Universidade Estadual Paulista "Júlio de Mesquita Filho"
Instituto de Biociências
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DÊ AOS ANIMAIS O QUE ELES PREFEREM: VALIDAÇÃO DE UM ÍNDICE DE PREFERÊNCIA POR MEIO DE TESTES DE ESFORÇO

Caroline Marques Maia

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Apoio
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Palavras-chave: Esforço físico; Esforço psicológico; Motivação; Preferência.

Dedico este trabalho a todos aqueles que lutam incansavelmente para que a voz dos animais seja ouvida e suas condições de vida não sejam ignoradas

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"Real respect for animals will come when we see them as sentient beings in their own right, with their own views and opinions, their own likes and dislikes. The animal voice should be heard."

- Marian Dawkins

SUMÁRIO

-Introdução-

A Ciência do bem-estar animal vem se desenvolvendo gradualmente desde a década de 60 (Duncan, 2006), provavelmente como consequência da publicação do livro *Animal Machines* de Ruth Harrison (1964), que denunciou uma série de práticas que desconsideravam o sofrimento animal. Nesse contexto, as pessoas começaram a voltar sua atenção para questões relacionadas ao bem-estar animal e tornou-se importante determinar as condições de bem-estar dos animais em determinadas situações e contextos. Assim, teve início uma incessante busca por indicadores de bem-estar, através dos quais se pudesse avaliar o estado do animal.

Motivados pelo sucesso no achado dos eixos fisiológicos de estresse (Selye, 1936), muitos pesquisadores deram início a uma busca por indicadores fisiológicos de bem-estar, inclusive considerando o próprio estresse como um contra-indicador dessa condição (Huntingford *et al.,* 2006; Van de Nieuwegiessen *et al.,* 2008). Entretanto, tal indicador não tem se mostrado inequívoco. O animal pode estar numa condição ruim mesmo não apresentando sinais de estresse (*e.g.* animal apresenta ferimento físico, mas não se encontra em estado de estresse). Ou seja, baixos níveis de estresse não devem ser considerados como sinônimos de bem-estar. Por outro lado, os animais podem estar numa boa condição quando em situação de estresse (*e.g.* evento reprodutivo). Ou seja, o estado de estresse deve fazer parte das considerações de bem-estar, mas não é sinônimo deste (Volpato *et al.,* 2009). Além disso, a resposta de estresse visa restabelecer a homeostase frente a uma situação perturbadora. Assim, há um nítido elemento de seleção envolvido, de forma que uma resposta de estresse ineficiente pode não ser suficiente para que o animal resolva a questão imposta pelo ambiente e a homeostase seja restabelecida. No caso do bem-estar, por outro lado, a situação é diferente. Há estados considerados de boas condições de bem-estar nos quais há alta demanda metabólica; em outros, a demanda é baixa (Volpato *et al*., 2009), como exemplificado acima. Assim, a evolução de respostas padronizadas (eixos) de bons estados de bem-estar parece pouco provável.

Por outro lado, alguns pesquisadores começaram a buscar indicadores comportamentais de bem-estar. Nesse caso passou-se a considerar que o animal que estivesse desenvolvendo comportamentos naturais da espécie estaria em bom estado de bem-estar e que aqueles que estivessem se comportando de forma estereotipada[1] estariam numa situação de pobre bem-estar (Volpato *et al.,* 2007 e 2009). Este indicador não parece adequado, pois pressupõe que todos os animais estão em boas condições na natureza, o que nem sempre é verdadeiro (Volpato *et al.,* 2009). Por exemplo, o comportamento de fuga da presa frente a um predador é um comportamento natural, mas não podemos dizer que a presa está em boas condições de bem-estar nesse momento. Além disso, alguns estudos mais antigos também contestaram a estereotipia como sinônimo de bem-estar pobre (Bildsøe *et al.,* 1991; De Passilé *et al.*, 1993). Nesse contexto, Swaisgood & Shepherdson (2005) argumentam que a expressão de um comportamento anormal, como a estereotipia, pode representar uma estratégia para lidar com as condições mais restritivas do ambiente cativo. Assim, segundo Hill & Broom (2009), comportamentos naturais não são necessariamente esperados em ambientes cativos.

Frente a esse impasse, Dawkins (2006, 2008) propõe que devemos voltar nossa atenção para as vontades e necessidades dos animais ao invés de buscar indicadores inequívocos de bem-estar. Assim, Volpato *et al.* (2007) incorporaram a definição de bem-estar como sendo o estado interno de um animal quando ele está numa situação a qual escolheu livremente. Dawkins (2008) ainda acrescenta a essa definição o estado de saúde, definindo o bem-estar como sendo o estado dos animais saudáveis que tem o que querem. Dessa forma, testes que determinam as vontades e necessidades passaram a ser utilizados visando detectar condições de conforto para os animais. Assim, testes nos quais o animal deve optar por uma entre duas (teste de pareamento) ou mais (teste de múltipla escolha) opções disponíveis, começaram a ser amplamente utilizados para determinar as preferências dos animais. Por exemplo, escolha por sacarose (Bartoshuk *et al.,* 1971), escolha por parceiro sexual (Basolo, 1990; Schlupp *et al.*, 1999; Braithwaite & Barber, 2000; Liao & Lu, 2009; Levy *et al.*, 2014), escolha por substrato (Webster & Hart*,* 2004; Galhardo *et al*., 2009) e escolha por temperatura (Girguis & Lee, 2006).

Nos estudos de preferência, geralmente os termos "escolha" e "preferência" são considerados como sinônimos, sendo essa última inferida diretamente a partir da primeira em poucos dias de teste (*e.g*. Schlupp *et al.*, 1999; Gonçalves & Oliveira, 2003; Webster & Hart*,* 2004;

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[1] Estereotipia é definida como uma série de movimentos regularmente repetidos sem função aparente (Dantzer e Mormède, 1983), provavelmente induzidos por frustração, tentativas repetidas de lidar com o ambiente ou ainda disfunção cerebral (Mason, 2006).

Galhardo *et al*., 2009; Liao & Lu, 2009; Levy *et al.*, 2014). Além disso, a maior parte das análises de dados dos estudos de preferência é feita ao nível de grupo e não de indivíduo (*e.g.* Soriguer *et al.,* 2002; Matsumoto *et al.,* 2008; Snowberg & Benkman, 2009; Zizzari *et al*., 2009; Graber *et al.*, 2015). Entretanto, alguns estudos demonstraram a existência de uma variabilidade individual significativa em respostas de preferência, mesmo com os testes tendo sido realizados em poucas triagens ou em poucos dias (*e.g.* Godin & Dugatkin, 1995; Johnsson *et al.,* 2000; Wolfgang & Birkhead, 2004; Browne *et al.,* 2010).

Recentemente, Maia & Volpato (2016; Anexo) demonstraram a existência de dois perfis distintos nas escolhas: a) não-preferência (escolhas inconsistentes ao longo de vários testes) e b) preferência (escolhas consistentes ao longo do tempo). Esses autores também relatam uma grande variabilidade individual nas respostas de preferência em testes realizados por 10 dias consecutivos e recomendam a aplicação do *Índice de Preferência* (IP) para determinação, a nível individual, das preferências e não-preferências, bem como de suas intensidades de resposta para identificar condições ambientais que melhorem o estado de bem-estar dos animais. Nesse contexto, reconhecidos esses dois perfis que já faziam parte das definições de bem-estar citadas acima (Dawkins, 2006, 2008; Volpato *et al.,* 2007), o passo relevante a seguir é avaliarmos o quanto esses perfis, identificados pelo IP, têm implicações para o estado de bem-estar e o quanto as respostas individuais, e não apenas a nível de grupo, são relevantes para os animais.

Uma das formas de avaliarmos as implicações para estados de bem-estar animal dos perfis de preferência e não-preferência, identificados com base nos cálculos do IP, seria inferir a intensidade da motivação dos animais para acessar tais perfis. De fato, segundo Duncan (2006), não basta identificar apenas quais são os itens preferidos dos animais, mas é necessário também determinar o quanto cada item é importante, ou seja, o quanto os animais estão motivados para acessá-los. Nesse contexto, se o IP é uma ferramenta confiável para detectar respostas de preferência e não preferência como fenômenos distintos, tais respostas devem gerar motivações diferentes aos animais, sendo que as preferências, por serem escolhas consistentes ao longo do tempo, devem fornecer melhores condições de bem-estar. Ou seja, os animais devem estar mais motivados para acessar preferências do que não preferências detectadas pelo IP. Além disso, embora os testes de motivação em animais tenham sido amplamente utilizados (*e.g.* Matthews

& Ladewig, 1994; Mason *et al.*, 2001; Sherwin, 2004; Albentosa & Cooper, 2005; Hovland *et al.*, 2006; Asher *et al.*, 2009; Houpt, 2012), a relação entre as respostas de preferência e aquelas de motivação pelos recursos ainda não está clara. Tal fato provavelmente é uma consequência das respostas de preferência e de motivação serem avaliadas em estudos independentes e por recursos diferentes. Por exemplo, há inúmeros trabalhos que avaliaram as preferências por parceiros sexuais em diversas espécies de peixes (*e.g.* Sekiya & Karino, 2004; O'Rourke & Mendelson 2010; Graber *et al.*, 2015), mas há apenas um trabalho que testou respostas de motivação em peixes, sendo que os recursos avaliados foram alimento e parceiro social (Galhardo *et al.*, 2011). Assim, considerando a nova proposta de Maia & Volpato (2016; Anexo), será que os animais estariam mais motivados para acessar itens preferidos do que aqueles não preferidos, que foram detectados com base em cálculos do IP? Será que a variabilidade individual nas respostas de preferência, por serem significativas, refletem em respostas individuais de motivação?

