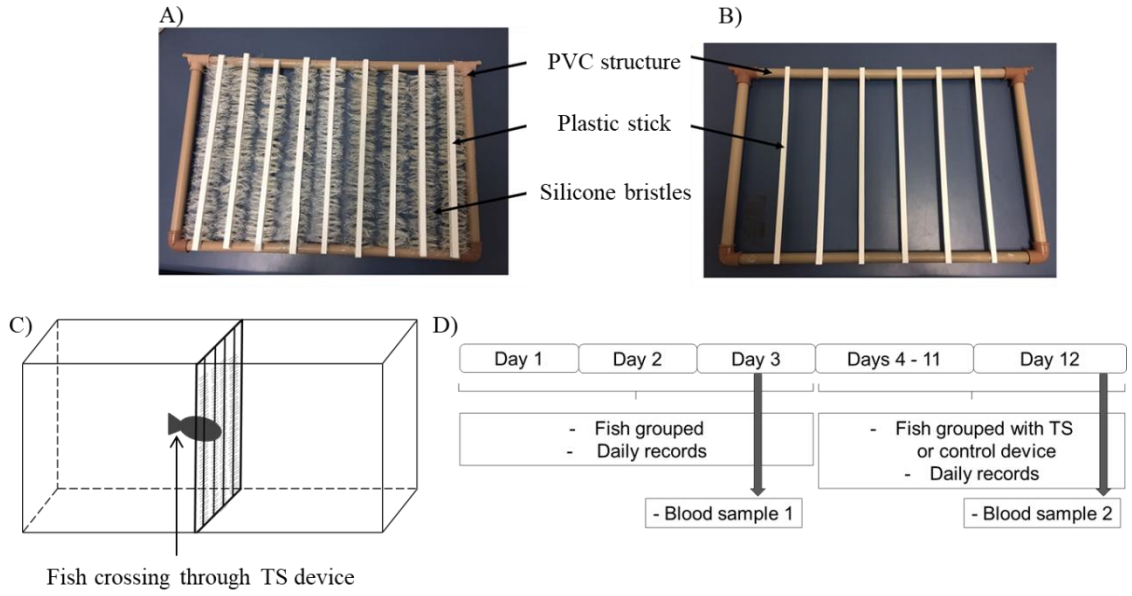

Figure 1. A) Photo of the TS device made by a PVC structure and plastic rods with silicone bristles. B) Photo of the TS device made by a PVC structure and plastic rods without silicone bristles. C) Scheme from a fish crossing through the TS device positioned in the center of the tank. D) Schematic design with the sequence of events.

Social rank

The rank (social position) of each fish was inferred by calculating the dominance index (DI) as used by Gonçalves-de-Freitas et al (2008) for Nile tilapia, it was calculated by the formula: DI = frequency of aggressive behavior emitted by the individual / total frequency of aggressive behavior in the group.

The DI value ranges from 0.0 to 1.0, the higher the DI, the higher the position in the social hierarchy occupied by the individual. Thus, we obtained four ranks in dominant-submissive order: alpha, beta, gamma and delta.

Aggressive interactions quantification

We quantified the frequency of crossings through the TS or control device of each fish. We also quantified the aggressive interactions of each fish based on the ethogram previously described for Nile tilapia (Carvalho, Gonçalves-de-Freitas, 2008; Boscolo, Morais, Gonçalves-de-Freitas, 2011). We divided aggressive interactions into attacks and threats, as used by Noleto-Filho et al (2017) and Gauy, Boscolo and Gonçalves-de-Freitas (2017).

The attacks quantified were: Lateral fight: The fish stand next to each other, facing the same or opposite directions, and flap their tails sideways. Mouth fight: Both fish

approach frontally with their mouths open and bite their opponent's mouth. Bite: The aggressor swims towards his opponent and bites his body.

The threat quantified was: Lateral threat: A fish with its open fins and open mouth approaches its opponent, who moves away.

Presence of ranks in each side of the tank

As the TS devices were positioned in the center of the tank, we needed to check whether it was acting as a landmark for the territory of the dominant fish, dividing the tank into two territories. Then we assessed whether the presence of each fish on tank sides A and B was associated with rank. The presence was quantified from the fourth to the twelfth day (135 min).

Specific Growth Rate – SGR

SGR expresses growth as the per cent increase in weight per unit of time (Crane, Ogle, Shoup, 2019). It was calculated by the formula $SGR = 100((W2/W1)^{1/\Delta t} - 1)$. Where W1 is the initial weight, W2 is the final weight and Δt is the time in days.

Cortisol assays

The physiological stress indicator was plasma cortisol. For blood sampling, the fish were individually anesthetized (immersion in benzocaine 0.09 g / L), hypodermic needles and heparinized syringes were used to puncture the tail vein and collect blood (approximately 1 ml). The procedure of each fish capture and anesthesia was performed in less than 2 minutes to avoid interfering with the results (e.g. Pottinger, 2008). Blood was centrifuged at 3.000 rpm for 10 minutes and plasma separated and frozen at -20 º C. Cortisol was measured using the ELISA method – Enzyme Linked Immunosorbent Assay, using commercial kits (Cortisol: IBL - Immuno Biological Laboratories, Hamburg, Germany), validated for Nile tilapia (e.g. Boscolo, Morais, Gonçalves-de -Freitas, 2011; Bolognesi, Gauy, Gonçalves-de-Freitas, 2019). The intra-assay coefficients of variation were: 7.85%, 5.5%, 4.6%, 8.64%, 5.17%, and 6.34%. The inter-assay coefficient of variation was 6.34%.

Data analysis

Data were analyzed using Statistica Software (StatSoft). Data were evaluated for normality by Shapiro-Wilk and for homoscedasticity by the Fmax test. The number of crossings through the TS device was transformed by $\sqrt{(x+0.5)}$ to fit homoscedasticity assumptions. We considered $p < 0.05$ for statistical significance.

The frequency of crossings through the TS devices over the days between treatments and by ranks was compared by General Linear Models (GLM) and Fisher LSD post hoc. The frequency of total aggressive interactions, attacks and threats by rank and between treatments was compared by independent t test. The position of ranks on each side of the tank, within and between treatments, was compared by dependent t test. The SGR between treatments and between ranks was compared by GLM. Plasma cortisol concentration before and after TS device use and between treatments were compared by GLM and Fisher LSD post hoc.

Ethics approval

All procedures in this study followed the ASAB / ABS Guidelines for the Use of Animals in Research and the ethical principles of the National Council for the Control of Animal Experiments (CONCEA). The present study was approved by the Ethics Committee in the Use of Animals of the Instituto de Biociências, Letras e Ciências Exatas da UNESP, São José do Rio Preto (Ceua – Ibilce/Unesp), protocol nº 180/2017.

Results

Frequency of crossings through the TS device or control device

The frequency of crossings was quantified from the 4th to the 12th day (15 min / day) in both treatments. The frequency of crossings was higher in all days in the treatment without TS than in the treatment with TS (GLM: $F_{1,28} = 201.61$, $p < 0.0001$, Figure 2A). The frequency of crossings was summed and presented by rank. The number of crossings was lower for all ranks of the treatment with TS than the treatment without TS (GLM: $F_{1.28} = 204.33$, $p < 0.0001$, Figure 2B). We observed difference between the ranks (GLM: $F_{3.84} = 4.56$, $p = 0.005$, Figure 2B). In the treatment with TS delta rank had higer number of crossings than beta rank (GLM: $F_{3.84} = 4.56$, $p = 0.04$, Figure 2B). In the treatment without TS we observed that the alpha rank had higher number of crossings than all the other ranks (GLM: $F_{3.84} = 4.56$, $p < 0.006$, Figure 2B).

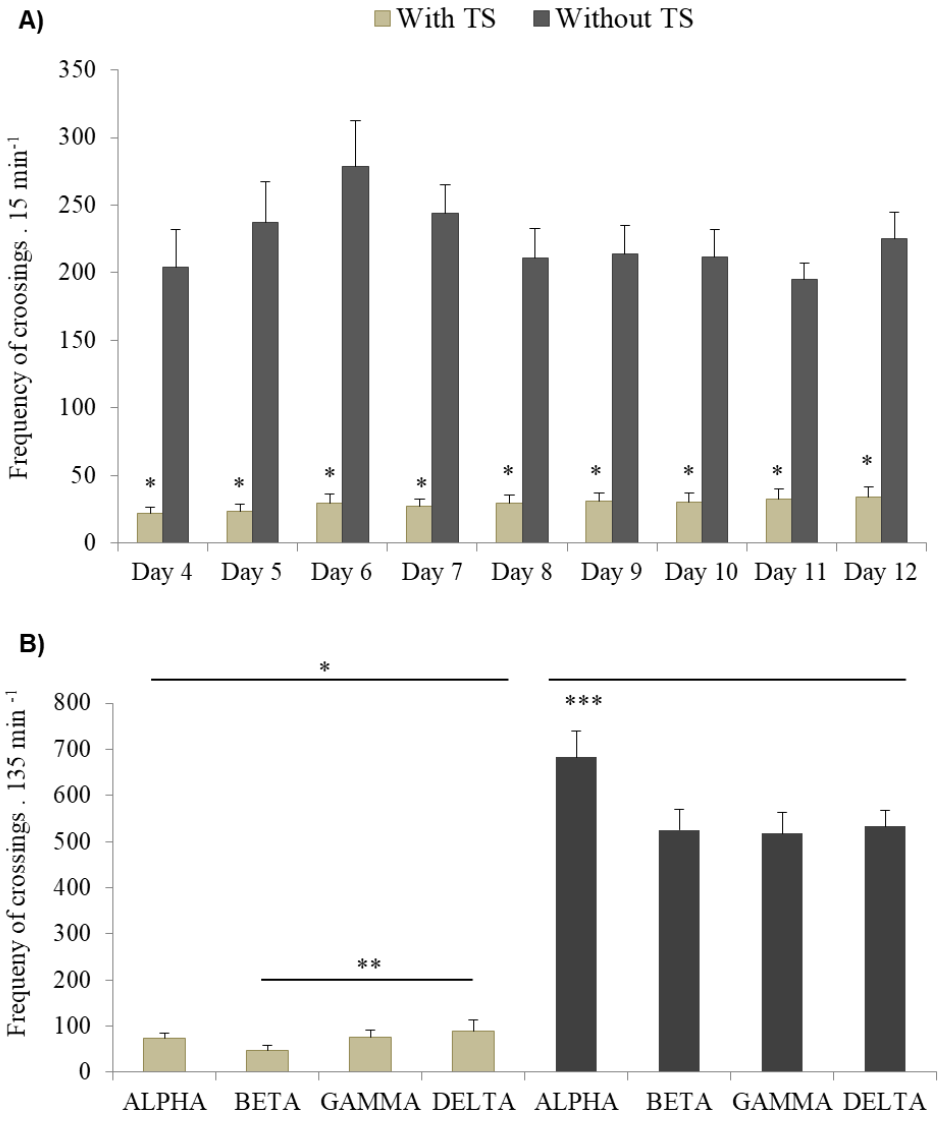

Figure 2. Mean ± standard error of the frequency of crossings by the TS device or control device A) per day. Asterisks indicate difference between treatments with TS and without TS. B) per rank. One asterisk indicates difference between treatments with and without TS. Two asterisks indicate difference between beta and delta ranks from treatment with TS. Three asterisks indicate difference among alpha rank and all the other ranks in treatment without TS. GLM and Fisher LSD.

Frequency of total aggressive interactions by rank

The total frequency of aggressive interactions (attacks + threats) from the 4th to the 12th day (135 min) was added. For alpha rank, it was lower in treatment with TS than in treatment without TS (Independent t test: $t_{28} = 4.39$, $p = 0.0001$, Figure 3A). For the other ranks, it was similar between treatments (beta: independent t test: $t_{28} = 1.60$, $p = 0.11$;

gamma: independent t test: $t_{28} = 1.18$, $p = 0.24$; delta: independent t test: $t_{28} = 0.62$, $p = 0.53$, Figure 3A).

Frequency of attacks by rank

The frequency of attacks from the 4th to the 12th day (135 min) was added. For alpha rank, it was lower in treatment with TS than in treatment without TS (Independent t test, $t_{28} = 3.21$, $p = 0.003$, Figure 3B). For the other ranks, it was similar between treatments (beta: independent t test: $t_{28} = 1.60$, $p = 0.11$; gamma: independent t test: $t_{28} = 1.18$, $p = 0.24$; delta: independent t test, $t_{28} = 0.62$, $p = 0.53$, Figure 3B).

Frequency of threats by rank

The frequency of threats from the 4th to the 12th day (135 min) was added. For alpha rank, it was lower in treatment with TS than in treatment without TS (Independent t test: $t_{28} = -2.98$, $p = 0.005$, Figure 3C). For the other ranks, it was similar between treatments (beta: independent t test: $t_{28} = -1.47$, $p = 0.15$; gamma: independent t test: $t_{28} = -1.92$, $p = 0.06$; delta: independent t test: $t_{28} = 0.70$, $p = 0.48$, Figure 3C).

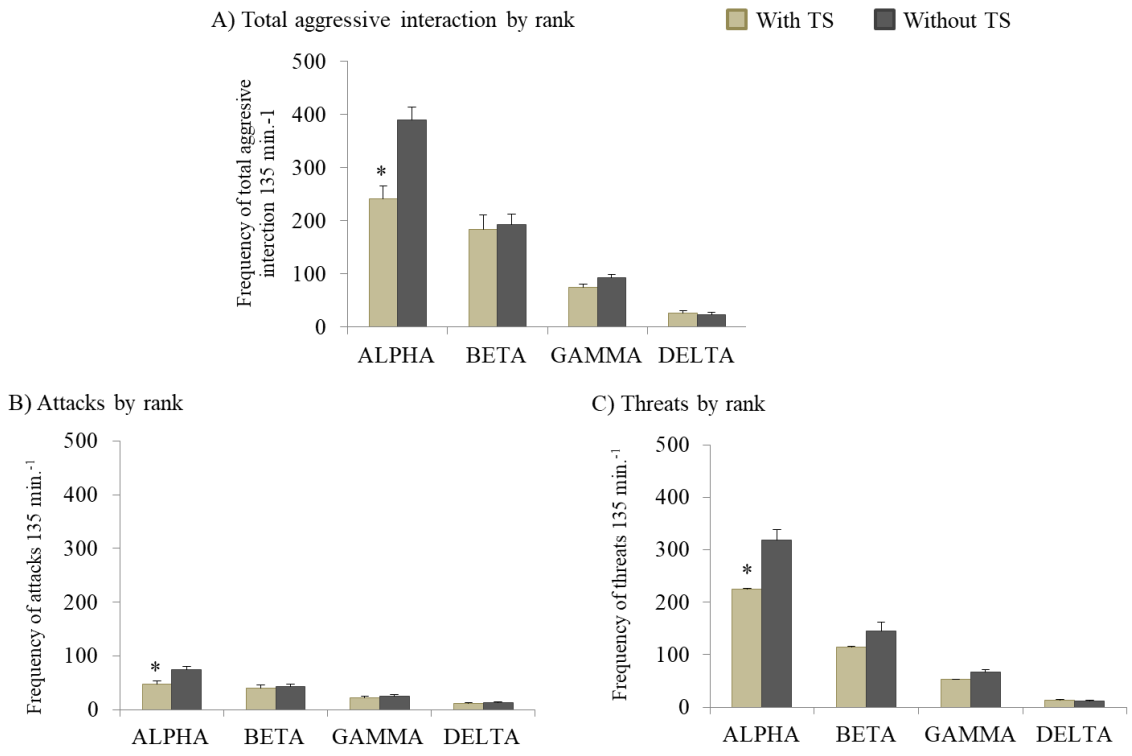

Figure 3. Mean ± standard error of frequency of: A) Total aggressive interaction (attacks + threats) by rank. B) Attacks by rank. C) Threats by rank. Independent t test compared between treatments with TS and without TS. Asterisk indicates significant difference between treatments with and without TS.

Presence of ranks in each side of the tank

In the treatment without TS the frequency of presence on each side of the tank was similar for all ranks (alpha: dependent t test: $t_{14} = 1.33$, $p = 0.20$; beta: dependent t test: $t_{14} = -0.89$, $p = 0.38$; gamma: dependent t test: $t_{14} = -0.03$, $p = 0.97$; delta: dependent t test: $t_{14} = -0.06$, $p = 0.94$, Figure 4).

In the treatment with TS for alpha and beta ranks, the frequency of presence on each side of the tank was similar (alpha: dependent t test: $t_{14} = -1.05$, $p = 0.30$; beta: dependent t test: $t_{14} = -1.82$, $p = 0.08$, Figure 4). For the gamma and delta ranks, the frequency was lower on the A side (gamma: dependent t test: $t_{14} = -3.67$, $p = 0.002$; delta: dependent t test: $t_{14} = -2.58$, $p = 0.02$, Figure 4).

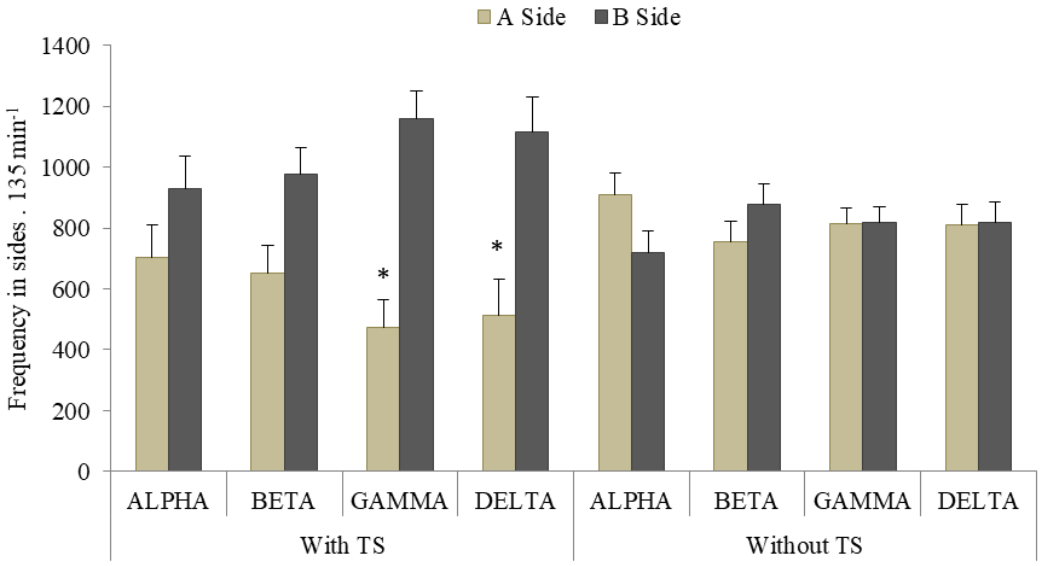

Figure 4. Mean ± standard error of frequency of presence on each side of the tank: With TS and Without TS. Dependent t test compare between sides. Asterisk indicates significant difference between sides.

SGR

In the treatment with TS the SGR was higher than in the treatment without TS (GLM: $F_{1,112} = 4.30$, $p = 0.04$, Figure 5). We did not observe difference among ranks (GLM: $F_{3,112} = 0.44$, $p = 0.72$, Figure 5).

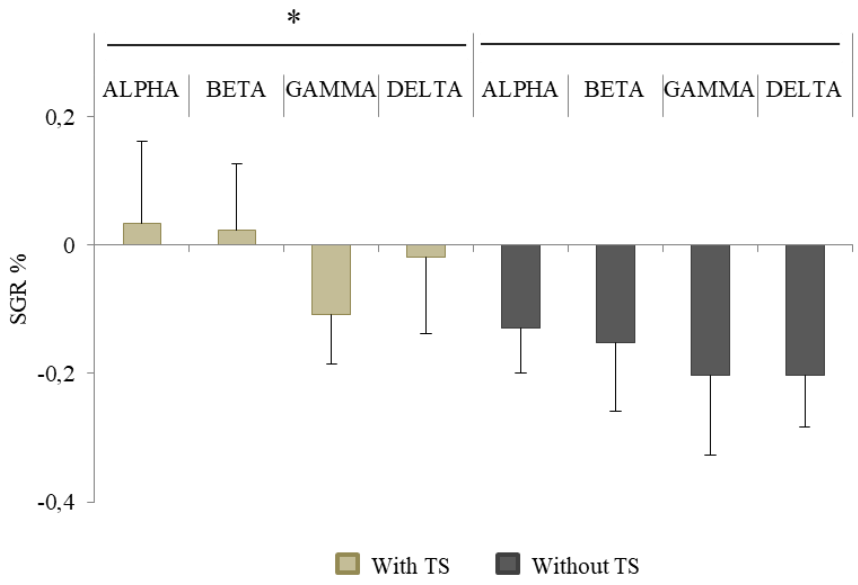

Figure 5. SGR (%) of fish in treatments with and without TS for each rank. GLM. Asterisk indicates significant difference between treatments with TS and without TS.

Plasma cortisol

Before the introduction of the TS device, we did not observe any difference in cortisol levels between treatments with and without TS (GLM: $F_{1,111} = 1.57$, $p = 0.21$, Figure 6A), nor between the ranks (GLM: $F_{3,111} = 0.18$, $p = 0.90$, Figure 6A) and neither in the interaction of treatments with ranks (GLM: $F_{3,111} = 1.51$, $p = 0.21$, Figure 6A). After 9 days with the TS or control device, we did not observe any difference in cortisol levels between treatments with and without TS (GLM: $F_{1,105} = 2.70$, $p = 0.10$, Figure 6B), nor between the ranks (GLM: $F_{3,105} = 0.22$, $p = 0.88$, Figure 6B) and neither in the interaction of treatments with the ranks (GLM: $F_{3,105} = 0.59$, $p = 0.62$, Figure 6B).

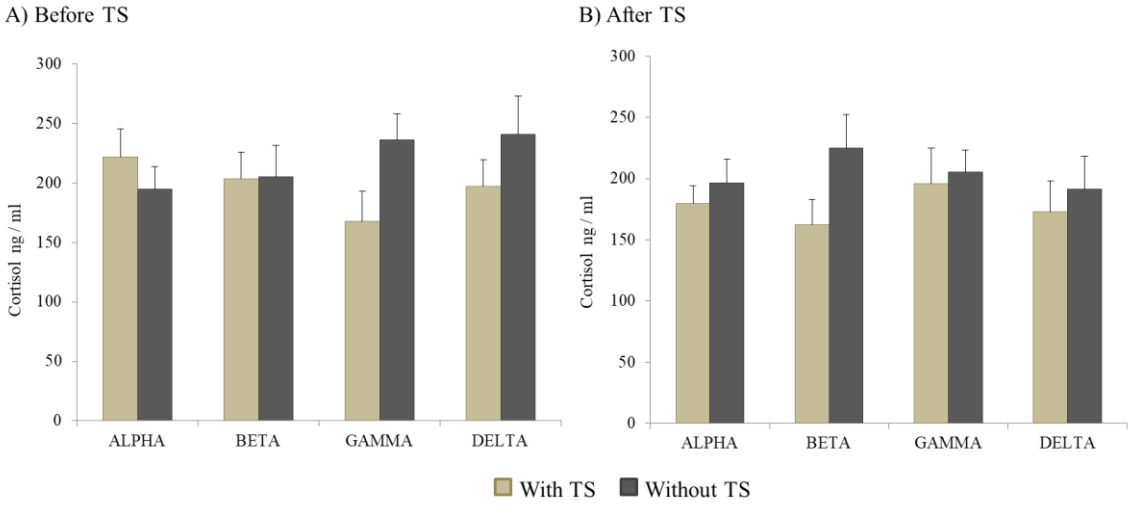

Figure 6. Mean ± standard error of plasma cortisol concentration in each rank. A) Before the introduction of the TS device (with or without TS). B) After 9 days with the TS or control device (with or without TS). GLM and Fisher LSD.

Discussion

We tested the effect of TS on aggressive interaction, growth and stress in Nile tilapia social groups. Firstly, we observed that grouped fish successfully crossed through the TS device. Then, we observed a reduction in aggressiveness for alpha ranked fish. Furthermore, TS reduced the growth of the group. However, there was no effect of TS on stress levels. Nevertheless, the effects on aggressiveness and growth indicate a positive influence of TS on the welfare of Nile tilapia social groups.

Frequency of crossings through the TS device or control device

The frequency of crossings through the device was higher in treatment without TS than in treatment with TS, as observed by Bolognesi, Gauy and Gonçalves-de-Freitas (2019) and Gauy et al (2021). In the treatment without TS the alpha rank crossed more times than the other ranks, this did not occur in the treatment with TS, where the alpha rank crossed similarly to the other ranks. This is in agreement with the alpha rank being less aggressive, as we present below. In the treatment with TS the delta rank crossed more than the beta rank. This could indicate that delta crossed repeatedly, as the delta's frequency of presence was mostly checked on a particular side of the tank.