Assim, aqui avaliamos se o IP é uma ferramenta confiável para detectar respostas de preferência e não preferência a nível individual, testando as respostas de motivação dos animais para acessarem recursos preferidos detectados pelo IP. Para isso, usamos como modelo de estudo o peixe tilápia-do-Nilo (*Oreochromis niloticus*) e como recurso a ser acessado a cor do ambiente. Baseamos nossa escolha no fato de que já foi demonstrado que diferentes cores ambientais afetam respostas fisiológicas e comportamentais dos peixes (Head & Malison, 2000; Ruchin, 2004; Karakatsouli *et al.*, 2008; Barcellos *et al.*, 2009; Maia & Volpato, 2013; Volpato *et al.*, 2013) e que a tilápia-do-Nilo é uma espécie cujas escolhas pela cor do ambiente temos estudado, o que nos permitiu um patamar inicial importante. Além disso, caso as preferências individuais realmente promovam melhores condições de bem-estar e, portanto, causem maior motivação dos animais para acessá-las, é natural esperar que isso não ocorra apenas numa espécie animal ou para um único tipo de recurso ambiental. Dessa forma, também testamos os peixes zebrafish (*Danio rerio*) e truta arco-íris (*Oncorhynchus mykiss*), por serem espécies pouco aparentadas da tilápia-do-Nilo. Ademais, além de avaliar as respostas de preferência e motivação pelo recurso cor ambiental, também avaliamos outros recursos considerados relevantes para duas das espécies em questão. No caso dos testes com zebrafish, também testamos o recurso planta artificial, que é comumente adicionado aos tanques de criação/manutenção dessa espécie em cativeiro como forma de enriquecimento ambiental. Para os testes com a truta arco-írirs, além da cor ambiental, testamos tocas e presença de coespecíficos como recursos a serem acessados, pois tais recursos têm sido demonstrados como relevantes para essa espécie (tocas: Conallin *et al.*, 2012; presença de conspecíficos: Dunlop *et al.*, 2006; Øverli *et al.*, 2006). Tanto a tilápia-do-Nilo quanto a truta arco-íris são espécies amplamente usadas na piscicultura, enquanto o zebrafish é uma espécie importante no ramo da aquariofilia e vem sendo utilizado como organismo modelo em diversas áreas da ciência. Dessa forma, conclusões específicas sobre as preferências e motivações dessas três espécies podem ter implicações mais rapidamente utilizadas nas condições de cultivo.

Objetivos

Avaliamos experimentalmente a seguinte tese e as respectivas hipóteses:

- <u>*TESE*</u>: *O Índice de Preferência (IP) é uma ferramenta confiável para indicar a vontade do animal em obter itens de recursos ambientais*.

- *hipótese 1*: A tilápia-do-Nilo está mais motivada e, portanto, se esforça fisicamente mais para acessar cores ambientais preferidas (obtidas pelo IP).

- *hipótese 2*: A variabilidade individual nas preferências obtidas pelo IP decorre de variabilidade individual nas motivações dos animais.

- *hipótese 3*: A validade do IP para indicar a vontade dos animais não é espécie-específica.

- *hipótese 4*: O IP indica não apenas motivação física, mas também psicológica, para a obtenção de itens.

- *hipótese 5*: A indicação da vontade do animal por meio do IP não depende do recurso.

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~Estrutura Geral da Tese~

Este texto é dividido em três capítulos, cada um correspondendo a um manuscrito preparado em língua inglesa e que será submetido a revistas científicas internacionais relevantes, ao menos nas áreas de comportamento e bem-estar animal. Cada capítulo (manuscrito) avalia parte das hipóteses propostas a partir da tese, como se segue:

CAPÍTULO 1 – Teste das hipóteses 1 e 2. Aqui avaliamos as respostas de preferência e não-preferência da tilápia-do-Nilo pela cor do ambiente por meio de cálculos do *Índice de Preferência*, com base em testes de múltipla escolha (4 opções disponíveis) realizados por 7 dias consecutivos. Tanto itens preferidos e não-preferidos bem como suas intensidades de resposta foram determinados, sendo que tais respostas foram avaliadas a nível de grupo e individualmente. Em seguida, testamos a motivação física dos mesmos indivíduos para acessar as cores ambientais anteriormente preferidas e não preferidas, registrando a frequência de tentativas dos indivíduos para acessar ambientes coloridos cujo acesso foi fisicamente, mas não visualmente, bloqueado. As respostas de motivação foram avaliadas a nível de grupo e também por indivíduo.

CAPÍTULO 2 – Teste das hipóteses 3, 4 e 5. Neste estudo avaliamos as respostas de preferência do zebrafish pela cor ambiental e também por plantas artificiais em testes de escolha independentes. As preferências e não-preferências individuais e suas intensidades de resposta foram determinadas pelo *Índice de Preferência*, com base em testes de múltipla escolha (4 opções disponíveis) realizados por 7 dias consecutivos. Em seguida, testamos a motivação física e psicológica dos mesmos peixes, a nível individual, para acessar cores ambientais ou plantas artificiais em opções anteriormente preferidas e não preferidas. Num primeiro teste, registramos as frequências de tentativas dos indivíduos para acessar ambientes com opções previamente preferidas ou não preferidas cujo acesso foi fisicamente, mas não visualmente, bloqueado, para inferir a motivação física dos indivíduos. Num segundo teste, registramos o compartimento (com opção previamente preferida ou não preferida) que os peixes acessaram logo após cruzarem um caminho longo e aversivo, para inferir a motivação psicológica dos indivíduos.

CAPÍTULO 3 – Teste das hipóteses 3, 4 e 5. Aqui avaliamos, em testes independentes, as respostas de preferência da truta arco-íris pela cor ambiental, por tocas artificiais e pela presença de coespecíficos. As preferências e não-preferências individuais, bem como suas intensidades de resposta, foram determinadas pelo *Índice de Preferência* com base em testes de múltipla escolha (4 opções disponíveis) realizados por 7 dias consecutivos. Em seguida, testamos a motivação física e psicológica dos mesmos peixes, a nível individual, para acessar cores ambientais, tocas artificiais ou coespecíficos em opções anteriormente preferidas comparadas àquelas não preferidas. Num primeiro teste, para avaliar a motivação física, registramos a frequência de tentativas dos indivíduos para acessar ambientes com opções anteriormente preferidas ou não preferidas cujo acesso foi fisicamente, mas não visualmente, bloqueado. Num segundo teste, para avaliar a motivação psicológica, registramos o compartimento (com opção previamente preferida ou não preferida) o qual os peixes acessaram primeiramente após cruzarem um caminho longo e aversivo.

~Procedimentos de Ética~

Os procedimentos experimentais desta tese realizados com a espécie de peixe tilápia-do-Nilo (*Oreochromis niloticus*) estão de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foram aprovados pela Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências de Botucatu, UNESP-SP (protocolo # 391-CEUA).

Os procedimentos experimentais desta tese realizados com as espécies de peixe zebrafish (*Danio rerio*) e truta arco-íris (*Oncorhynchus mykiss*) estão de acordo com o Animal Welfare Insurance arquivado com o Office for Laboratory Animal Welfare e foram aprovados pelo Institutional Animal Care and Use Committee (IACUC) da Pennsylvania State University, University Park campus, USA (protocolo # 36902).

~ Capítulo 1 ~

A tilápia-do-Nilo é fisicamente motivada para acessar cores preferidas, determinadas por cálculos do IP? Essa resposta está presente a nível individual?

Preference Index as an expression of motivation for a resource

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Abstract

Preference and motivation methods are used to identify better environmental conditions for animals, but whether there is correspondence between these methods has not previously been shown. Here we investigated this by contrasting preference and motivation for environmental colors in the fish Nile tilapia. Correspondence was demonstrated, as well as consistent individual variability for fish preferences and non-preferences, which were consistent with motivation tests. Visitation frequencies of 34 isolated fish to colored compartments (red, yellow, green and blue) were registered over 7 consecutive days. From these data we calculated values of a Preference Index, PI, as described in Maia & Volpato (A history-based method to estimate animal preference. *Scientific Reports*, 2016; Attachment). Then, we measured the number of pushes fish made on transparent hinged doors that blocked access to the colored compartments. Fish tended to avoid yellow color in the preference tests and were less motivated to access yellow and red colors. In contrast, they were more motivated to reach colors that they usually preferred. This shows that the preferred options indicate fish motivation. Despite this general pattern, color preference varied among fish. Thus, the PI indicates motivation for access to a resource, but in terms of which kind of resource is sought after, individuality seems to be the rule.

Introduction

Preference and motivation tests have been widely applied to investigate the wants of animals to try identify what might improve captive environmental conditions. Preference tests (*e.g.* Basolo, 1990; Braithwaite & Barber, 2000; Girguis & Lee, 2006; Liste *et al.*, 2014) are usually based on multiple-choice tests. Recently, a Preference Index (PI) has been developed to separate choice from preference, by determining preferred and non-preferred items in multiple-choice tests conducted over time. In contrast, motivation tests (*e.g.* Matthews & Ladewig, 1994; Mason *et al.*, 2001; Sherwin, 2004; Asher *et al*., 2009; Houpt, 2012), measured in terms of effort to reach a goal, can identify the importance of specific environmental resources for the animals. These approaches help identify factors that contribute to good welfare conditions for animals, and improve on assays that use physiological and other behavioral indicators of welfare, which are often challenging to interpret unequivocally (Volpato *et al*., 2007; Volpato 2009; Volpato *et al*., 2009).