This difference on crossing between treatments could be due to some factors, such as: (1) Fish may interpret the TS device as a physical barrier in the environment, even if they can cross it. The silicone bristles of the TS device may have caused some resistance to the fish passage, unlike the control device, which did not have bristles and could allow access to the other side of the tank more easily. However, Bolognesi, Gauy and Gonçalves-de-Freitas (2019) observed that isolated Nile tilapia had numbers of crossings by the TS device or tank center similar to the ones found here. Furthermore, the frequency of crossings was lower in isolated fish (see Bolognesi, Gauy, Gonçalves-de-Freitas, 2019) than in grouped fish. This demonstrates that the TS device with bristles did not represent a totally insurmountable barrier to fish. We can argue that in the study by Bolognesi, Gauy and Gonçalves-de-Freitas (2019) there may have been an effect of social isolation, a situation in which individuals of a social species are kept isolated. In teleost fish, social

isolation can reduce the locomotion of individuals (Gómez-Laplaza, Morgan, 2003), and decrease learning (Brandão, Braithwaite, Gonçalves-de-Freitas, 2015) this would explain a smaller number of crossings in isolated fish than in grouped fish. (2) The TS device may have functioned as a spatial reference of territory. In this way, the available space of the tank could have been delimited in two distinct territories. The Nile tilapia is a territorial species (Carvalho, Gonçalves-de-Freitas, 2008) and could have delimited territory on only one side of the tank, reducing the number of crossings by the TS device. But as we see below, the alpha and betta ranks (higher ranks) occupied both sides of the tank equally, demonstrating that the division of the territory did not occur. (3) The TS device may have acted as a visual barrier, preventing vision from the other side of the tank. This would have led to less exploration of the tank, reducing movement and, consequently, reducing the number of crossings. However, Nile tilapia has good vision (Castro et al., 2009) and also communicates by chemical signals (Gonçalves-de-Freitas et al., 2008). Thus, even so the TS device had decreased visual communication of individuals, chemical communication could not be impaired by the TS device and could encourage the fish to explore the other side. (4) The TS device may have meant a new object in the environment and caused a neophobia effect (aversion to new objects or situations) in fish (Ashley, Sneddon, 2008). Sneddon, Braithwaite and Gentle (2003), for example, demonstrated that rainbow trout took a relatively long time to get close to a new object soon after it was introduced into the environment, and stayed close to it for a short time, showing neophobia. However, we observed that on the first day with the TS device the Nile tilapia explored and crossed it. Thus, even if the device represented a new object in the environment, it had little neophobic effect, since the frequency of crossings was constant from the first day on. In addition, crossings in both treatments remained constant over the days, indicating fish habituation to the device, probably as a consequence of environmental exploration. Since fish daily sought contact with TS, we concluded that the TS device did not represent an aversive object to fish. This is in agreement with Gauy et al (2021), who demonstrated that Nile tilapia does not prevent TS even after a stressful situation and is motivated to access it. Even considering all these possibilities, it is important to emphasize that grouped fish, under social stress, spontaneously sought TS during all days of the experiment.

Frequency of aggressive interactions in each rank

We expected that ranks in the TS treatment would present reduction of aggressive behavior, as demonstrated by Bolognesi, Gauy and Gonçalves-de-Freitas (2019). In fact TS

decreased aggressiveness in grouped fish with the effect on alpha rank. In the study made by Bolognesi, Gauy and Gonçalves-de-Freitas (2019), the effect of TS in reducing aggression in pairs of Nile tilapia was due to the reduction of attacks, which are considered aggressive confrontations of high intensity (Ros, Becker, Oliveira, 2006). In the present study, we observe the effect of TS in alpha rank with the reduction in attacks and threats (which are considered low intensity confrontations, Ros, Becker, Oliveira, 2006). In the Nile tilapia social group, the dominant fish is more aggressive and may present a higher level of social stress in heterogeneous groups (Boscolo, Morais, Gonçalves-de-Freitas, 2011; Barreto, Boscolo, Gonçalves-de-Freitas, 2015). Thus, the effect of TS on aggressiveness is very relevant, acting precisely on the dominant fish, which probably promote better levels of welfare for the whole group.

Does the reduction of aggressive interaction is due to TS?

A main question can rise from these results is "does the reduction of aggressive interaction showed by dominant fish is really an effect of TS or can be due to other effects from the TS device in the center of the tank?" As mentioned in the beginning of this section, the TS device could be acting as a physical and visual barrier that can reduce movements and sight of fish and, as a consequence, reduce opportunities of encounters to fight. To clarify this idea the fish position in the fish tank can bring us some answer.

Presence of ranks in each side of the tank

The frequency of presence of each fish on the sides of the tank showed us the behavior of the social group in relation to the TS device, since it was positioned in the center of the tank. In the treatment without TS, fish of all ranks remained equally on both sides, demonstrating that the control device, without the silicone bristles, did not prevent the group from moving through the entire available space in the tank. However, in the treatment with TS, the fish stayed longer on one side of the fish tank than on the other. This could have happened because the TS device with silicone bristles could have functioned as a visual barrier, which would make it difficult to see the other side and could make the fish move less. However, when analyzing the ranks we observed that only the gamma and delta ranks remained longer on one side of the tank, while the alpha and beta ranks remained equally on both sides. Dominant animals tend to be more active than submissive animals, as they have priority access to environmental resources (Ang, Manica, 2010), which explains the alpha and beta ranks remain on both sides, due to greater movement, and also gamma and delta ranks remain more on a single side, due to moving

less. Besides, the delta rank crossed more through the TS device than beta, which does not indicate that a lower rank has moved less than a higher rank. Also, the gamma and delta ranks may have moved less as they need to flee less from the confrontations coming from the higher ranks. This is in accordance with the alpha rank of the treatment with TS showing less aggressive interaction.

In the treatment with TS, the alpha and beta ranks remain equally on the sides of the tank, which suggests that the TS device with silicone bristles did not act as a visual barrier, isolating the fish on one side, which could have directly influenced the dynamics of aggressive interactions in the social group. For the dominance hierarchy to be established and maintained, fish must recognize visual (Castro et al., 2009) and chemical (Keller-Costa; Canário, Hubbard, 2015) cues to emit aggressive confrontations. Thus, we infer that TS specifically had an effect on decreasing aggressiveness.

SGR

The SGR indicates the growth per day (Crane, Ogle, Shoup, 2019), and then it can be used as an additional welfare indicator. We expected that fish in the TS treatment would show a higher growth rate, as a consequence of less stress, less aggressiveness and better welfare conditions reached by the TS. In fact, fish from the TS treatment had a higher SGR rate than fish from treatment without TS. This is in accordance with alpha rank in the TS treatment showing less aggressiveness. This was expected, as in conditions where there is lower frequency of aggressive interaction, individuals have a greater amount of energy available for their growth (Johnsson, Winberg, Sloman, 2006). In addition, a great energy input is necessary to maintain the dominance hierarchy for Nile tilapia (Alvarenga, Volpato, 1995), which can make the energy not available for other activities such as growth, for example.

In our study, we have to consider that the SGR was negative in all ranks in treatment without TS and for gamma and delta ranks in treatment with TS, meaning weight loss. For alpha and beta ranks the SGR was positive, indicating weight gain. This indicates that, even though aggressive interactions were reduced by TS, it was still costly for the group. Thus, although we used the amount of feed commonly offered to Nile tilapia (i.e. 3% of biomass / day, according to Long et al., 2015; Fattah et al., 2021), this amount of food seems to have been insufficient to provide energy needs. On the other hand, alpha and beta rank animals under TS showed less growth and, as they maintain a higher SGR, they are possibly under better welfare conditions.

Plasma Cortisol

We expected that fish in the TS treatment would present a lower level of stress (cortisol), as demonstrated by Soares et al (2011) for the marine fish *Ctenochaetus striatus*. But we did not observe any effect of TS on the cortisol of grouped Nile tilapia. As observed in other studies, TS can reduce stress in humans (Field et al., 2005), cattle (Probst et al., 2012) and marine fish (Soares et al., 2011), and this effect has been demonstrated by decreasing cortisol levels. In Nile tilapia dominance hierarchy, the dominant fish has a higher level of stress, indicated by the higher level of cortisol (Boscolo, Morais, Gonçalves-de-Freitas, 2011; Barreto, Boscolo, Gonçalves-de-Freitas, 2015), so we expected that the TS could act reducing the stress of the dominant fish, as occurs in client fish *C. striatus* (Soares et al., 2011) and in several mammals (e.g. Field et al., 2005; Probst et al., 2012). However, fish plasma cortisol levels were similar between treatments with and without TS.

Although cortisol is one of the main indicators of stress, it is involved with other functions such as the immune response, inflammatory and functions that require metabolic changes (Mommsen, Vijayan, Moon, 1999). Bolognesi, Gauy and Gonçalves-de-Freitas (2019) argue that a greater number of physiological indicators, in addition to gene expression in certain brain areas, may be necessary to assess stress in a broader sense. Furthermore, analyzing cortisol alone may limit the understanding of the complex scenario related to the stress response in relation to TS in Nile tilapia (Bolognesi, Gauy, Gonçalves-de-Freitas, 2019).

Based on different approaches to accessing welfare (Fraser et al 1997; Huntingford et al 2012), the functioning approach assumes that biological functions must be adequate, thus, we assume that high levels of cortisol would indicate a disturbance in physiological function. However, Ellis et al (2012) argue that what is important in the functioning approach is whether or not the animal is managing to deal with changes in its environment and that the cortisol response, by itself, does not represent the animal's ability in dealing with these changes. Martins et al (2012) mentions that the variation in cortisol is not only related to the social position that the individual occupies, but to the stability of the social hierarchy. For example, Fox et al (1999) demonstrated that cortisol levels are dependent on social position as well as social stability in the cichlid *Haplochromis burtoni*.

It has been observed that TS can elevate serotonin levels in the human brain (Field et al., 2005). Elevated serotonin reduces aggressive behavior in lizards (Larson, Summers,

2001), mammals (Edwards, Kavitz, 1997) and salmonid fish (Winberg, Tornqvist, 2016). Thus, TS could increase the level of serotonin in fish, reducing certain aggressive behaviors, however, this mechanism remains to be tested.

Conclusions

The TS device was an effective way to provide TS to grouped fish. The TS device may have caused some resistance to the fish passage, even if they managed to cross it, and could interfere in fish locomotion. TS may have decreased locomotion in the gamma and delta ranks, which may suggest less escape as a result of fewer confrontations received by the higher ranks.

We did observe effects of TS on aggressiveness and growth, although we did not observe change in plasma cortisol levels in grouped Nile tilapia. Reducing aggressiveness in social species in captivity is a way to promote better levels of welfare (Gonçalves-de-Freitas et al., 2019). The less growth demonstrated by SGR is also a way to infer better level of welfare, considering growth as a welfare indicator.

We emphasize that the fish remained in contact with TS for nine days. Thus, studies with access to long-term TS are needed to understand how it acts in fish and its possible benefits. Recently Cohen et al (2020) commented that fish mechanoreception could be more complex than we thought, this open pathways for further studies regarding tactile stimulation and its effect on fish.

We conclude that TS reduces aggressiveness, with an evident effect on the dominant fish, reduces growth and has no effect on cortisol levels in Nile tilapia groups. Thus, the positive effects of TS can contribute to the increase animal welfare in this species.

References

ALVARENGA, CMD; VOLPATO, GL. Agonistic profile and metabolism in alevins of the Nile tilapia. **Physiology & Behavior**, v. 57, n. 1, p. 75-80, 1995. DOI: https://doi.org/10.1016/0031-9384(94)00206-K

ANG, TZ; MANICA, A. Unavoidable limits on group size in a body size-based linear hierarchy. **Behavior Ecology**, v. 21, p. 819–825, 2010. DOI: https://doi.org/10.1093/beheco/arq062

ASHLEY, PJ. Fish welfare: current issues in aquaculture. **Applied Animal Behaviour Science**, v. 104, n. 3-4, p. 199-235, 2007. DOI: https://doi.org/10.1016/j.applanim.2006.09.001

ASHLEY, PJ; SNEDDON, LU. Pain and fear in fish. In: Branson, EJ (Ed). **Fish Welfare**. Oxford, Blackwell Publishing Ltd, 2008. p. 49– 77.

BARTON, BA; MORGAN, JD. VIJAYAN MM. Physiological and condition-related indicators of environmental stress in fish. In: Adams, SM (Ed). **Biological Indicators of Aquatic Ecosystem Stress.** American Fisheries Society: 2002. p. 111-148.

BOLOGNESI, MC; GAUY, ACS; GONÇALVES-DE-FREITAS, E. Tactile stimulation reduces aggressiveness but does not lower stress in a territorial fish. **Scientific reports**, v. 9, n. 1, p. 1-10, 2019. DOI: https://doi.org/10.1038/s41598-018-36876-1

BOSCOLO, CNP; MORAIS, RN; GONCALVES-DE-FREITAS, E. Same-sized fish groups increase aggressive interaction of sex-reversed males Nile tilapia GIFT strain. **Applied Animal Behaviour Science**, v. 135, n. 1-2, p. 154-159, 2011. DOI: https://doi.org/10.1016/j.applanim.2011.10.003

BRANDÃO, ML; BRAITHWAITE, VA; GONÇALVES-DE-FREITAS, E. Isolation impairs cognition in a social fish. **Applied Animal Behaviour Science**, v. 171, p. 204-210, 2015. DOI: https://doi.org/10.1016/j.applanim.2015.08.026

BSHARY, R; WÜRTH, M. Cleaner fish Labroides dimidiatus manipulate client reef fish by providing tactile stimulation. **Proceedings of the Royal Society of London. Series B: Biological Sciences**, v. 268, n. 1475, p. 1495-1501, 2001. DOI: https://doi.org/10.1098/rspb.2001.1495

BUTLER JM; MARUSKA, KP. Mechanosensory signaling as a potential mode of communication during social interactions in fishes. **Journal of Experimental Biology**, v. 219, p. 2781-2789, 2016a. DOI: 10.1242/jeb.133801. PMID: 27655819.

BUTLER JM; MARUSKA, KP. The Mechanosensory Lateral Line System Mediates Activation of Socially-Relevant Brain Regions during Territorial Interactions. **Frontiers in Behavioral Neuroscience**, v. 13, p. 01-18, 2016b. DOI: 10.3389/fnbeh.2016.00093. PMID: 27242462.

CARVALHO, TB; GONÇALVES-DE-FREITAS, E. Sex group composition, social interaction, and metabolism in the fish Nile tilapia. **Brazilian Journal of Biology**, v. 68, n. 4, p. 807-812, 2008. DOI: 10.1590/s1519-69842008000400015

CASTRO, ALS et al. Visual communication stimulates reproduction in Nile tilapia, Oreochromis niloticus (L.). **Brazilian Journal of Medical and Biological Research**, v. 42, n. 4, p. 368-374, 2009. DOI: 10.1590/s0100-879x2009000400009

COHEN, KE; FLAMMANG, BE; CRAWFORD, CH; HERNANDEZ, LP. Knowing when to stick: touch receptors found in the remora adhesive disc. **Royal Society Open Science**, v. 7, p. 190990, 2020. DOI: 10.1098/rsos.190990. PMID: 32218935.

COOMBS, S. Nearfield detection of dipole sources by the goldfish (Carassius auratus) and the mottled sculpin (Cottus bairdi). **Journal of Experimental Biology**, v. 190, p. 109-129, 1994. DOI: https://doi.org/10.1242/jeb.190.1.109

DIJKGRAAF, S. The functioning and significance of the lateral-line organs. Biological reviews of the Cambridge Philosophical Society, v. 38, p. 51-105, 1963. DOI: 10.1111/j.1469-185x.1963.tb00654.x

EDWARDS, DH; KRAVITZ, EA. Serotonin, social status and aggression. **Current Opinion in Neurobiology**, v. 7, p. 812-819, 1997. DOI: https://doi.org/10.1016/S0959-4388(97)80140-7

ELLIS, T et al. Cortisol and finfish welfare. **Fish Physiology and Biochemistry**, v. 38, n. 1, p. 163-188, 2012. DOI: https://doi.org/10.1007/s10695-011-9568-y

FATTAH, AFA; AHMED, FA; SAID, EN; FARAG, MR. Impact of feeding system on the behaviour and performance of Nile tilapia (Oreochromis niloticus), Aquaculture. v. 538, p. 736514, 2021. DOI: https://doi.org/10.1016/j.aquaculture.2021.736514.

FIELD, T et al. Cortisol decreases and serotonin and dopamine increase following massage therapy. **International Journal of neuroscience**, v. 115, n. 10, p. 1397-1413, 2005. DOI: https://doi.org/10.1080/00207450590956459

FOX, HE et al. Stress and dominance in a social fish. **Journal of Neuroscience**, v. 17, n. 16, p. 6463-6469, 1997. DOI: https://doi.org/10.1523/JNEUROSCI.17-16-06463

FRASER D et al. A scientific conception of animal welfare that reflects ethical concerns. **Animal Welfare**, v. 6, p. 187-205, 1997.

GAUY, ACS; BOLOGNESI, MC; MARTINS, GD; GONÇALVES-DE-FREITAS, E. Preference and motivation tests for body tactile stimulation in fish. **Animals**, 11, 2021. DOI: https://doi.org/10.3390/ani11072042

GAUY, ACS; BOSCOLO, CNP; GONÇALVES-DE-FREITAS, E. Less water renewal reduces effects on social aggression of the cichlid Pterophyllum scalare. **Applied Animal Behaviour Science**, v. 198, p. 121-126, 2018. DOI: https://doi.org/10.1016/j.applanim.2017.10.003

GILMOUR, KM; DIBATTISTA, JD; THOMAS, JB. Physiological causes and consequences of social status in salmonid fish. **Integrative and Comparative Biology**, v. 45, n. 2, p. 263-273, 2005. DOI: https://doi.org/10.1093/icb/45.2.263

GÓMEZ-LAPLAZA, LM; MORGAN, E. The influence of social rank in the angelfish, Pterophyllum scalare, on locomotor and feeding activities in a novel environment. **Laboratory Animals**, v. 37, n. 2, p. 108-120, 2003. DOI: https://doi.org/10.1258/00236770360563741

GONÇALVES-DE-FREITAS, E et al. Effect of water renewal on dominance hierarchy of juvenile Nile tilapia. **Applied Animal Behaviour Science**, v. 112, n. 1-2, p. 187-195, 2008. DOI: https://doi.org/10.1016/j.applanim.2007.07.002

GONÇALVES-DE-FREITAS, E et al. Social behavior and welfare in Nile tilapia. **Fishes**, v. 4, n. 2, p. 23, 2019. DOI: https://doi.org/10.3390/fishes4020023

GUIDELINES FOR THE TREATMENT OF ANIMALS IN BEHAVIOURAL RESEARCH AND TEACHING, **Animal Behaviour**, v. 159, p. I-IX, 2020. DOI: https://doi.org/10.1016/j.anbehav.2019.11.002.

HERNANDEZ-REIF, M; DIEGO, M; FIELD, T. Preterm infants show reduced stress behaviors and activity after 5 days of massage therapy. **Infant Behavior and Development**, v. 30, n. 4, p. 557-561, 2007. DOI: https://doi.org/10.1016/j.infbeh.2007.04.002

HUNTINGFORD, FA et al. Current issues in fish welfare. **Journal of fish Biology**, v. 68, n. 2, p. 332-372, 2006. DOI: https://doi.org/10.1111/j.0022-1112.2006.001046.x

HUNTINGFORD, FA. Fish Behaviour: Determinants and Implications for Welfare. In: Kristiansen, TS, Ferno, A, Pavlidis, MA, van de Vis, H. (Eds), **The Wealfare of Fish**. Springer: 2020. p. 73-110.

HUNTINGFORD, FA. The physiology of fish behaviour: a selective review of developments over the past 40 years. **Journal of Fish Biology**, v. 81, n. 7, p. 2103-2126, 2012. DOI: https://doi.org/10.1111/j.1095-8649.2012.03480.x

IMANAKA, A et al. Neonatal tactile stimulation reverses the effect of neonatal isolation on open-field and anxiety-like behavior, and pain sensitivity in male and female adult Sprague–Dawley rats. **Behavioural Brain Research**, v. 186, n. 1, p. 91-97, 2008. DOI: https://doi.org/10.1016/j.bbr.2007.07.039

JOHNSSON, JI; WINBERG, S; SLOMAN, KA. Social interactions. In: Sloman, KA, Wilson, RW, Balshine, S. (Eds), **Behaviour and Physiology of Fish**. San Diego, Academic Press, 2006. p. 151–196.

KAZUMYAN, AO. Tactile reception and behavior of fish. **Journal of Ichthyology**, v. 51, p. 1035–1103, 2011. DOI: https://doi.org/10.1134/S003294521111004X

KELLER-COSTA, T; CANÁRIO, AVM; HUBBARD, PC. Chemical communication in cichlids: a mini-review. **General and Comparative Endocrinology**, v. 221, p. 64-74, 2015. DOI: https://doi.org/10.1016/j.ygcen.2015.01.001

LARSON, ET; SUMMERS, CH. Serotonin reverses dominant social status. **Behavioural Brain Research**, v. 121, p. 95-102, 2001. DOI: https://doi.org/10.1016/S0166-4328(00)00393-4

LONG, L; YANG, J; LI, Y; GUAN, C; WU, F. Effect of biofloc technology on growth, digestive enzyme activity, hematology, and immune response of genetically improved farmed tilapia (Oreochromis niloticus). **Aquaculture**. V. 448, p. 135-141. 2015. DOI: https://doi.org/10.1016/j.aquaculture.2015.05.017

MARTINS, CIM et al. Behavioural indicators of welfare in farmed fish. Fish **Physiology and Biochemistry**, v. 38, n. 1, p. 17-41, 2012. DOI: https://doi.org/10.1007/s10695-011-9518-8

MCCONNACHIE, E et al. Cows are highly motivated to access a grooming substrate. **Biology Letters**, v. 14, n. 8, 2018. DOI: https://doi.org/10.1098/rsbl.2018.0303

MOMMSEN, TP. VIJAYAN, MM; MOON, TW. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. **Reviews in Fish Biology and Fisheries**, v. 9, n. 3, p. 211-268, 1999. DOI: https://doi.org/10.1023/A:1008924418720

NOLETO-FILHO, EM et al. Bayesian analysis improves experimental studies about temporal patterning of aggression in fish. **Behavioural Processes**, v. 145, p. 18-26, 2017. DOI: https://doi.org/10.1016/j.beproc.2017.09.017

POTTINGER, T. The Stress Response. in Fish: Mechanisms, Effects and Measurement. In: Branson, EJ (Ed). **Fish Welfare**. Oxford, Blackwell Publishing Ltd, 2008. p. 32-48.

PROBST, JK et al. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. **Applied Animal Behaviour Science**, v. 139, n. 1-2, p. 42-49, 2012. DOI: https://doi.org/10.1016/j.applanim.2012.03.002

ROS, AFH; BECKER, K; OLIVEIRA, RF. Aggressive behaviour and energy metabolism in a cichlid fish, Oreochromis mossambicus. **Physiology & Behaviour**, v. 89, p. 164–170, 2006. DOI: https://doi.org/10.1016/j.physbeh.2006.05.043

SCHMIED, C et al. Effect of previous stroking on reactions to a veterinary procedure: Behaviour and heart rate of dairy cows. **Interaction studies**, v. 11, n. 3, p. 467-481, 2010. DOI: https://doi.org/10.1075/is.11.3.08sch

SHAHIN, M. The effects of positive human contact by tactile stimulation on dairy cows with different personalities. **Applied Animal Behaviour Science**, v. 204, p. 23-28, 2018. DOI: https://doi.org/10.1016/j.applanim.2018.04.004

SLOMAN, KA et al. Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. **Physiological and Biochemical Zoology**, v. 74, n. 3, p. 383-389, 2001. DOI: https://doi.org/10.1086/320426

SNEDDON, LU; BRAITHWAITE, VA; GENTLE, MJ. Novel object test: examining nociception and fear in the rainbow trout. **The Journal of Pain**, v. 4, n. 8, p. 431-440, 2003. DOI: https://doi.org/10.1067/S1526-5900(03)00717-X

SOARES, MC et al. Tactile stimulation lowers stress in fish. **Nature Communications**, v. 2, n. 1, p. 1-5, 2011. DOI: https://doi.org/10.1038/ncomms1547

SØRENSEN, C et al. Social stress reduces forebrain cell proliferation in rainbow trout (Oncorhynchus mykiss). **Behavioural Brain Research**, v. 227, n. 2, p. 311-318, 2012. DOI: https://doi.org/10.1016/j.bbr.2011.01.041

VERA-CRUZ, EM; BROWN, CL. The influence of social status on the rate of growth, eye color pattern and insulin-like growth factor-I gene expression in Nile tilapia, Oreochromis

niloticus. **Hormones and Behavior**, v. 51, n. 5, p. 611-619, 2007. DOI: https://doi.org/10.1016/j.yhbeh.2007.02.010

VOLPATO, GL; BARRETO, RE. Environmental blue light prevents stress in the fish Nile tilapia. **Brazilian Journal of Medical and Biological Research**, v. 34, p. 1041-1045, 2001. DOI: https://doi.org/10.1590/S0100-879X2001000800011

WINBERG, S; THÖRNQVIST, P-O. Role of brain serotonin in modulating fish behavior. **Current Zoology**, v. 62, n. 3, p. 317-323, 2016. DOI: https://doi.org/10.1093/cz/zow037

CHAPTER III

Tactile stimulation improves learning ability in cichlid fish

Marcela Cesar Bolognesi[1,2], Ana Carolina dos Santos Gauy[1,2], Sara Nicolau Flores[2], Eliane Gonçalves-de-Freitas[1,2]

[1] Centro de Aquicultura da Unesp - UNESP. Universidade Estadual Paulista. Via de Acesso Prof. Paulo Donato Castellane s/n, 14884-900, Jaboticabal/SP, Brasil.

[2] Instituto de Biociências, Letras e Ciências Exatas – UNESP. Universidade Estadual Paulista. Rua Cristóvão Colombo 2265, 15055-000, São José do Rio Preto/SP, Brasil.

Abstract

Positive conditions are associated with better learning ability in vertebrates. One way to provide positive conditions is by the body tactile stimulation (TS), a type of sensory enrichment associated with relaxing conditions that can reduce stress and aggressiveness in fish and also increase memory capacity in mammals. So we tested, for the first time, the effect of this stimulus on the cognitive ability of fish. Male Nile tilapias were subjected to TS by using a device made with silicone bristles, through which the fish pass and receive physical stimulation. Isolated individuals were submitted to two treatments: with TS and without TS for seven days. On days 8 and 9 they underwent a detour reaching test that measures learning and inhibitory control, components of cognitive ability. In phase 1 of the test, the fish needed to learn to detour an opaque barrier to reach food (reward). In phase 2, the opaque barrier was replaced by a transparent one to test whether the fish would inhibit the impulse to go directly to the food, and remember to make the detour to reach the reward. In phase 1, 60.9% of TS + fish learned to detour in the opaque barrier, while only 32% of TS - fish learned. In phase 2, fish from both treatments that learned in the opaque barrier were tested in the transparent barrier. All showed inhibitory control, but TS + fish were faster and made fewer mistakes to perform the detour. We conclude that TS improves fish learning and has a slight effect on cognitive flexibility, therefore, being a way to counteract detrimental effects of artificial environment on fish cognition.

Key-words: inhibitory control, cognitive flexibility, detour reaching test.