As suggested by Duncan (2006), analyses of motivation should be used to complement the findings of preference tests. Linking the tests is important because preference indicates what animals want, while motivation indicates how much they want it. However, the relationship between animal preference and motivation is not clear. This is probably because preferred items and motivational responses to access resources have been evaluated separately and for different resources. For example, although there are many studies testing the mating preferences of both male and female fish (*e.g.* Sekeija & Karino, 2004; O'Rourke & Mendelson 2010; Graber *et al.*, 2014), as far as we know, there is only one peer-reviewed study that evaluated motivation in fish and this tested motivation to obtain food and a social partner (Galhardo *et al.*, 2011). Moreover, most preference and motivation studies have ignored individual variability, even though considerable individual variation of preference is known to exist (Godin & Dugatkin, 1995; Wolfgang & Birkhead, 2004; Browne *et al*., 2010; Johnsson *et al*., 2000), even when tests were applied over several days (Maia & Volpato, 2016; Attachment). Thus, here we tested the correspondence between preference and motivation responses for a same resource. This allowed us to confirm the biological importance of Maia & Volpato (2016)'s PI and emphasizes the importance of individual variability in preference and motivation within an animal welfare context.

We focused on choice tests of the fish Nile tilapia, *Oreochromis niloticus*, to different background colors. Environmental colors seem to be an important resource for fish, as some colors have been demonstrated to influence physiology and behavior of fish, affecting, for example, stress response (Karakatsouli *et al.*, 2008; Barcellos *et al.*, 2009; Maia & Volpato, 2013), growth (Head & Malison, 2000; Ruchin, 2004; Karakatsouli *et al.*, 2008), reproduction (Volpato *et al.*, 2004) and feeding (Spence & Smith, 2008; Volpato *et al*., 2013).

Methodology

Animals and Holding Conditions

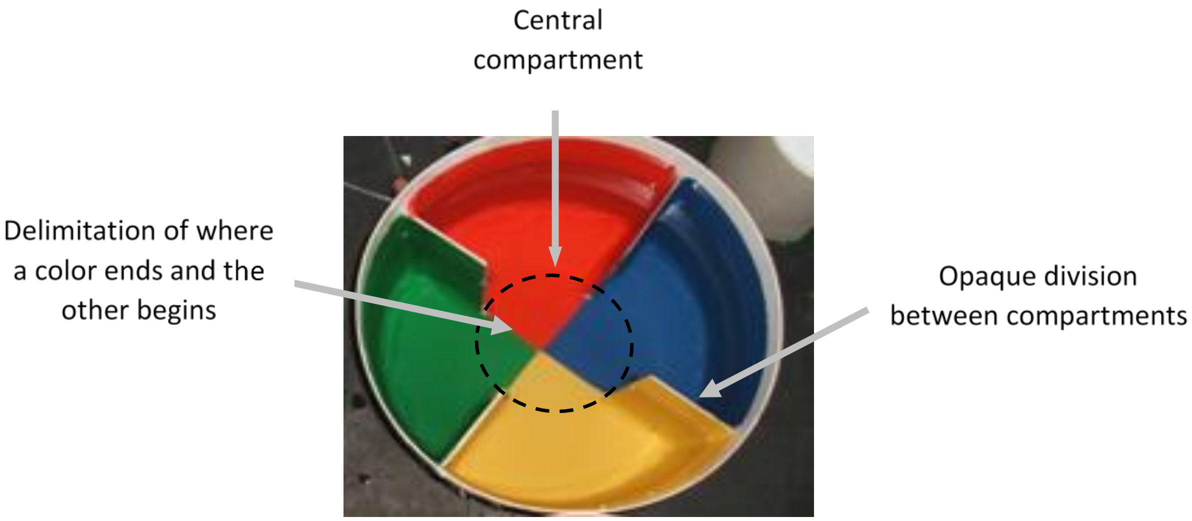
Adult Nile tilapia (*Oreochromis niloticus*) from a monoculture hatchery were held in indoor tanks (from 1000 to 1700 L) at a maximum density of 1 fish/5 l. Water quality was maintained with temperature ranging from 23.0 to 28.5°C; pH ≅ 7.0; ammonium < 0.3 ppm; and nitrite < 0.05 ppm. Photoperiod was from 6:00 h to 18:00 h under white light; food (36% crude protein) was offered once a day (∼ 5% of biomass). All tanks were equipped with biological filters, constant aeration and a water circulation system. Fish stayed at least 1 month in these holding conditions prior to experimentation.

Experimental Design

We evaluated the momentary choice responses of isolated fish (n = 34 fish) for background colors during 10 consecutive days to calculate their individual PI values (based on Maia & Volpato, 2016; Attachment). Individual fish were able to swim between four colored compartments (red, yellow, green or blue compartments) in each daily test, and visitation frequency to the compartments was registered each 30 s for 1h/day during the last 7 days (test days), as the first 3 days were considered as an acclimation period to the experimental conditions. From these choice frequencies, the calculated PIs indicated the preferred and non-preferred colors and the intensity of these responses for each fish. On the 11th day, a 5-min assay was used to test the motivation responses of the same fish to access the colored compartments by registering the frequency of pushes fish gave to transparent, hinged doors that blocked access to each colored compartment.

Specific Procedures

After being measured (mean ± SD: 7.37 ± 0.17 cm, standard body length) and weighed, (12.08 ± 1.11 g), the fish were isolated (08:00 h to 09:00 h, a.m.) in glass aquaria (40 x 20 x 25 cm) equipped with mechanical and chemical filters. Twenty-four hours later, we started preference tests only for fish with clearer eyes, which indicates low level or lack of stress (Suter & Huntingford, 2002), a technique recently adapted for Nile tilapia (Freitas *et al.,* 2014). Individual fish were placed in the central compartment of a circular aquarium (Figure 1) divided into four compartments of the same size and of similar structure, but of different colors.



Central compartment

Delimitation of where a color ends and the other begins

Opaque division between compartments

Figure 1: Top view of a circular test aquarium showing the four compartments of the same volume and area. Each compartment was internally covered by adhesive paper of different colors (red, yellow, green or blue). The central compartment was formed by the junction of the background colors of the other compartments. Aquarium diameter = 40 cm; water column height = 15 cm. The outer walls and the internal partitions were opaque with the respective colors.

Each fish was placed inside a 10-cm diameter transparent cylinder placed in the center of each circular aquarium (central compartment, Figure 1). After 5 min, we carefully removed the cylinder, releasing fish to swim freely over the four compartments as they were filmed from above for 1 h. Then, we returned fish to their respective isolation aquarium, where they were fed and stayed until the test in the next day. These procedures were repeated for 10 consecutive days, but fish were filmed just on the last 7 days (test days). From filming, we registered the visitation frequency of fish in each colored compartment every 30 s, totaling 120 observation points during each daily test.

In the morning of the 11th day, we tested the fish motivation to reach the different colored compartments using an opaque, white chamber (Figure 2) in the center of each circular aquarium. Each chamber had four transparent hinged doors, which faced each of the four colored compartments. An individual fish was placed inside the chamber and filmed from above for 5 min (a sufficient period to motivate fish to push on the doors, based on pilot tests). From video analyses, we registered the number of pushes on each transparent door.

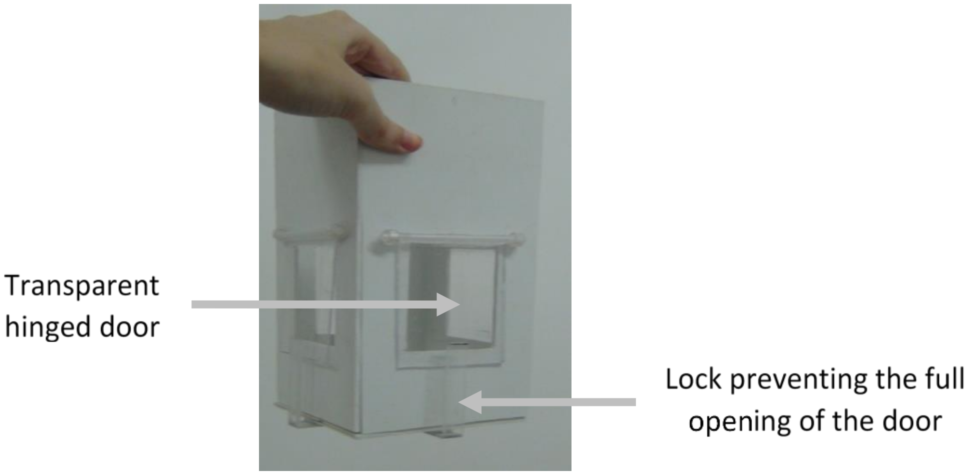


Figure 2: The white and opaque chamber used in the motivation test. Transparent locks attached to the bottom of the chamber prevented the complete opening of the transparent doors. Thus, fish could push and move the doors, but not completely open them and get outside the chamber.

During all experimental procedures, the mechanical and chemical filters were kept constant in isolation aquaria and we siphoned and performed partial water changes in the circular test aquaria twice a week, always after the morning tests. These procedures maintained water quality at levels similar to those of holding conditions. A white light (~ 150 Lx) was placed ~ 2.5 m above the test aquaria. Temperature varied from 24.5°C to 29.0°C, with a homogeneous distribution of variation over all test aquaria. All fish were tested in the morning, as Nile tilapia were found to be more decided (for substratum resources) in this period of the day (Freitas & Volpato, 2013).

Preference Index (PI) Calculations

We calculated the PI values to determine individual preference and non-preference responses as described in Maia & Volpato (2016; Attachment). Thus, we calculated the cumulative frequency of visitation over the test days and, based on this, the cumulative area above the cumulative-frequency line. According to Maia & Volpato (2016; Attachment), the calculated areas increase with the value of frequencies obtained in the most recent tests, a step based on the assumption that the most recent choices may have a higher impact for the calculations, as the preference responses may vary over time. All these calculations were made for each color option and for each individual fish. Thus, we obtained PI values. Positive PIs represent preferences and negative PIs represent non-preferences, while each PI value indicates the intensity of such responses, thus identifying the most preferred and the most non-preferred colors for each fish (for details, see Maia & Volpato, 2016; Attachment).

Statistical Analyses

We compared the number of preferences for the different colors considering all fish (n = 34), and also computing only fish with one preferred color (one-preference fish, n = 11), by Goodman's proportion test (1964, among multinomials). To compare PI values for different colors, we had to discard data from 8 fish because there was a problem with the videos of their preference tests. As the data of the remaining fish (n = 26), including the data of one-preference fish (n = 7) were not homogeneously distributed (Levene's test, $P < 0.05$), we used a Friedman's test for these comparisons among colors. Considering the motivation test data, we opted to use proportional data of frequency of pushes, because the intra-fish variation of this response was high. Thus, we compared the proportions of pushes among different colors considering data of all tested fish and also considering only data of one-preference fish (n = 33 and 10, respectively; one fish did not push at all). As these data were not normally distributed (Kolmogorov-Smirnov test, $P < 0.05$) and were not homogeneously variable (Levene's test, $P < 0.05$), we used a Friedman's test for these comparisons.

To determine whether individual fish were more motivated to access their own preferred colors, we compared proportions of pushes between the most preferred and the most non-preferred color of each individual ($n = 33$) by Wilcoxon test, as these data were not normally nor homogeneously distributed (Kolmogorov-Smirnov and Levene's tests, $P < 0.05$, respectively). We also compared proportions of pushes between all preferred and all non-preferred colors of each fish ($n = 33$) by a Dependent-T test, as these data were normally and homogeneously distributed (Kolmogorov-Smirnov and Levene's tests, $P > 0.05$, respectively). For all comparisons, we fixed $\alpha = 0.05$.

Results

Considering all tested fish, blue, green and red colors were detected as the most preferred options, with the highest PI median values (Figure 3A, B). On the other hand, yellow was the most non-preferred option (Figure 3A, B). A similar profile occurred when we considered only one-preference fish (Figure 3C, D). However, red and green were more similar with yellow when considering the most preferred color responses (Figure 3C), and red was more similar with yellow both for most non-preferred color responses and also PI values (Figure 3D).

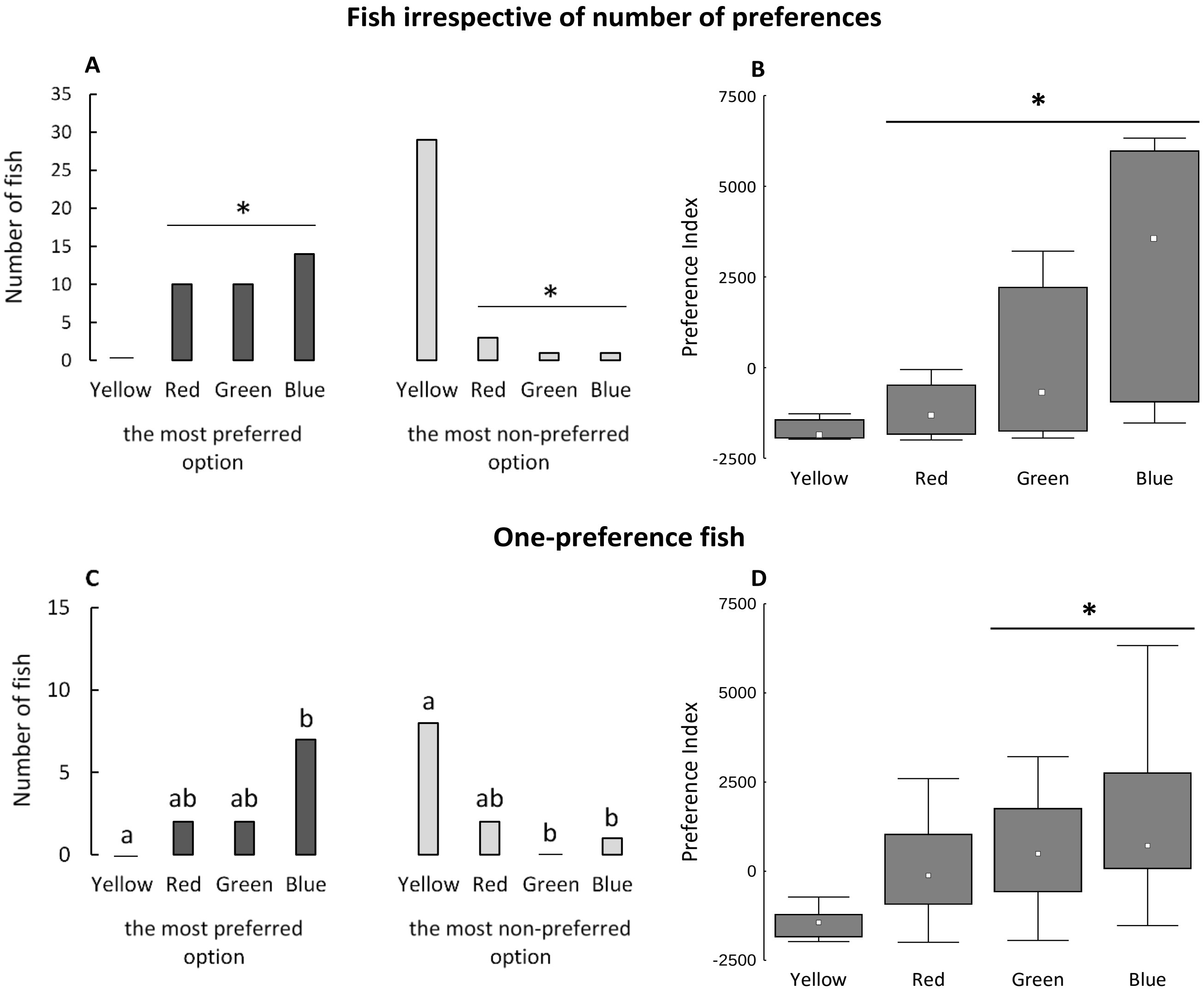


Figure 3: Background colors selected by fish as their most preferred or most non-preferred options, both in terms of frequency of preference responses and PI values considering all tested fish (A and B, respectively) and just fish that preferred only one color (C and D, respectively). In B and D, box-plot: □ indicates median; columns show quartile (Q1 and Q3) and bars indicate maximum values. * indicates significant differences between colors in A (Goodman's proportion test, $P < 0.05$), B (Friedman test, $P < 0.00$, Fr = 37.06) and D (Friedman test, $P < 0.01$, Fr = 12.09). Numbers of fish with different small letters are significantly different in C (Goodman's proportion test, $P < 0.05$). N = 34 in A and C; n = 25 in B; n = 7 in D.

All tested fish, except one, pushed on the hinged doors during the 5-min trial of motivation test (n = 33). Fish, irrespective of number of preference responses, pushed more to access green and blue colors than to access red and yellow colors (Figure 4A). Moreover, one-preference fish also expressed this same pattern, but with blue being more similar with yellow color (Figure 4B).

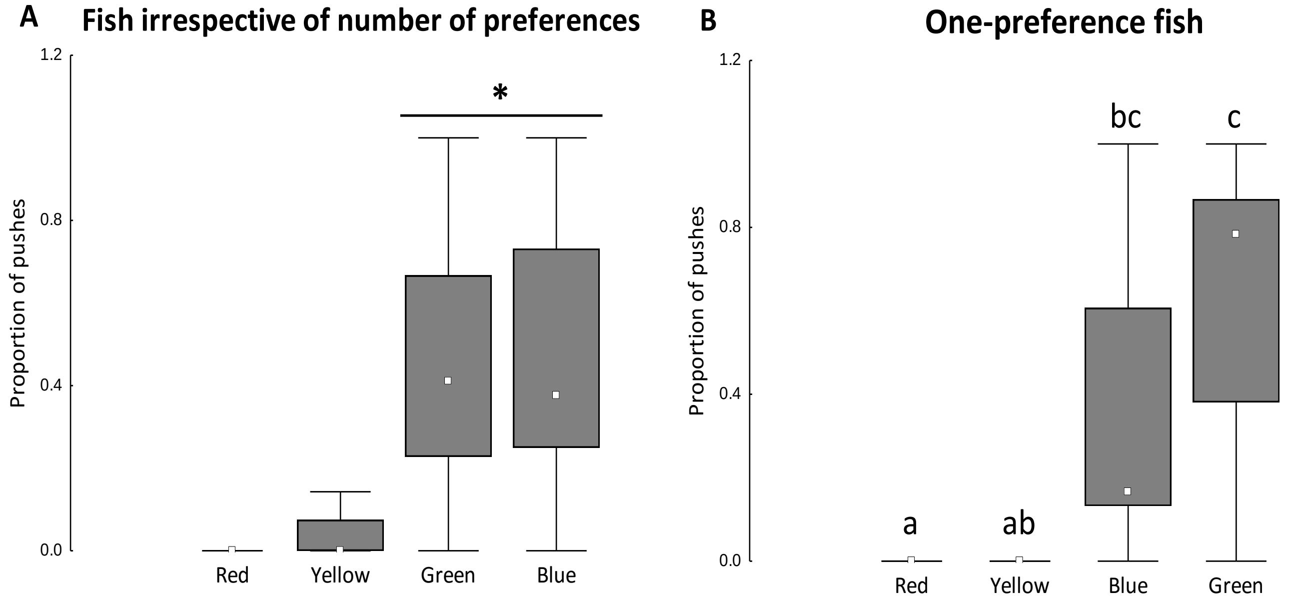


Figure 4: Proportion of pushes on the transparent hinged doors to access each background color, when considering all tested fish (A) or just fish that preferred only one color (B). Box-plot: □ indicates median; columns show quartile (Q1 and Q3) and bars indicate maximum values. * indicates significant differences between colors in A (Friedman test, P < 0.00, Fr = 61.47); in B, proportions of pushes with different small letters are significantly different (Friedman test, P < 0.00, Fr = 18.95). N = 33 in A; n = 10 in B.