Introduction

Cognitive ability is a term that encompasses various abilities, such as learning, memory, judgment, decision making, cognitive flexibility and self-recognition. Stressful and impoverished environments can impair these abilities (Näslund, Johnsson, 2016). On the other hand, enriched environments can reduce the detrimental effects on vertebrate cognition (e.g. Salvanes et al., 2013) and bring positive aspects on animal welfare. According to Kotrschal and Taborsky (2010) adding structures to animals' environments is one way to stimulate cognitive development. For example, zebrafish raised in an enriched environment (with artificial plants and gravel) showed greater proliferation of cells in the telencephalon, the cognitive center region (von Krogh et al., 2010). Atlantic salmon raised in a structure enriched environment (with pebbles, cobbles and vertically floating plastic structures) showed increased neurogenesis and better learning (Salvanes et al., 2013). Thus, structural environmental enrichment can improve cognitive ability by improving learning and adjustment to new conditions (Näslund, Johnsson, 2016). This behavior adjustment in response to environmental new conditions is called cognitive flexibility (Highgate, Schenk 2020).

Tactile stimulation (TS) is a type of sensory enrichment made on an animal's body, which can generate positive effects on the organism. For example, massage has a positive effect on humans, by reducing stress, measured by cortisol levels (Field et al., 2005). Farm mammals such as cows (Schmied et al., 2010), piglets (Tallet et al., 2014) and lambs (Coulon et al., 2015) have been found to lower their heart rate when petted by their caretakers, an effect that is related to states of calm. Cognitive effects of TS were observed by De Los Angeles et al (2016) and Antoniazzi et al (2017), who documented an improvement in memorization capacity in newborn rats that received TS. Positive effects of TS have also been observed in fish. Soares et al (2011) demonstrated a decrease in cortisol in the striated surgeon fish, *Ctenochaetus striatus*, which had access to artificial TS, and Bolognesi, Gauy and Gonçalves-de-Freitas (2019) observed a reduction in aggressiveness in TS treated Nile tilapia. Gauy et al (2021) found that TS may have a positive meaning for Nile tilapia, since fish are motivated to access TS and face an aversive area to reach it, even after stressful stimulus. Nonetheless, there are no studies regarding the potential positive association of TS and cognitive ability in fish.

According to Brown (2015) fish form long-term memories, show social learning and use tools. Fish also have a complex nervous system and complex cognitive ability (Braithwaite, 2006). Given this context, we here hypothesize that TS might improve fish

cognitive abilities. We test this hypothesis with the cichlid Nile tilapia, *Oreochromis niloticus*, a species widely used in aquaculture worldwide (FAO, 2017) and which is usually subjected to stressful and stimuli deprived environments (Ashley, 2007). In a prior test of TS on aggressive behavior in this cichlid, Bolognesi, Gauy and Gonçalves-de-Freitas (2019) developed a TS device made with silicone bristles, through which the fish passes spontaneously, thus receiving body TS, the authors found that TS decreases aggressive interactions in pair staged fights of fish. Furthermore, Gauy et al (2021) demonstrated that Nile tilapia spontaneously seek for TS and are motivated to guarantee access to TS, showing the positive meaning of TS for this species. Therefore, Nile tilapia appears to be a key species to test effects of TS enrichment on cognitive ability and flexibility.

One test used to measure cognitive ability is the detour reaching test. For example, we can use a transparent barrier containing the food visible to the animal, the animal must inhibit the impulse to go directly to the food and make a detour to reach it. In the first attempts, the animal may not perform the detour and hit the barrier (Kralik, Hauser, Zimlicki, 2002). An opaque barrier can also be used initially to teach the animal to detour (e.g. Kralik, Hauser, Zimlicki, 2002). The detour reaching test allows measuring different cognitive abilities such as motor capacity, inhibitory control, insight, learning, social learning and memory (Kabadayi, Bobrowicz, Osvath, 2018). Inhibitory control works by blocking impulsive acts and seeking an alternative response. Repeated testing trials can be used to quantify learning processes in terms of decreases in the latency to perform the detour, and decreases in the number of errors (Kabadayi, Bobrowicz, Osvath, 2018). This test is widely used to measure cognitive ability in primates (e.g. Kralik, Hauser, Zimlicki, 2002), birds (e.g. Booguert et al., 2011; Vernouillet et al., 2016), and has also been used more recently in fish. Lucon-Xicatto, Gatto and Bisazza (2017) tested guppies (*Poecilia reticulata*) in an inhibitory control task. The guppies learned to make a detour to reach the food in an opaque cylinder, performed the detour correctly in the transparent cylinder and decreased the time to perform the detour, demonstrating learning and inhibitory control. Sovrano, Baratti and Potrich (2018) observed that the fishes *Danio rerio*, *Xenotoca eiseni*, *Carassius auratus*, and *Pterophyllum scalare* detoured barriers to approach conspecifics (reward). Brandão, de Almeida-Fernandes and Gonçalves-de-Freitas (2019) showed that male and female Nile tilapia learned to detour an opaque barrier, but females performed the detour more quickly in the transparent barrier, showing better performance in this task

than males. Here we tested for the first time, the effect of TS on cognition by using the detour reaching paradigm. Following our hypothesis, we predict that TS treated fish (TS +) will prove more proficient at the detour reaching task than non-treated (TS -) fish.

Methods

Subjects and Housing

We used 48 sex reverted adult males of Nile tilapia, *O. niloticus* (GIFT – Genetically Improved Farmed Tilapia variety) from a commercial supplier. The fish were acclimated for at least 15 days in blue polypropylene water tanks (capacity 500 L, 1 fish / 10 L). The water temperature was maintained at 27 º C by heaters and thermostats, the photoperiod was 12 h of light (7:00 a. m. - 19:00 p. m.) controlled by timer. Fish were fed with tropical fish food (28 % crude protein; to apparent satiety). Water quality was maintained using Canister biological filters (400L / h filtration) with water tanks siphoned to remove waste. The TS + fish presented mean ± standard error of 15.52 ± 0.24 cm in length and 114.80 ± 4.73 g, and TS – fish presented mean ± standard error of 16.33 ± 0.29 cm in length and 138.17 ± 6.65 g (Independent t test, $p = 0.12$).

Tactile Stimulation (TS) device

To provide TS in our experimental treatment, we constructed a rectangular PVC device (40 x 30 cm) with plastic sticks containing silicone bristles against which fish could swim and receive stimulation (Figure 1A). As a control, we constructed a similar device but without the silicone bristles (Figure 1B). Both devices were positioned in the center of fish tanks, so that fish swimming from one side of the tank to the other would pass through (Figure 1C).

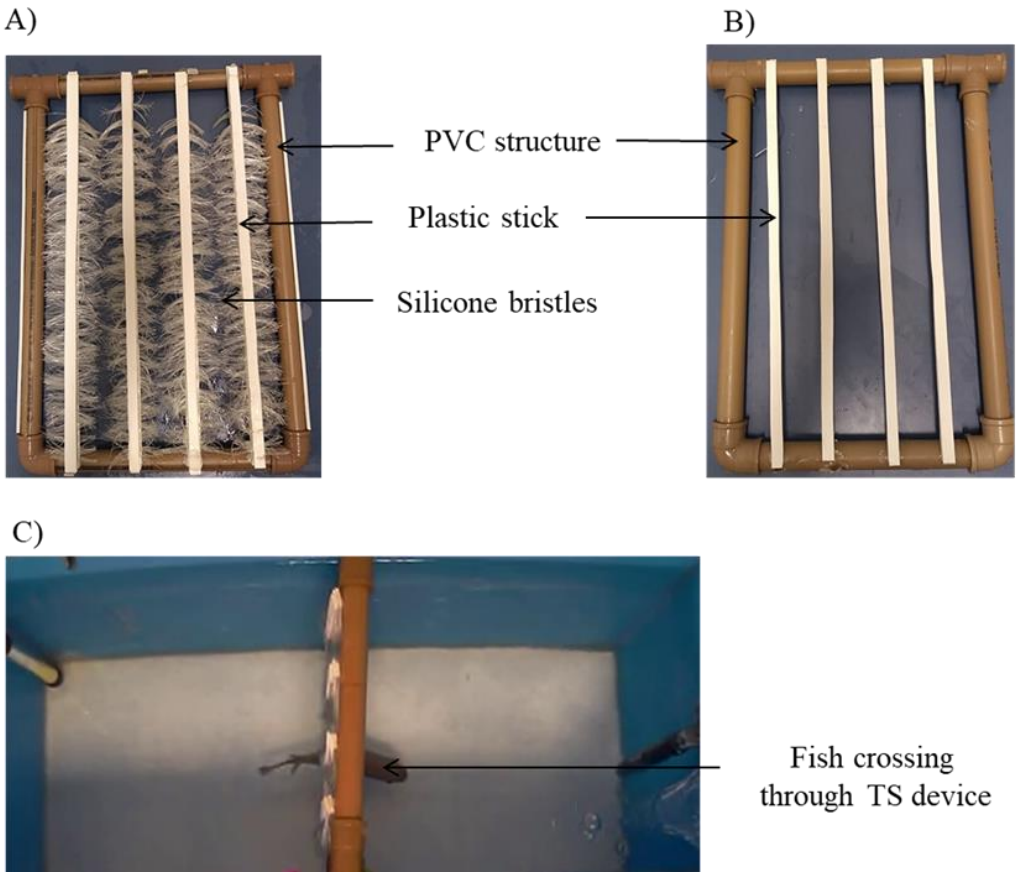

Figure 1. Tactile stimulation device. A) Device showing the rectangular PVC structure (40 x 30 cm) with plastic sticks with bristles each side. B) The similar device without bristles, used as a control. C) View from above the fish tank, with a fish receiving tactile stimulation as it swims through the TS device.

Feed habituation

Before the experimental design, fish were grouped together for two days in glass fish tanks (10 fish per tank, 60 x 60 x 60 cm, 216 L capacity, covered with blue plastic) to habituate to eat from a feeder, a structure that keeps the food (a sall piece of sausage) attached to one tank wall. The fish were fed eight times a day, which provides enough time for them to learn to feed from the feeder (Bolognesi, Gauy, Gonçalves-de-Freitas, 2019).

Experimental design

The fish were allocated randomly to two treatments: TS + (23 replicates / fish) and TS - (25 replicates / fish). Fish were anesthetized (Benzocaine 0.03 g / L) and then measured and weighed. Then, they were isolated for seven days in individual glass fish tanks (120 x 40 x 30 cm; 140 L capacity, covered with blue plastic) containing either the TS device or the control device in the center. Fish were fed once a day using a feeder, that

fixed the food, placed on the opposite side of the fish related to the TS or control device. Thus, the fish needed to cross through the TS device to obtain food. At each feeding session, the fish were filmed for 30 minutes: 10 minutes before feeding, 10 minutes during and 10 minutes after feeding, to tally the number of crossings through the TS or control device. On the 8th and 9th days, they were submitted to the detour reaching test (adapted from Brandão, de Almeida-Fernandes, Gonçalves-de-Freitas, 2019).

Detour reaching test

Phase 1 – Detour training: On the 8th day of the experiment, the TS device or control was removed from the fish tank. We then presented a black opaque U-shaped barrier with food attached behind it (Figure 2A) and conducted a series of trials. At the start of each trial, the fish was confined temporarily to one of the sides of the tank using a plastic plate, thus standardizing the location of fish at the trail start. Then the opaque barrier with food was introduced to the other side of the tank (Figure 2A). The task for the fish, once released, was to learn to detour the barrier to find the food.

For each fish we ran 25 consecutive trials, divided into 5 test blocks, with 5 trials each. Each trial lasted 10 minutes. The learning criterion used for advancing to the next experimental phase was 4 successful trials in 5, within one block at least (Figure 2B). All trials were filmed to review the detour outcomes from each fish.

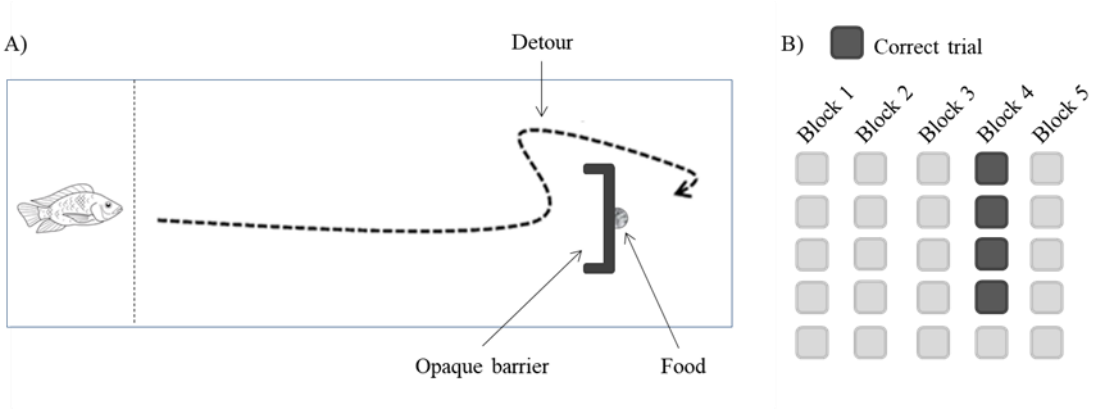

Figure 2. A) Detour reaching set up. Schematic view of a fish tank containing the fish temporarily confined on one side (15 x 40 x 30 cm, 18 L capacity), and the opaque barrier with food behind it on the opposite side. Dashed line corresponds to the trajectory that the fish must learn to obtain food, which is as a detour around the barrier. B) Scheme of a random example of the learning criterion fulfilled.

Phase 2 – Detour test: In phase 2 we used again a U-shaped barrier with food attached behind it, but in this case the barrier was transparent glass, so that fish could see the food before routing around the detour. On the 9th day we repeated the 25 trials per fish: the fish was again confined temporarily in one side of the tank using a plastic plate, and again the barrier plus food was introduced in the other side of the tank. The fish was then released; the task in this case was to remember that it had to detour the barrier to find the food. As with phase 1, there were 25 trials divided into 5 test blocks, with 5 trials each, and each trial lasting 10 minutes. All trials were filmed for later review of trial outcomes for each fish, latency to perform the detour, and number of hits on the barrier (errors). The latency to perform the detour was counted in seconds from the moment the fish was released until the moment the detour was performed (swam around the barrier). The number of hits on the barrier corresponds to the number of times the fish touched the transparent barrier, without making the detour.

Data analysis

Data were analyzed in Statistica (StatSoft). Data were assessed for normality by Kolmogorov-Smirnov and for homoscedasticity by the Fmax test. Analysis of the number of crossings by the TS device (or control) were performed with the period without food (20 minutes of observations per day). The number of crossings through TS device over the days was analyzed between treatments with and without TS by General Linear Models (GLM).

In phase 1, we quantified the number of fish that performed the detour in each treatment. These percentages were analyzed by Goodman's Binomial. In phase 2, we quantified for both treatments: the number of successful trials, the latency to perform the detour and the number of hits in the barrier before performing the detour. These variables were analyzed by GLM and Tuckey post hoc. The treatments were categorical variables and the five test blocks, repeated variables. The first and fifth test blocks were contrasted by planned comparisons. When necessary, the data were transformed to achieve normality and homoscedastic criteria (the data on the percentage of fish that performed the detour were transformed by 2*arcosen.$\sqrt{(x)}$; the latency data to perform the detour were transformed by log; and the data on the number of hits in the barrier were transformed by $\sqrt{(x+0.5)}$. We used a threshold of $p < 0.05$ for inferences about statistical significance.

Ethics approval

All procedures followed the ethical principles of the National Council for the Control of Animal Experiments (CONCEA). This study was approved by the Ethics Committee on the Use of Animals of the Instituto de Biociências, Letras e Ciências Exatas, UNESP, São José do Rio Preto (CEUA-IBILCE/UNESP), protocol nº 201/2019.

Results

Crossings through the TS or control device - Comparison between treatments

We did not observe any significant difference between the TS + and TS - treatments in the number of crossings through the device (GLM, $F_{(1,46)} = 0.28$, $p = 0.59$).

Phase 1 – Detour training

According to the learning criterion, we observed that 60.83% of TS + fish learned to detour, as compared to 32% of TS - fish (significant difference by Goodman's Binomial, alpha < 0.05).

Phase 2 – Detour test

Percentage of fish that performed the detour: We did not observe statistical differences across treatments in the percentage of fish that successfully performed the detour (GLM, $p > 0.10$, Figure 3A). We did observe learning in both treatments: in both cases, the percentage of fish that succeeded with the detour task increased over time in the comparison between the first and fifth test blocks (Planned comparisons, $F_{(1,19)} = 9.37$, $p = 0.006$, Figure 3B).

Latency to perform the detour: TS + fish detoured faster than did TS - fish in the second test block (GLM, $p < 0.048$, Figure 3C). In both treatments, we again found evidence for learning: we observed a decrease in latency to perform the detour between the first and fifth test blocks (Planned comparisons, $F_{(1,19)} = 81.62$, $p = 0.0000001$, Figure 3D).

Number of hits in the barrier: The number of hits in the barrier decreased from the second test block for both treatments (GLM, $p < 0.03$, Figure 3E). It also decreased from the first to the fifth test block (Planned comparisons, $F_{(1,19)} = 93.83$, $p = 0.00000001$; Figure 3F) in both treatments.

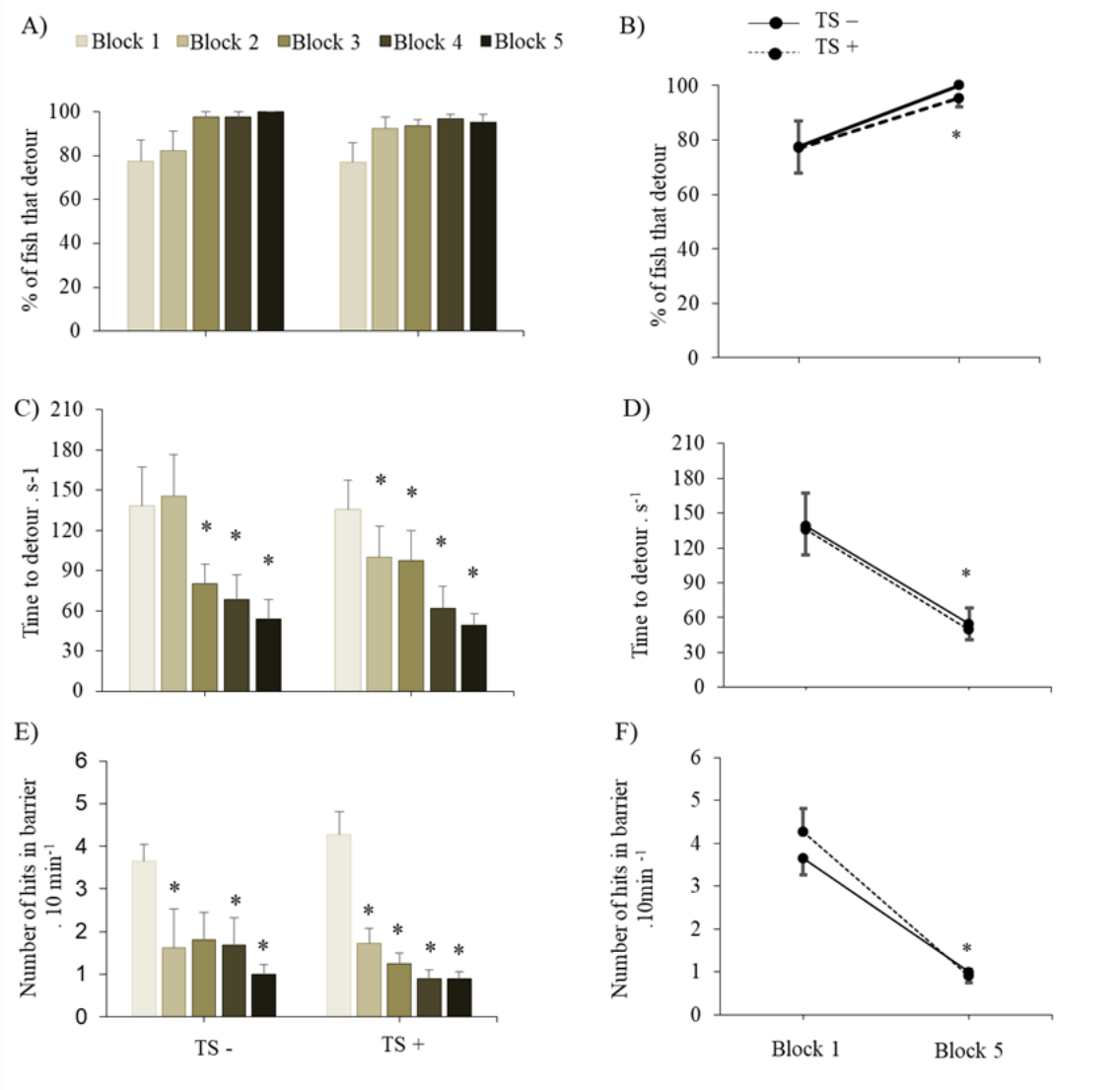

Figure 3. Fish performance on Phase 2 – Detour test. Percentage of fish that successfully detoured the barrier (A, B), latency to detour (C, D), and number of hits in the barrier (E, F). Each block equals 5 trials (25 in total). In A, B C asterisks indicate significant differences among block 1 and other blocks, GLM. In B, D, F there are the contrast between block 1 and block 5 by planned comparisons, asterisks indicate significant differences among block 1 and block 5 in both treatments. Data are shown as mean ± standard error.

Discussion

We tested the cognitive ability of male Nile tilapia that had or had not access to TS using the detour reaching test. We observed a slight effect of TS on cognitive flexibility and a strong effect on learning showing a positive effect of TS on fish cognitive skills.

TS was already tested in other vertebrate animals and had positive effects on reducing stress (Probst et al., 2012; Soares et al., 2011), behaviors linked to depression and anxiety (Freitas et al., 2015), aggressiveness (Bolognesi, Gauy, Gonçalves-de-Freitas, 2019) and memory (Antoniazzi et al., 2017). As memory is related to learning and other cognitive abilities (Gevins, Smith, 2000), we tested here whether TS would have an effect on the cognitive ability of male Nile tilapia. We used the detour reaching test, which is a way to infer learning and inhibitory control, that infers cognitive flexibility (MacLean et al., 2014; Lucon-Xicatto, Gatto, Bisazza, 2017).

The fish crossed through the TS device or control device, exploring the entire fish tank. The number of crossings through the TS device was similar between the two treatments over the experiment days. This indicates that the device (TS + or TS -) does not reduce fish locomotion or form an obstruction in the available space, as well is not perceived as an aversive object, as observed by Bolognesi, Gauy and Gonçalves-de-Freitas (2019) and by Gauy et al (2021).

Some cognitive functions influence the ability to perform a detour, such as spatial learning, reasoning and motor control (Kabadayi, Bobrowicz, Osvath, 2018). These functions can be investigated by the detour reaching test and its variations (Santacà et al., 2019). Brandão, de Almeida-Fernandes and Gonçalves-de-Freitas (2019) had shown that Nile tilapia has the motor capacity to perform a detour in this kind of test. To perform the detour the fish must move the whole body, which can be difficult compared to barrier tests in which the animal only needs to move a part of the body such as the arms or hands, for example primates (Brandão, de Almeida-Fernandes, Gonçalves-de-Freitas, 2019). The detour reaching test with transparent barriers is used in several vertebrate groups to infer inhibitory control and consequently cognitive flexibility (MacLean et al., 2014; Lucon-Xicatto, Gatto, Bisazza, 2017). However, Vernouillet et al (2016) mentions that there are species that need a training period to learn to detour a barrier. In addition, a transparent barrier represents an object that animals have never come into contact with in nature or rearing condition (Lucon-Xicatto, Gatto, Bisazza, 2017) and its use can increase the animal's difficulty in performing the detour, as noted by Kralik (2005). The opaque barrier allows habituation to a new object in the environment (Kabadayi, Bobrowicz, Osvath, 2018; Brandão, de Almeida-Fernandes, Gonçalves-de-Freitas, 2019) and shows that the animal has the motor capacity to perform a detour (e.g. Brandão, de Almeida-Fernandes, Gonçalves-de-Freitas, 2019). Therefore, we used an opaque barrier in the first test phase.

This phase ensures that the fish learn to detour (Kabadayi, Bobrowicz, Osvath, 2018). In the TS + treatment, 60.86 % of the fish match the criterion of detouring the opaque barrier, which represents almost double the number of fish that learned in the TS - treatment (32 %). Thus, TS + were more successful in performing the detour and, therefore, learned more than TS - fish, which demonstrates a positive effect of TS on learning. Other studies used a similar criterion such as Vernouillet et al (2016), the authors made learning graphic curves using the detour reaching test with a bird, using a criterion of 4 out of 5 successful trials on the opaque barrier and 10 trials on the transparent barrier. The same criterion was used by MacLean et al (2014) in the detour test with mammals and birds to measure self-control.

Having cognitive flexibility, measured here through inhibitory control, can bring advantages to animals. In some moments it is necessary for the animal to change its behavior (by inhibiting it) to deal with a situation that will guarantee its survival (Diamond, 2013). For example, in a natural environment, fish are faced with conditions in which they must avoid an obstacle present in the environment to achieve a certain goal, be it shelter, prey, or even a sexual partner (Lucon-Xicatto, Gatto, Bisazza, 2017). Nile tilapia females exhibit a parental care behavior in which they carry eggs and larvae in their mouths (Falter, 1983) and need to inhibit the behavior of swallowing them (Brandão et al., 2019). Males do not show this behavior, but they need the inhibitory capacity for decision making, to reduce the risk of losing their territory and reproductive site when chasing an intruder male (Brandão, de Almeida-Fernandes, Gonçalves-de-Freitas, 2019). Therefore, the success of social behavior in Nile tilapia should be related with the selection of inhibitory control.

Some studies have shown that individuals who are trained with an opaque barrier tend to do better in the transparent barrier detour test (e.g. Santos, Ericson, Hauser, 1999 with primate; Vernouillet et al., 2016 with bird). Transparent barriers pose a greater challenge than opaque ones because when the reward (food) can be seen, this stimulus predominates over other cues and animals tend to ignore them and go directly to the reward (Diamond, 1990). In this work, all fish that reach the criterion in the phase 1 with the opaque barrier, successfully performed the detour in phase 2 with the transparent one, demonstrating a likely effect of the first phase of the detour test on inhibitory control. It means that the individual probably recalls the detour previously learned and used it when the characteristic of the way to reach the reward changed, consequently showing a cognitive flexibility.