Regardless of colors, Nile tilapia pushed more to access their individually most preferred item, and pushed less for their individually most non-preferred item (Figure 5A). This same pattern occurred when all the preferred or the non-preferred items were considered (Figure 5B).

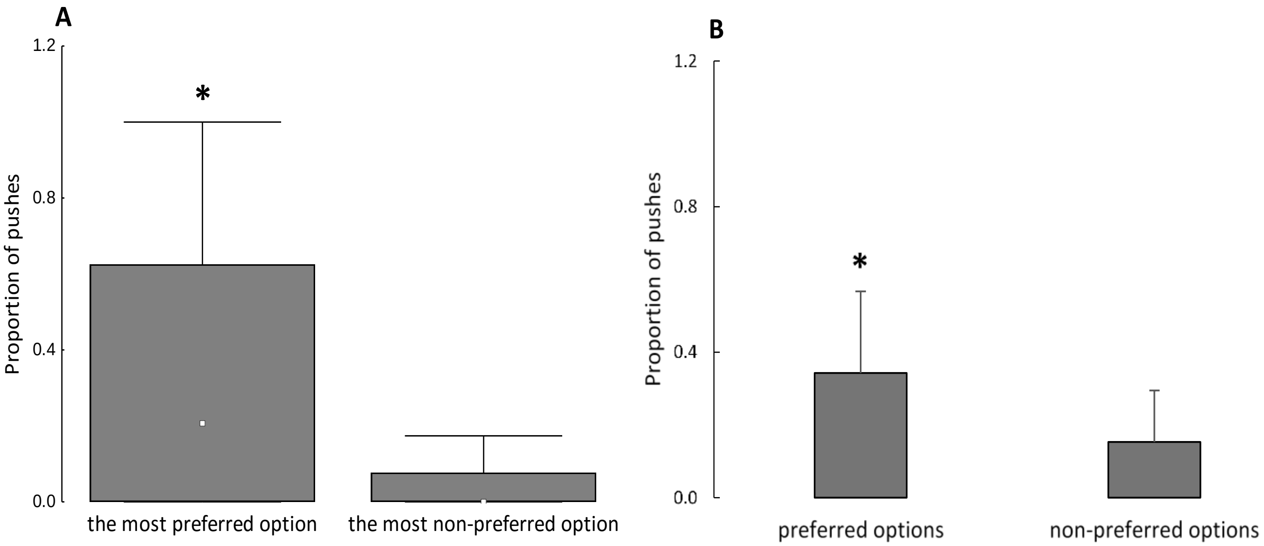


Figure 5: Proportion of pushes made on the transparent hinged doors to reach the individually most preferred and most non-preferred colors (A) or to access all the individually preferred and non-preferred colors (B). In A, box-plot: □ indicates median; columns show quartile (Q1 and Q3) and bars indicate maximum values. In B, mean + SD; * indicates significant differences (Wilcoxon test, P = 0.00, Z = 3.03; Dependent-T test, P = 0.00, t = 3.16, respectively). N = 33 in A and B.

Discussion

Here we demonstrated that the motivation test corroborates the PI to identify preferred and non-preferred items. This provides a tool to assess a key animal welfare issue: how to determine what animals prefer so that they can be housed and reared in better conditions (base on Dawkins (2006)'s theory). Our tests detected similar responses for motivation and PI. This validates PI as an animals' preference measure, supporting the importance of both the preferred and non-preferred classification of resource items with separate biological relevance. Considering specifically the Nile tilapia preference for background colors, we reinforced blue as a color with welfare value for this species, while yellow should not be considered. Despite this general pattern for the Nile tilapia, individual preferences should also be considered rather than group responses.

Taking into account that fish rejected yellow color as their most preferred option and selected it as their most non-preferred option (Figure 3A), Nile tilapia showed that they do not want to be on a yellow background color, at least in the context of the colors tested here. Such a response is also detected by the intensity of preferences, as the values of the PIs for yellow were the lowest ones (Figure 3B). This makes sense because the fish itself is more contrasted and so more visible against the yellow background – the lightest color in our tests. Thus, fish in yellow compartment may be concerned about being more visible to potential predators; however, this assumption needs further investigation.

On the other hand, Luchiari *et al.* (2007) demonstrated that Nile tilapia prefer yellow light. This difference, compared to our results, may be explained by the fact that these authors evaluated the preference responses for light color (incident color), and not background colors as tested here. However, methodological differences might better explain these contradictory results. We evaluated the preferences for 7 consecutive days after 3 days of adjustment in test conditions, while Luchiari *et al.* (2007) tested the preference responses immediately and over only 2 days, which translates to the period of adjustment that we gave the animals in the test conditions (as shown in Maia & Volpato, 2016; Attachment). This highlights the need to evaluate preference consistency over time and for a longer period than 2 or 4 days that is typically the time over which these assays are scored. On the other hand, blue color was detected here as one of the most preferred colors with also the highest PI values (Figure 3). Considering that blue light reduces stress response (cortisol: Volpato & Barreto, 2001; ventilatory frequency: Maia & Volpato, 2013) and improves reproduction (Volpato *et al.*, 2004) in the Nile tilapia, we emphasize blue as a relevant color to improve welfare in the Nile tilapia and this supports our methodological approach.

Considering the motivation response of fish, Nile tilapia pushed less on the hinged doors to access yellow and red colors (Figure 4A), indicating that this species is less motivated to access the color avoided in preference tests (yellow), and also the red color. In fact, red color impairs growth of some fish species (Ruchin, 2004), including the Nile tilapia (Luchiari & Freire, 2009; Volpato *et al.*, 2013), possibly explaining why fish were less motivated to access the red background color, as shown here. Our approach agrees with Duncan (2006)'s proposal that evaluating animals' preferences for environmental resources can be combined with test for motivation to access each specific resource. Here, the differences between preference and motivation responses may have been a consequence of context. That is, in the preference tests fish could freely access any colored compartment, while in the motivation tests fish were restricted to a white and small chamber (Figure 2) and were able just to try access to the blocked colored compartments.

However, although there were some differences between preference and motivation responses for the colors, as explained above, our findings also show that the motivation to access items can distinguish preferred from non-preferred items. In fact, the main pattern (preference for blue and non-preference for yellow) was still maintained between preference and motivation tests (compare Figures 3 and 4). More importantly, as the animals showed that they were more motivated to access their previously preferred items identified here by PI calculations, these findings demonstrate that motivation tests empirically corroborate the PI, proposed by Maia & Volpato (2016; Attachment), as a key tool to determine preferred and non-preferred options. In this way, our results validate preference and non-preference classification, as proposed by Maia & Volpato (2016; Attachment), as two biological patterns with different significance for the animals. It should be more important for the animal to access a previously preferred item than to reach a non-preferred one. Therefore, the PI indicates animal motivation for resources, which is of importance for the welfare issue.

Considering the above conclusion, results from one-preference fish should be considered more carefully. These represent the fish that are most decided when given multiple-choice options. These fish expressed the same avoidance response for yellow and low motivation to access yellow and red colors. However, some variations of response were observed (compare Figures 3A,B and 4A with Figures 3C,D and 4B). For example, one-preference fish avoided not only yellow but also red color in preference tests, considering the intensity of preference responses (Figure 3D). Moreover, these fish selected red color less often as their most preferred color and more often as their most non-preferred color, compared with analyses including all tested fish (contrast Figures 3C and 3A). This means that the one-preference fish expressed a more similar response between preference and motivation tests, by avoiding and being less motivated to access both yellow and red colors (Figures 3C,D and 4B). This reinforces that motivational responses indicate preferred options shown in PI calculations.

Nile tilapia also expressed individual variability in color preferences. They were more motivated not only to access their individually most preferred color, but also to reach all their own preferred colors than to reach all their non-preferred options (Figure 5). Despite this, studies often evaluate preference (*e.g.* Basolo, 1990; Girguis & Lee, 2006; Liste *et al*., 2014) and motivation responses (*e.g.* Matthews & Ladewig, 1994; Mason *et al.*, 2001; Sherwin, 2004; Asher *et al*., 2009; Houpt, 2012) to detect better species conditions irrespective of individual variability. However, our results highlight the need to consider individual variability. This is consistent with the significant individual variation for preferences demonstrated for other choice resources (Godin & Dugatkin, 1995; Wolfgang & Birkhead, 2004; Browne *et al*., 2010; Johnsson *et al*., 2000). Thus, our findings support the PI (Maia & Volpato, 2016; Attachment) as a reliable and useful method to identify individually preferred and non-preferred options as two distinct biological responses.