In phase 2, all fish detoured the transparent barrier. We expected that more TS + fish would perform better in inhibitory control than TS - fish, but we saw that the same percentage of fish in both treatments performed the detour with the transparent barrier. This can be explained as they had learned to detour the barrier in the previous phase and remembered the detour in this phase. As fish with better cognitive ability were selected in phase 1 in both treatments, we did not see any difference in phase 2 between TS + ad TS - fish. Testing individuals repeatedly can infer learning, indicated by improved performance over the trials (Kabadayi, Bobrowicz, Osvath, 2018; Gatto, Lucon-Xiccato, Bisazza, 2018; Brandão, de Almeida-Fernandes, Gonçalves-de-Freitas, 2019). Fish from both treatments showed a decrease over time in the latency to perform the detour and in the number of hits on the barrier (errors), which demonstrates that there was also learning in this phase. In addition, TS + fish reduced the latency to perform the detour more quickly, indicating the effect of TS on the readiness of flexibility process.

Environmental enrichment can increase neurogenesis in the hippocampus and is correlated with improved learning and memory (van Praag, Kempermann, Gage, 2000; Veena et al., 2009). Salvanes et al (2013) show that salmon in an enriched environment had better spatial learning and better learning performance. Furthermore, they showed an increase in gene expression related to neurogenesis and indicative of changes associated with learning and memory (Salvanes et al., 2013). Zebrafish reared with enrichment showed faster learning (Spence, Magurran, Smith, 2011). Strand et al (2010) showed that the enriched environment improved social learning in fish. Then, the present study joins others that show that enriched environments can improve learning and enhance cognitive ability in fish.

The mechanism by which TS has an effect on Nile tilapia learning remains to be tested. But we can propose that it would be through stress reduction. This effect of TS was observed in a marine fish (Soares et al., 2011). High levels of cortisol can impair cognitive processes (Law, Clow, 2020), such as memory and attention (Vedhara et al., 2000). Sørensen et al (2011), for example, found that cortisol reduces cell proliferation in the trout telencephalon. The loss of cognitive abilities in stressed animals can interfere with their ability to adjust to the environment (Mendl, 1999). Thus, cortisol reduction through TS could improve learning. However, Bolognesi, Gauy and Gonçalves-de-Freitas (2019) did not observe a reduction in cortisol after a confinement stress situation in Nile tilapia exposed to TS. Another possibility is that TS acts on the gene expression of factors that

favor the appearance of synapses, neuron survival and neurogenesis in the hippocampus, as demonstrated for rats (Antoniazzi et al., 2017). In mammals, the hippocampus is a neural area related to memory, learning, spatial navigation (Bird, Burgess, 2008) and cognitive flexibility (Rubin et al., 2014). This relationship of TS with the hippocampus could be another way through which TS works. In fish, the TS effect could act on structures with similar function to the mammalian hippocampus. This possibility remains to be tested. Overall, we found that TS improves learning and has a slight effect on fish cognitive flexibility. We also conclude that TS has a positive effect on fish cognitive performance, therefore, is a way to counteract detrimental effects of artificial environment on fish cognition.

References

ANTONIAZZI, CTD et al. Tactile stimulation during different developmental periods modifies hippocampal BDNF and GR, affecting memory and behavior in adult rats. **Hippocampus**, v. 27, n. 2, p. 210-220, 2017. DOI: https://doi.org/10.1002/hipo.22686

ASHLEY, PJ. Fish welfare: current issues in aquaculture. **Applied Animal Behaviour Science**, v. 104, n. 3-4, p. 199-235, 2007. DOI: https://doi.org/10.1016/j.applanim.2006.09.001

BIRD, CM; BURGESS, N. The hippocampus and memory: insights from spatial processing. **Nature Reviews Neuroscience**, v. 9, n. 3, p. 182-194, 2008. DOI: https://doi.org/10.1038/nrn2335

BOLOGNESI, MC; GAUY, ACS; GONÇALVES-DE-FREITAS, E. Tactile stimulation reduces aggressiveness but does not lower stress in a territorial fish. **Scientific Reports**, v. 9, n. 1, p. 1-10, 2019. DOI: https://doi.org/10.1038/s41598-018-36876-1

BOOGERT, NJ et al. Song repertoire size in male song sparrows correlates with detour reaching, but not with other cognitive measures. **Animal Behaviour**, v. 81, n. 6, p. 1209-1216, 2011. DOI: https://doi.org/10.1016/j.anbehav.2011.03.004

BRAITHWAITE, VA. Cognitive Ability in Fish. In: Sloman, KA.; Wilson, RW; Balshine, S. (Org). **Behaviour and Physiology of Fish**. Academic Press, 2006. v. 24, p. 1-37.

BRANDÃO, ML; DE ALMEIDA FERNANDES, AMT; GONÇALVES-DE-FREITAS, E. Male and female cichlid fish show cognitive inhibitory control ability. **Scientific Reports**, v. 9, n. 1, p. 1-8, 2019. DOI: https://doi.org/10.1038/s41598-019-52384-2

BROWN, C. Fish intelligence, sentience and ethics. **Animal Cognition**, v. 18, n. 1, p. 1-17, 2015. DOI: https://doi.org/10.1007/s10071-014-0761-0

COULON, M et al. Do lambs perceive regular human stroking as pleasant? Behavior and heart rate variability analyses. **PLoS One**, v. 10, n. 2, p. e0118617, 2015. DOI: https://doi.org/10.1371/journal.pone.0118617

DE LOS ANGELES, GAM et al. Tactile stimulation effects on hippocampal neurogenesis and spatial learning and memory in prenatally stressed rats. **Brain Research Bulletin**, v. 124, p. 1-11, 2016. DOI: https://doi.org/10.1016/j.brainresbull.2016.03.003

DIAMOND, A. Executive functions. **Annual Review of Psychology**, v. 64, p. 135-168, 2013. DOI: https://doi.org/10.1146/annurev-psych-113011-143750

DIAMOND, A. **The development and neural bases of higher cognitive functions**. New York Academy of Sciences, 1990.

FALTER, U. Les comportements agonistiques de Sarotherodon niloticus (Pisces, Cichlidae) et la signification évolutive de l'incubation buccale. **Bulletins de l'Académie Royale de Belgique**, v. 69, n. 1, p. 566-594, 1983.

FAO. World Aquaculture 2015: A Brief Overview; **FAO Fisheries and Aquaculture Report**, n. 1140. Rome, Italy, 2017. http://www.fao.org/3/i7546e/i7546e.pdf.

FIELD, T et al. Cortisol decreases and serotonin and dopamine increase following massage therapy. **International Journal of neuroscience**, v. 115, n. 10, p. 1397-1413, 2005. DOI: https://doi.org/10.1080/00207450590956459

FREITAS, D et al. Neonatal tactile stimulation decreases depression-like and anxiety-like behaviors and potentiates sertraline action in young rats. **International Journal of Developmental Neuroscience**, v. 47, p. 192-197, 2015. DOI: https://doi.org/10.1016/j.ijdevneu.2015.09.010

GATTO, E; LUCON-XICCATO, T; BISAZZA, A. Factors affecting the measure of inhibitory control in a fish (Poecilia reticulata). **Behavioural Processes**, v. 157, p. 11-17, 2018. DOI: https://doi.org/10.1016/j.beproc.2018.08.003

GAUY, ACS et al. Preference and motivation tests for body tactile stimulation in fish. **Animals**, 11, 2021. DOI: https://doi.org/10.3390/ani11072042

GEVINS, A; SMITH, ME. Neurophysiological measures of working memory and individual differences in cognitive ability and cognitive style. **Cerebral Cortex**, v. 10, n. 9, p. 829-839, 2000. DOI: https://doi.org/10.1093/cercor/10.9.829

HIGHGATE, Q; SCHENK, S. Cognitive flexibility in humans and other laboratory animals. **Journal of the Royal Society of New Zealand**, p. 1-31, 2020. DOI: https://doi.org/10.1080/03036758.2020.1784240

KABADAYI, C; BOBROWICZ, K; OSVATH, M. The detour paradigm in animal cognition. **Animal Cognition**, v. 21, n. 1, p. 21-35, 2018. DOI: https://doi.org/10.1007/s10071-017-1152-0

KOTRSCHAL, A; TABORSKY, B. Environmental change enhances cognitive abilities in fish. **PLoS Biology**, v. 8, n. 4, p. e1000351, 2010. DOI: https://doi.org/10.1371/journal.pbio.1000351

KRALIK, JD. Inhibitory control and response selection in problem solving: How cotton-top tamarins (Saguinus oedipus) overcome a bias for selecting the larger quantity of food. **Journal of Comparative Psychology**, v. 119, n. 1, p. 78, 2005. DOI: https://doi.org/10.1037/0735-7036.119.1.78

KRALIK, JD; HAUSER, MD; ZIMLICKI, R. The relationship between problem solving and inhibitory control: Cotton-top tamarins (Saguinus oedipus) performance on a reversed contingency task. **Journal of Comparative Psychology**, v. 116, n. 1, p. 39, 2002. DOI: https://doi.org/10.1037/0735-7036.116.1.39

LAW, R; CLOW, A. Stress, the cortisol awakening response and cognitive function. **International Review of Neurobiology**, v. 150, p. 187-217, 2020. DOI: https://doi.org/10.1016/bs.irn.2020.01.001

LUCON-XICCATO, T; GATTO, E; BISAZZA, A. Fish perform like mammals and birds in inhibitory motor control tasks. **Scientific Reports**, v. 7, n. 1, p. 1-8, 2017. DOI: https://doi.org/10.1038/s41598-017-13447-4

MACLEAN, EL et al. The evolution of self-control. **Proceedings of the National Academy of Sciences**, v. 111, n. 20, p. E2140-E2148, 2014. DOI: https://doi.org/10.1073/pnas.1323533111

MENDL, M. Performing under pressure: stress and cognitive function. **Applied Animal Behaviour Science**, v. 65, n. 3, p. 221-244, 1999. DOI: https://doi.org/10.1016/S0168-1591(99)00088-X

NÄSLUND, J; JOHNSSON, JI. Environmental enrichment for fish in captive environments: effects of physical structures and substrates. **Fish and Fisheries**, v. 17, n. 1, p. 1-30, 2016. DOI: https://doi.org/10.1111/faf.12088

PROBST, JK et al. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. **Applied Animal Behaviour Science**, v. 139, n. 1-2, p. 42-49, 2012. DOI: https://doi.org/10.1016/j.applanim.2012.03.002

RUBIN, RD et al. The role of the hippocampus in flexible cognition and social behavior. **Frontiers in Human Neuroscience**, v. 8, p. 742, 2014. DOI: https://doi.org/10.3389/fnhum.2014.00742

SALVANES, AGV et al. Environmental enrichment promotes neural plasticity and cognitive ability in fish. **Proceedings of the Royal Society B: Biological Sciences**, v. 280, n. 1767, p. 20131331, 2013. DOI: https://doi.org/10.1098/rspb.2013.1331

SANTACÀ, M et al. Sensory differences mediate species variation in detour task performance. **Animal Behaviour**, v. 155, p. 153-162, 2019. DOI: https://doi.org/10.1016/j.anbehav.2019.05.022

SANTOS, LR; ERICSON, BN; HAUSER, MD. Constraints on problem solving and inhibition: Object retrieval in cotton-top tamarins (Saguinus oedipus oedipus). **Journal of Comparative Psychology**, v. 113, n. 2, p. 186, 1999. DOI:https://doi.org/10.1037/0735-7036.113.2.186

SCHMIED, C et al. Effect of previous stroking on reactions to a veterinary procedure: Behaviour and heart rate of dairy cows. **Interaction Studies**, v. 11, n. 3, p. 467-481, 2010. DOI: https://doi.org/10.1075/is.11.3.08sch

SOARES, MC et al. Tactile stimulation lowers stress in fish. **Nature Communications**, v. 2, n. 1, p. 1-5, 2011. DOI: https://doi.org/10.1038/ncomms1547

SØRENSEN, C et al. Cortisol reduces cell proliferation in the telencephalon of rainbow trout (Oncorhynchus mykiss). **Physiology & Behavior**, v. 102, n. 5, p. 518-523, 2011. DOI: https://doi.org/10.1016/j.physbeh.2010.12.023

SOVRANO, VA; BARATTI, G; POTRICH, D. A Detour Task in Four Species of Fishes. **Frontiers in Psychology**, v. 9, p. 2341, 2018. DOI: https://doi.org/10.3389/fpsyg.2018.02341

SPENCE, R; MAGURRAN, AE; SMITH, C. Spatial cognition in zebrafish: the role of strain and rearing environment. **Animal Cognition**, v. 14, n. 4, p. 607-612, 2011. DOI: https://doi.org/10.1007/s10071-011-0391-8

STRAND, DA et al. Enrichment promotes learning in fish. **Marine Ecology Progress Series**, v. 412, p. 273-282, 2010. DOI: https://doi.org/10.3354/meps08682

TALLET, C et al. Behavioural and physiological reactions of piglets to gentle tactile interactions vary according to their previous experience with humans. **Livestock Science**, v. 167, p. 331-341, 2014. DOI: https://doi.org/10.1016/j.livsci.2014.06.025

VAN PRAAG, H; KEMPERMANN, G; GAGE, FH. Neural consequences of enviromental enrichment. **Nature Reviews Neuroscience**, v. 1, n. 3, p. 191-198, 2000. DOI: https://doi.org/10.1038/35044558

VEDHARA, K et al. Acute stress, memory, attention and cortisol. **Psychoneuroendocrinology**, v. 25, n. 6, p. 535-549, 2000. DOI: https://doi.org/10.1016/S0306-4530(00)00008-1

VEENA, J et al. Enriched environment restores hippocampal cell proliferation and ameliorates cognitive deficits in chronically stressed rats. **Journal of Neuroscience Research**, v. 87, n. 4, p. 831-843, 2009. DOI: https://doi.org/10.1002/jnr.21907

VERNOUILLET, A et al. Inhibition in Clark's nutcrackers (Nucifraga columbiana): results of a detour-reaching test. **Animal Cognition**, v. 19, n. 3, p. 661-665, 2016. DOI: https://doi.org/10.1007/s10071-016-0952-y

VON KROGH, K et al. Forebrain cell proliferation, behavior, and physiology of zebrafish, Danio rerio, kept in enriched or barren environments. **Physiology & Behavior**, v. 101, n. 1, p. 32-39, 2010. DOI: https://doi.org/10.1016/j.physbeh.2010.04.003

Final Considerations

Fish are sentient beings and those under human influence, for example in aquaculture systems, must have access to increased welfare. When welfare is classified as poor or low, we must think of ways to improve it. There are two main groups of welfare indicators, physiological and behavioral. Among the physiological indicators are cortisol, which infers stress; and growth. Among the behavioral indicators are aggressiveness and cognitive performance. Knowing the level of stress, growth, aggression and cognitive performance, we can measure the welfare.