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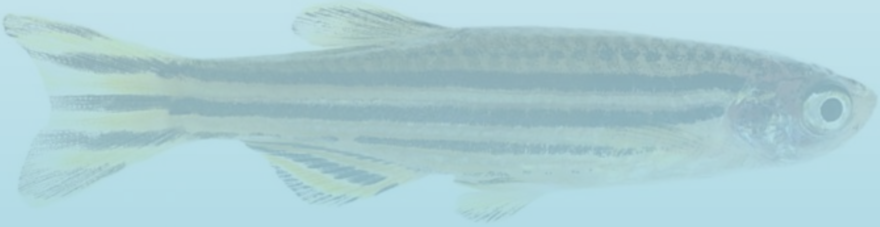
Imagem da tilápia-do-Nilo: adaptação de foto retirada do site www.ittiofauna.org

- Capítulo 2 -



O zebrafish é física e psicologicamente motivado para acessar cores ou plantas artificiais preferidas, determinadas individualmente por cálculos do IP?

Individual preference for environmental conditions increases zebrafish physical and psychological motivation to access these

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Abstract

Animal preferences are often inferred from choice tests, while the motivation to access preferred items is measured in terms of effort tests. What animals prefer tends to vary among individuals, but assessment of preference is often performed on groups of animals. Here, we investigated the kind of environment individual zebrafish prefer and whether they are motivated to access the preferred options. Individual fish were tested in circular aquaria evenly divided into 4 compartments. Twelve fish were allowed to choose between compartments with different background colours, while 12 other fish chose between compartments with different kinds of artificial plant. Visitation frequency in the compartments was registered every 30 s during 1 h trials that were performed over 7 consecutive days. These observations were used to calculate a preference index score for each fish. Individual fish were then tested in two kinds of motivation test. (i) A physical effort test: fish were put into a dark chamber with four transparent, hinged doors that faced the different choice compartments, and the frequency of pushes on each door was quantified to infer the motivation of the fish to access different options. (ii) A psychological effort test: fish had to swim across an open, brightly lit area to access compartments that were their primary preference; secondary preference (or the least non-preferred item); or the compartment that they had shown least interest in. The fish showed significant individual variability in their preferred options and in the strength of preference for these options. Moreover, fish were willing to push more on hinged doors that had access to their most preferred compartments compared to other available choices. When forced to cross a brightly lit open area,

more fish selected to enter their primary preference compartment. We conclude that there is variation among individuals in terms of their preferences and that zebrafish are physically and psychologically motivated to gain access to their most preferred options.

Introduction

Preference tests have been intensely used in animal welfare research to try to detect conditions that provide a better captive environment for animals. This approach has become popular because it enables us to determine the "wants" and "desires" of animals, and it improves on other physiological and behavioural welfare indicators that are often challenging to interpret (Volpato 2009; Volpato *et al*., 2009). In preference tests, animals must choose between one of two (binary tests) or more choice options (multiple choice tests). Such tests were pioneered by Dawkins (Dawkins 2006, 2008; reviewed in Galhardo *et al*., 2009), who emphasized the importance of applying such tests to determine animal preference. In this context, many studies have now evaluated animal preferences for various environmental characteristics (*e.g.* sucrose: Bartoshuk *et al.,* 1971; sexual partner: Ryan, 1980; Basolo, 1990; Braithwaite & Barber, 2000; Liao & Lu, 2009; substrate: Webster & Hart, 2004; Galhardo *et al.,* 2009; temperature: Girguis & Lee, 2006; food: Jung & Kralik, 2013; space to feed: Rioja-Lang *et al.,* 2012).

Although preference tests have been widely applied, there are a number of underlying issues with respect of their use and interpretation. First of all, preference is generally inferred qualitatively (preference does or does not exist) from a momentary choice made by the animals. Thus, choice and preference have been treated synonymously (*e.g.* Schluter *et al.*, 1998; Schlupp *et al.*, 1999; Webster & Hart, 2004; Galhardo *et al.,* 2009; Liao & Lu, 2009), and the intensity of the response is often overlooked. Moreover, studies of choice and preference are usually made on group responses rather than at an individual level (*e.g.* Soriguer *et al.*, 2002; Matsumoto *et al.*, 2008; Burfeind *et al.*, 2009; Snowberg & Benkman, 2009; Zizzari *et al.*, 2009), although individual variability can be found when animals are in choice tests repeated over a few days (Godin & Dugatkin, 1995; Johnsson *et al.*, 2000; Wolfgang & Birkhead, 2004; Browne *et al.*, 2010).

Recently, Maia & Volpato (2016; Attachment) demonstrated two distinct profiles concerning choice: a) non-preference (inconsistent choices across tests conducted on several consecutive days) and b) preference (consistent choices over time). To explore these different responses, Maia and Volpato developed the use of a *Preference Index* (PI), which identifies not only individual preferences and non-preferences, but also the intensity of these responses. Over 10 consecutive tests, their study found significant individual variability in preferred and non-preferred items and in the intensity of such responses. This variability reinforces the need to evaluate the profiles of individual preferences and non-preferences, as well as the intensity associated with these.

Motivation tests have been proposed (Hursh *et al.*, 1988; Cooper & Appleby, 1997; Duncan, 2006; Hovland *et al.*, 2006; Asher *et al.*, 2009) and used (*e.g.* Matthews & Ladewig, 1994; Mason *et al.*, 2001; Sherwin, 2004; Albentosa & Cooper, 2005; Hovland *et al.*, 2006; Kruschwitz *et al.*, 2008; Asher *et al.*, 2009) as a way to determine how much animals want to obtain certain resources. Duncan (2006) suggested measuring animal preference using choice tests, while also applying motivation tests to gauge how much effort the animal is willing to spend to gain access to what it prefers. To differentiate between preference and non-preference, while also taking into account variation in individual response, it is possible to test whether animals strive more to reach items that were previously the most preferred ones compared to the most non-preferred items. From an animal welfare perspective, finding out a consistent preference as opposed to a non-preference represented by momentary and weak choices could be a more informative means of determining what animals want. If animals are more motivated to attain their most preferred items rather than their weak choices as non-preferred items, this would reinforce the differentiation proposed by Maia & Volpato (2016; Attachment), and would help validate the PI as a useful tool to detect intensity of preferences and non-preferences.

To investigate this, we tested whether zebrafish (*Danio rerio*) display individual variability in their response to preference and non-preference, and in their intensity of such responses. In addition, we tested whether these animals apply more effort and, therefore, are more motivated to obtain an item that was previously the most preferred. We chose to work with zebrafish, because this species is increasingly used as a model organism in several areas of science, as well as being an extremely common ornamental fish, thus, conclusions about zebrafish preferences, non-preferences and their motivation to reach them, are highly relevant for improving maintenance in captivity.

Methodology

Animals and Holding Conditions

We used adult zebrafish that were bred and maintained in aquaria at Pennsylvania State University, USA. Fish were fed twice a day, with commercial flake food in the morning and with live artemia larvae in the afternoon. Water temperature was maintained at 25.0 ± 1.0 ^{0}C; pH ~ 8.5; ammonium < 0.3 ppm and nitrite < 0.05 ppm. Aeration and mechanical, chemical and biological filtration were provided, and partial water changes were carried out fortnightly. The photoperiod was maintained from 8:00 h a.m. to 8:00 h p.m. (12:12), with white light.

Experimental Design

Fish were selected by first isolating them for 24 h (in aquaria: 35 x 19 x 27cm), this was done to find fish that were actively swimming throughout the tank and helped avoid selecting fish with a strong tendency to freeze (Levin *et al.,* 2007; Egan *et al.,* 2009; Wong *et al.,* 2010). Two groups of 12 fish were created this way.

Two kinds (conditions) of preference test were given: a series of four compartments of different colours (Figure 1A; n = 12), or three kinds of artificial plants and one empty compartment with no plants (Figure 1B; n = 12). Individual fish were exposed to one of these conditions for 1 h at the same time of day for 3 consecutive days as an acclimation period and then for 7 consecutive test days, when they were filmed. A PI (Maia & Volpato, 2016; Attachment) was used to determine individual preferences, non-preferences and the intensity of these responses. Then, on the 11th day, a physical effort test was given to the individuals tested in both conditions (colours, n = 12; plants, n = 11, one video was lost) to investigate the motivation of the fish to reach their most preferred option identified during the 7 days of preference tests. On the next day (12thday), a psychological effort test (colours or plants, n = 10; four videos were lost) was used to investigate how willing the fish were to swim over an exposed, brightly lit area to gain access to compartments similar to those experienced during the 7 days of preference tests.

Specific Procedures

The isolation aquaria were equipped with aeration and mechanical, chemical and biological filtration, and a tube heater that stabilized the water temperature (~ 25.0^{o}C). Photoperiod was maintained from 8:00 h a.m. to 8:00 h p.m. (12:12), with white light, during all experimental procedures. The weight (g) and total length (cm) of tested individuals (mean ± sd) did not vary between the test groups (weight: 0.48 ± 0.08 g and 0.48 ± 0.09 g for background colour and artificial plant conditions, respectively; length 3.72 ± 0.14 cm and 3.71 ± 0.17 cm for background colour and artificial plant conditions, respectively; unpaired Student's t test, $P > 0.05$).

Preference tests

Four choice compartments were available in the experimental aquaria. For the background colour condition these were red, yellow, blue and green made by attaching coloured self-adhesive tape to the walls (see Figure 1A); for the artificial plant condition these were 2 branches of a same sized orange, red or green plant in compartments or an empty compartment with no plants (see Figure 1B).

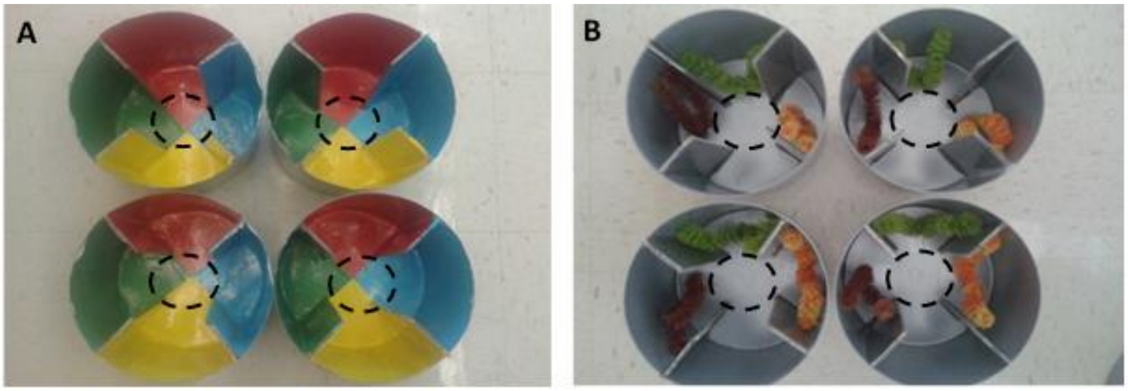

Figure 1: Aerial view of the cylindrical aquaria (27 cm of diameter and 10 cm of water column) used for preference tests. (A) the background colours, and (B) the artificial plant options. The doted circles represent the area created by the central compartment.

On each day of a preference test, individual fish were placed inside a transparent plastic cylinder (diameter ~ 7 cm) in the central compartment of the experimental aquaria (Figure 1). After 5 min, during which the fish could settle and survey the choice options through the clear walls, the cylinder was slowly raised to give the fish full access to the experimental aquaria. Fish were filmed for 1 h, before they were returned to their respective isolation aquaria, where they were then fed. These procedures were repeated each morning for 10 consecutive days, but fish were filmed just on the last 7 days (test days). From the videos, we noted the position of fish every 30 s (120 records per hour of filming) to determine the visitation frequency for each compartment. Every day, before starting the preference tests, the position of the plants was adjusted, so that they stayed in a similar position throughout the trials. To maintain a stable temperature (25.0 ± 1.0^{o}C), each experimental aquaria contained a tube heater. The heater was placed in a different compartment each observation day, so that any possible effect on fish choice would be similarly distributed among compartments.

Physical and psychological effort tests

After the final preference test, a physical effort test was applied the next morning (day 11). These trials were run in the same experimental aquaria, but now, a dark chamber with transparent and hinged doors was placed in the central compartment of each experimental aquaria. The chamber was positioned in such a way that each door faced a choice compartment (Figure 2). Individual fish were placed inside the chamber and filmed from above for 5 min. In a pilot test, zebrafish were found to push on the transparent doors but they could not open them, so we used the number of pushes on the doors as a measure of their motivation to access the different compartments. During the pilot trials the fish continued to push on the doors without giving up, for more than 10 min, so a trial time of 5 min was used. The number of pushes the fish made on each door was recorded. The heaters were removed for these 5-min trials, but the water temperature remained stable at 25.0° ± 1.0°C. At the end of the trials, the fish were returned to their respective isolation aquaria and were fed.

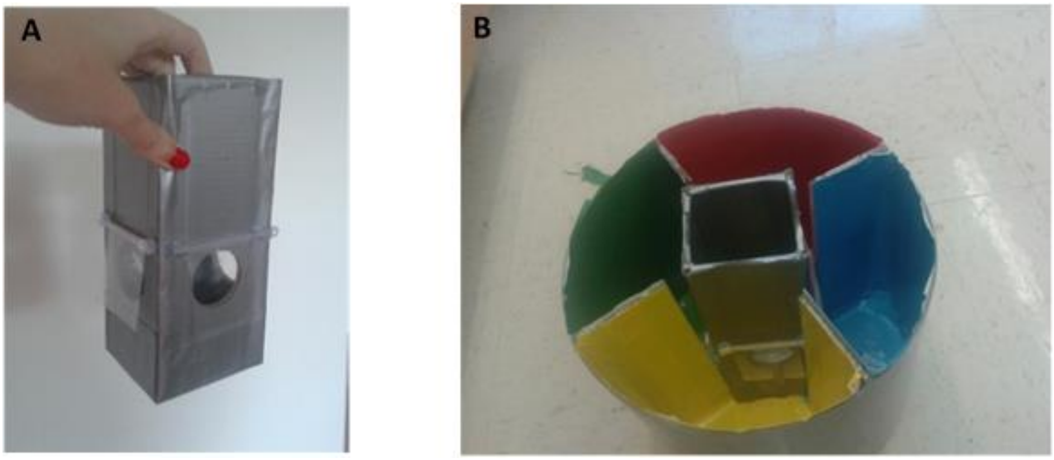

Figure 2: Chamber (7 x 8 x 20 cm) used in the physical effort test, (A) in a front view and (B) inserted in the central compartment of one experimental aquarium.

On the morning of the 12thday, a psychological effort test was applied. This test was performed in experimental aquaria with a start chamber at one end and 3 compartments, of the same size and containing choice options, at the other end (Figure 3). The start chamber had a single exit that led out onto an expansive area with a long (~ 58 cm) and white base in a "V" shape, that the fish find aversive, *i.e.* avoid moving over (Brown *et al.*, 2005). At the opposite end of the tank there were 3 choice compartments containing (i) the primary preference, (ii) the secondary preference (or the least non-preferred item, for fish with just one preference response) or (iii) the most non-preferred item. The positioning of these compartments was alternated between test individuals. Each experimental aquaria had a heater that was removed before testing, but the temperature did not vary between the test conditions (25.0 ± 1.0°C).

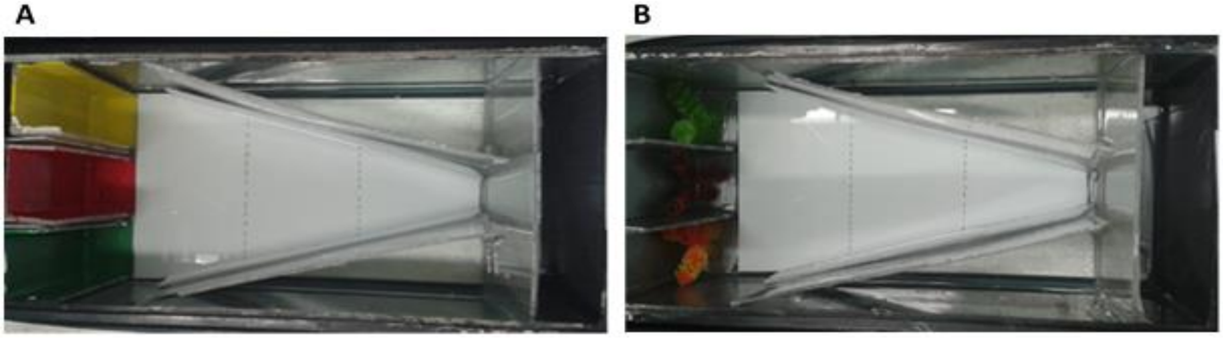

Figure 3: Aerial view of the experimental aquaria (76 x 30 x 33 cm; ~ 5 cm of water depth) used in the psychological effort test for (A) background colour condition, and (B) artificial plat condition. The start chamber is on the far right side (7.5 x 30 cm). On the left side, 3 choice compartments were available (8.5 x 6.5 x 27.5 cm).

During a trial, a single fish was placed into the start chamber and given 5 min to settle. A raisable and transparent trap door was then opened giving the fish access to the whole tank. Trials lasted 15 min. We recorded the first choice compartment a fish entered after crossing the white background. After the trials, the fish were returned to their respective isolation aquaria, where they were fed. On day 13, all test fish were anesthetized using a buffered MS222 solution and measures of weight (g) and total length (cm) were made.

Preference Index (PI) Calculations

It is possible in preference tests for the fish to express more than one preference, or non-preference, because they may select to visit more than one compartment a similar number of times, making it difficult to say that they prefer one of those options over the other. To resolve this, we applied a PI to evaluate preference and non-preference responses over time based on the momentary choices made daily. PI was calculated as described in Maia & Volpato (2016; Attachment). This involves, first summing the daily visitation frequencies (cumulative-frequency) for each choice option for each individual fish and then calculating the area above the cumulative-frequency line. This area increases with the value of frequencies obtained in the most recent tests, a step based on the assumption that the preference response may vary over time and, therefore, the most recent choices may have a higher impact for the calculations. Next, the cumulative area is measured by summing the calculated areas over time. Positive PIs represent preference responses and negative PIs represent non-preference responses. Each individual value of PI represents the intensity of preference/non-preference responses, thus identifying the most preferred and the most non-preferred items per individual fish.