According to Gonçalves-de-Freitas et al (2019), a solution to improve Nile tilapia welfare is providing them with a more enriched environment, and tactile stimulation could be a way to do it. Therefore, this thesis proposed to test the effect of tactile stimulation as a way to improve fish welfare. Nile tilapia (*O. niloticus*) was the species used because of its importance in world aquaculture and for being an excellent model for several types of research in behavior, physiology, and also animal welfare.

We conclude that tactile stimulation reduces aggressiveness and promotes less growth in Nile tilapia groups. It also improves learning in isolated fish. However, tactile stimulation does not reduce stress in both isolated and grouped fish. Unlike what was seen for other animals, where tactile stimulation decreased stress. We can say that tactile stimulation can have different meanings in different species, that it is a topic that is still poorly understood. Overall, these positive effects on aggression, growth and learning allow us to infer that tactile stimulation promotes positive states and, therefore, increases welfare in this species. Thus, tactile stimulation can be a new strategy to increase the welfare of fish in aquaculture systems.

Complementary References

ANTONIAZZI, CTD et al. Tactile stimulation during different developmental periods modifies hippocampal BDNF and GR, affecting memory and behavior in adult rats. **Hippocampus**, v. 27, n. 2, p. 210-220, 2017. DOI: https://doi.org/10.1002/hipo.22686

ASHLEY, PJ. Fish welfare: current issues in aquaculture. **Applied Animal Behaviour Science**, v. 104, n. 3-4, p. 199-235, 2007. DOI: https://doi.org/10.1016/j.applanim.2006.09.001

BARLOW, G. **The Cichlid Fishes: Nature's Grand Experiment in Evolution**, 1 ed.; Basic Books: New York, 2002.

BOLOGNESI, MC; GAUY, ACS; GONÇALVES-DE-FREITAS, E. Tactile stimulation reduces aggressiveness but does not lower stress in a territorial fish. **Scientific reports**, v. 9, n. 1, p. 1-10, 2019. DOI: https://doi.org/10.1038/s41598-018-36876-1

BOSCOLO, CNP; MORAIS, RN; GONCALVES-DE-FREITAS, E. Same-sized fish groups increase aggressive interaction of sex-reversed males Nile tilapia GIFT strain. **Applied Animal Behaviour Science**, v. 135, n. 1-2, p. 154-159, 2011. DOI: https://doi.org/10.1016/j.applanim.2011.10.003

BRAITHWAITE, VA. Cognitive Ability in Fish. In: Sloman, KA.; Wilson, RW; Balshine, S. (Org). **Behaviour and Physiology of Fish**. Academic Press, 2006. v. 24, p. 1-37.

BRANDÃO, ML; BRAITHWAITE, VA; GONÇALVES-DE-FREITAS, E. Isolation impairs cognition in a social fish. **Applied Animal Behaviour Science**, v. 171, p. 204-210, 2015. DOI: https://doi.org/10.1016/j.applanim.2015.08.026

BROOM, DM. Indicators of poor welfare. **British Veterinary Journal**, v. 142, n. 6, p. 524-526, 1986. DOI: https://doi.org/10.1016/0007-1935(86)90109-0

BSHARY, R; GRUTTER, AS. Asymmetric cheating opportunities and partner control in a cleaner fish mutualism. **Animal Behaviour**, v. 63, n. 3, p. 547-555, 2002. DOI: https://doi.org/10.1006/anbe.2001.1937

BSHARY, R; WÜRTH, M. Cleaner fish Labroides dimidiatus manipulate client reef fish by providing tactile stimulation. **Proceedings of the Royal Society of London. Series B: Biological Sciences**, v. 268, n. 1475, p. 1495-1501, 2001. DOI: https://doi.org/10.1098/rspb.2001.1495

CARVALHO, TB; GONÇALVES-DE-FREITAS, E. Sex group composition, social interaction, and metabolism in the fish Nile tilapia. **Brazilian Journal of Biology**, v. 68, n. 4, p. 807-812, 2008. DOI: 10.1590/s1519-69842008000400015

CHANDROO, KP; DUNCAN, IJH; MOCCIA, RD. Can fish suffer? Perspectives on sentience, pain, fear and stress. **Applied Animal Behaviour Science**, v. 86, n. 3-4, p. 225-250, 2004. DOI: https://doi.org/10.1016/j.applanim.2004.02.004

CONTE, FS. Stress and the welfare of cultured fish. **Applied Animal Behaviour Science**, v. 86, n. 3-4, p. 205-223, 2004. DOI: https://doi.org/10.1016/j.applanim.2004.02.003

COULON, M et al. Do lambs perceive regular human stroking as pleasant? Behavior and heart rate variability analyses. **PLoS One**, v. 10, n. 2, p. e0118617, 2015. DOI: https://doi.org/10.1371/journal.pone.0118617

DAWKINS, MS. From an animal's point of view: motivation, fitness, and animal welfare. **Behavioral and Brain Sciences**, v. 13, n. 1, p. 1-9, 1990. DOI: https://doi.org/10.1017/S0140525X00077104

DIBATTISTA, JD et al. Growth depression in socially subordinate rainbow trout Oncorhynchus mykiss: more than a fasting effect. **Physiological and Biochemical Zoology**, v. 79, n. 4, p. 675-687, 2006. DOI: https://doi.org/10.1086/504612

ELLIS, T et al. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. **Journal of Fish Biology**, v. 65, n. 5, p. 1233-1252, 2004. DOI: s://doi.org/10.1111/j.0022-1112.2004.00499.x

FAO. World Aquaculture 2015: A Brief Overview; **FAO Fisheries and Aquaculture Report**, n. 1140. Rome, Italy, 2017. http://www.fao.org/3/i7546e/i7546e.pdf.

FIELD, T et al. Cortisol decreases and serotonin and dopamine increase following massage therapy. **International Journal of neuroscience**, v. 115, n. 10, p. 1397-1413, 2005. DOI: https://doi.org/10.1080/00207450590956459

FRASER D et al. A scientific conception of animal welfare that reflects ethical concerns. **Animal Welfare**, n. 6, p. 187-205, 1997.

FREITAS, RHA; VOLPATO, GL. Motivation and time of day affect decision-making for substratum granulometry in the Nile tilapia Oreochromis niloticus. **Journal of Applied Ichthyology**, v. 29, n. 1, p. 239-241, 2013. DOI: https://doi.org/10.1111/jai.12026

GAUY, ACS; BOLOGNESI, MC; MARTINS, GD; GONÇALVES-DE-FREITAS, E. Preference and motivation tests for body tactile stimulation in fish. **Animals**, 11, 2021. DOI: https://doi.org/10.3390/ani11072042

GONÇALVES-DE-FREITAS, E et al. Social behavior and welfare in Nile tilapia. **Fishes**, v. 4, n. 2, p. 23, 2019. DOI: https://doi.org/10.3390/fishes4020023

GRUTTER, AS. Relationship between cleaning rates and ectoparasite loads in coral reef fishes. **Marine Ecology Progress Series**, v. 118, p. 51-58, 1995. DOI: 10.3354/meps118051

HERNANDEZ-REIF, M; DIEGO, M; FIELD, T. Preterm infants show reduced stress behaviors and activity after 5 days of massage therapy. **Infant Behavior and Development**, v. 30, n. 4, p. 557-561, 2007. DOI: https://doi.org/10.1016/j.infbeh.2007.04.002

HUNTINGFORD, FA et al. Current understanding on fish welfare: a broad overview. **Journal of Fish Biology**, v. 68, p. 332-372, 2006. DOI: https://doi.org/10.1111/j.0022-1112.2006.001046.x

HUNTINGFORD, FA et al. Defining, assessing and promoting the welfare of farmed fish. **Revue scientifique et technique (International Office of Epizootics)**, v. 33, p. 233-244, 2014. DOI: 10.20506/rst.33.1.2286

JOHNSSON, JI; WINBERG, S; SLOMAN, KA. Social interactions. In: Sloman, KA, Wilson, RW, Balshine, S. (Org). **Behaviour and Physiology of Fish,** Academic Press, 2006. v. 24, p. 151–196.

MARTINS, CIM et al. Behavioural indicators of welfare in farmed fish. **Fish Physiology and Biochemistry**, v. 38, p. 17-41, 2012. DOI: https://doi.org/10.1007/s10695-011-9518-8

MCCONNACHIE, E et al. Cows are highly motivated to access a grooming substrate. **Biology Letters**, v. 14, n. 8, p. 20180303, 2018. DOI: https://doi.org/10.1098/rsbl.2018.0303

PANKHURST, N. W. The endocrinology of stress in fish: an environmental perspective. **General and Comparative Endocrinology**, v. 170, n. 2, p. 265-275, 2011. DOI: https://doi.org/10.1016/j.ygcen.2010.07.017

POTTINGER, T. The Stress Response in Fish: Mechanisms, Effects and Measurement. In: Branson, EJ (Ed). **Fish Welfare**. Oxford, Blackwell Publishing Ltd, 2008. p. 32-48.

POTTINGER, TG; PRUNET, P; PICKERING, AD. The effects of confinement stress on circulating prolactin levels in rainbow trout (Oncorhynchus mykiss) in fresh water. **General and Comparative Endocrinology**, v. 88, n. 3, p. 454-460, 1992. DOI: https://doi.org/10.1016/0016-6480(92)90240-K

PROBST, JK et al. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. **Applied Animal Behaviour Science**, v. 139, n. 1-2, p. 42-49, 2012. DOI: https://doi.org/10.1016/j.applanim.2012.03.002

SCHMIED, C et al. Effect of previous stroking on reactions to a veterinary procedure: Behaviour and heart rate of dairy cows. **Interaction studies**, v. 11, n. 3, p. 467-481, 2010. DOI: https://doi.org/10.1075/is.11.3.08sch

SCHRAM, E et al. Stocking density-dependent growth of Dover sole (Solea solea). **Aquaculture**, v. 252, n. 2-4, p. 339-347, 2006. DOI: https://doi.org/10.1016/j.aquaculture.2005.07.011

SNEDDONS, LU; WOLFENDEN, DCC; THOMSON, J.S. Stress Management and Welfare. In: Schreck, CB; Tort, L; Farrell, AP; Brauner, CJ (Eds). **Biology of Stress in Fish** – Fish Physiology. Academic Press. 2016. p. 464-541.

SOARES, MC et al. Tactile stimulation lowers stress in fish. **Nature Communications**, v. 2, n. 1, p. 1-5, 2011. DOI: https://doi.org/10.1038/ncomms1547

SØRENSEN, C et al. Social stress reduces forebrain cell proliferation in rainbow trout (Oncorhynchus mykiss). **Behavioural Brain Research**, v. 227, n. 2, p. 311-318, 2012. DOI: https://doi.org/10.1016/j.bbr.2011.01.041

TALLET, C et al. Behavioural and physiological reactions of piglets to gentle tactile interactions vary according to their previous experience with humans. **Livestock Science**, v. 167, p. 331-341, 2014. DOI: https://doi.org/10.1016/j.livsci.2014.06.025

VOLPATO, GL; GONÇALVES-DE-FREITAS, E; FERNANDES-DE-CASTILHO, M. Insights into the concept of fish welfare. **Diseases of Aquatic Organisms**, v. 75, n. 2, p. 165-171, 2007. DOI: 10.3354/dao075165

WEBSTER, J. International standards for farm animal welfare: Science and values. **The Veterinary Journal**, v. 1, n. 198, p. 3-4, 2013. DOI: 10.1016/j.tvjl.2013.08.034