Statistical Analyses

For the physical effort test, the frequencies of pushes on the transparent doors were first proportionally transformed, as fish expressed high individual variation in the total number of pushes applied during the 5 min of test. We compared these proportional frequencies of pushes between the most preferred and the most non-preferred items by dependent T-test when data presented normal distribution and were homoscedastic (Kolmogorov-Smirnov and Levene test,

$P > 0.05$; respectively). If the assumptions for parametric analysis were not achieved, we used a Wilcoxon test.

A Goodman proportion test (1964, among multinomials) was used to analyze the psychological effort test, and compare the frequencies of fish that first entered their primary preference, their secondary preference (or the least non-preferred item) or their most non-preferred compartment. As only frequencies (proportions) of fish were compared here, there was no need to test the data for normality or homoscedasticity. For all these analyzes, we considered $\alpha = 0.05$.

Results

Preferred options varied among individuals in both test conditions, both in terms of the preferred (positive PIs) or non-preferred (negative PIs) options and also in relation to the intensity of each response, measured by individual values of PI (Figures 4 and 5). In relation to background colours, there were 8 preferences for green, 6 for red, 4 for blue and 1 for yellow (Figure 4; positive PIs). In the plant condition, there were 8 preferences for no plant, 6 for the red, 4 for the green and 3 for the orange plant (Figure 5; positive PIs). In both conditions, there were fish that expressed more than one preference (Figures 4 and 5; graphs with more than one positive PI value). Only 1 fish (number 4, Figure 4) had 3 preferences in relation to the 4 options available in the tests. Individual profiles of preference and non-preference responses over the days of test are shown in Figures 4 and 5.

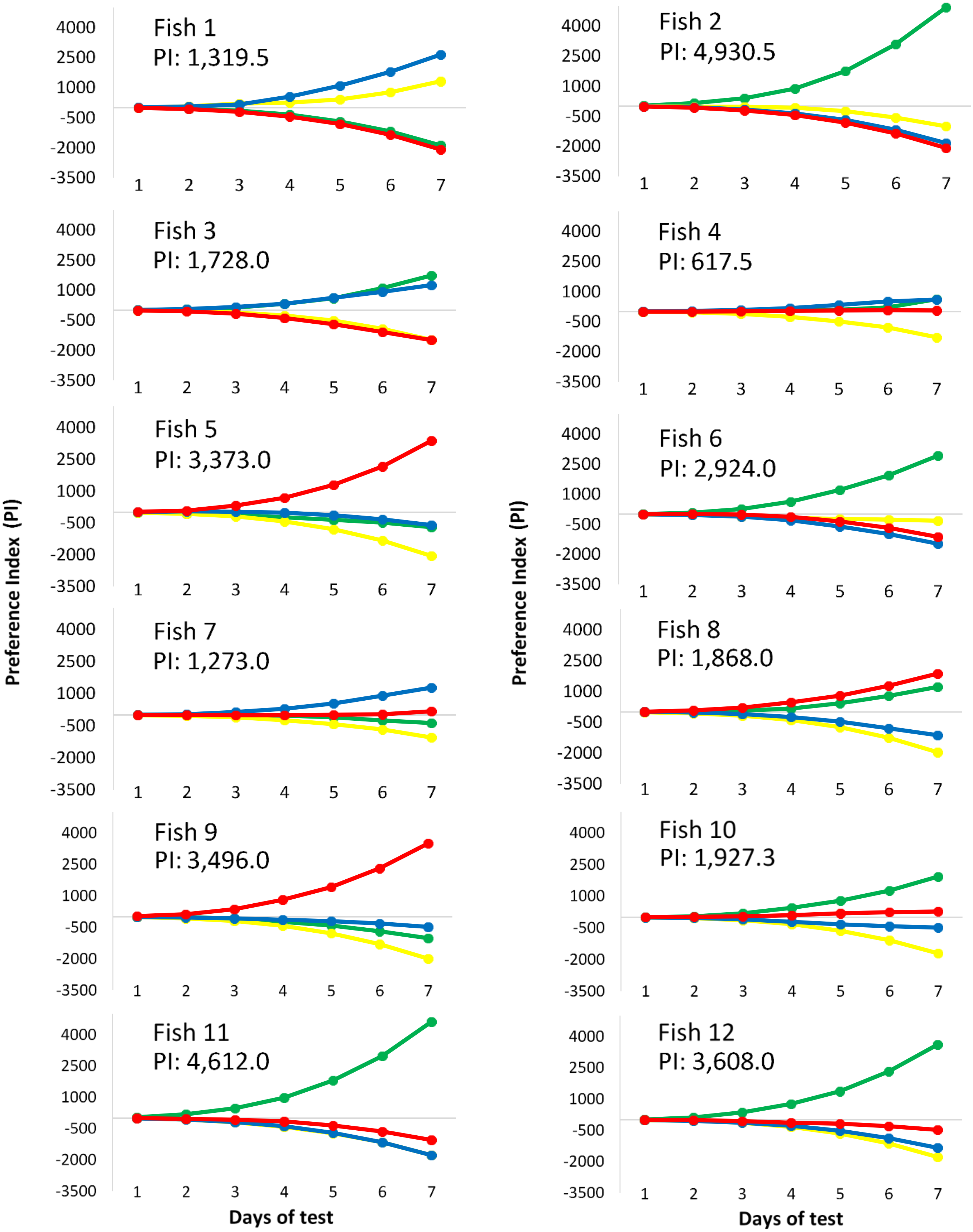

Figure 4: Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses in background colour condition. PI values for the most preferred option for the last test are indicated above lines for each fish.

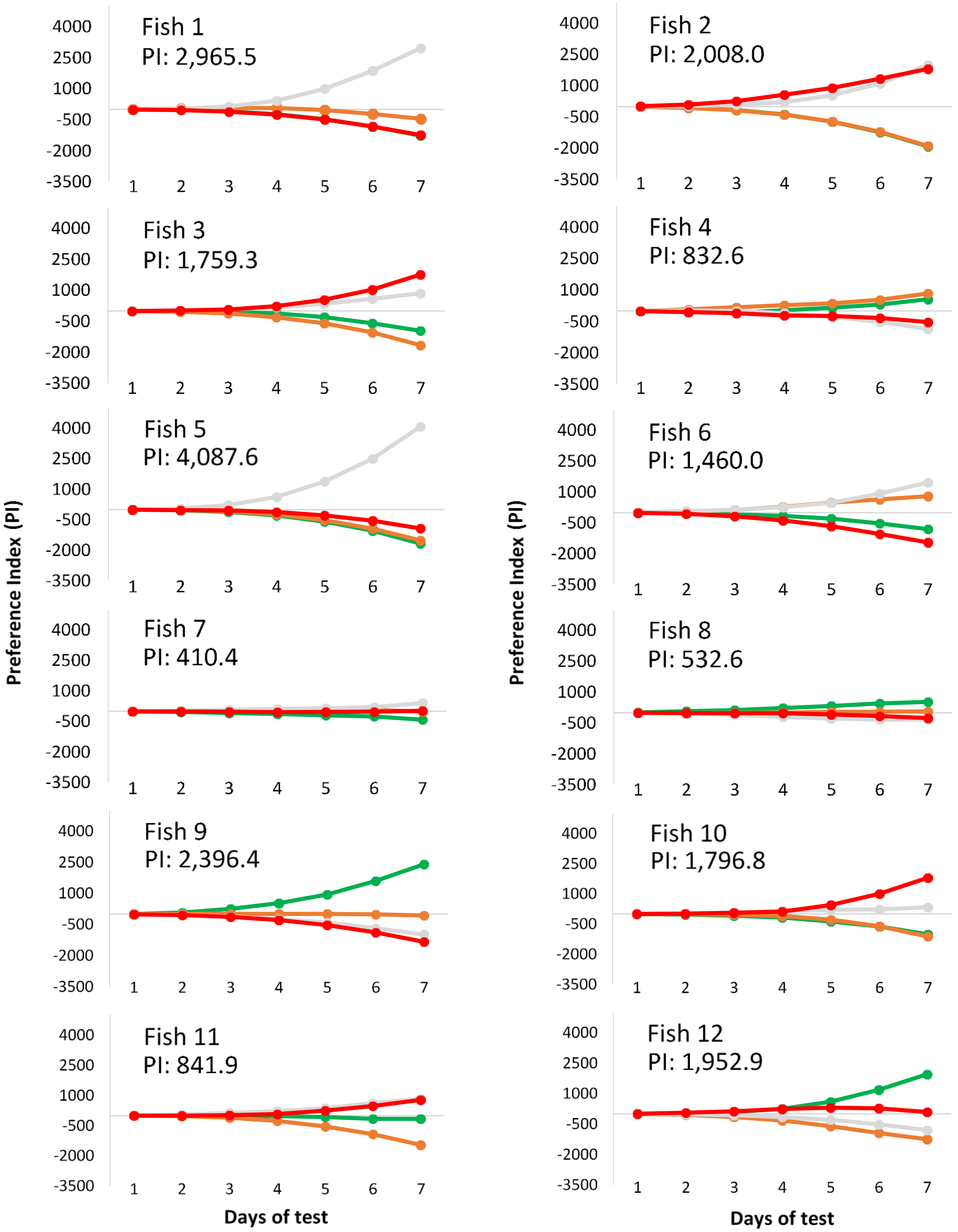

Figure 5: Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses in artificial plant condition. PI values for the most preferred option for the last test are indicated above lines for each fish. —●— = no plant option

In the physical effort test, all fish pushed on the doors during the 5-min test period. Fish pushed significantly more to access their most preferred compartment than to access their most non-preferred compartment both in background colour condition (Figure 6A) and in artificial plant condition (Figure 6B).

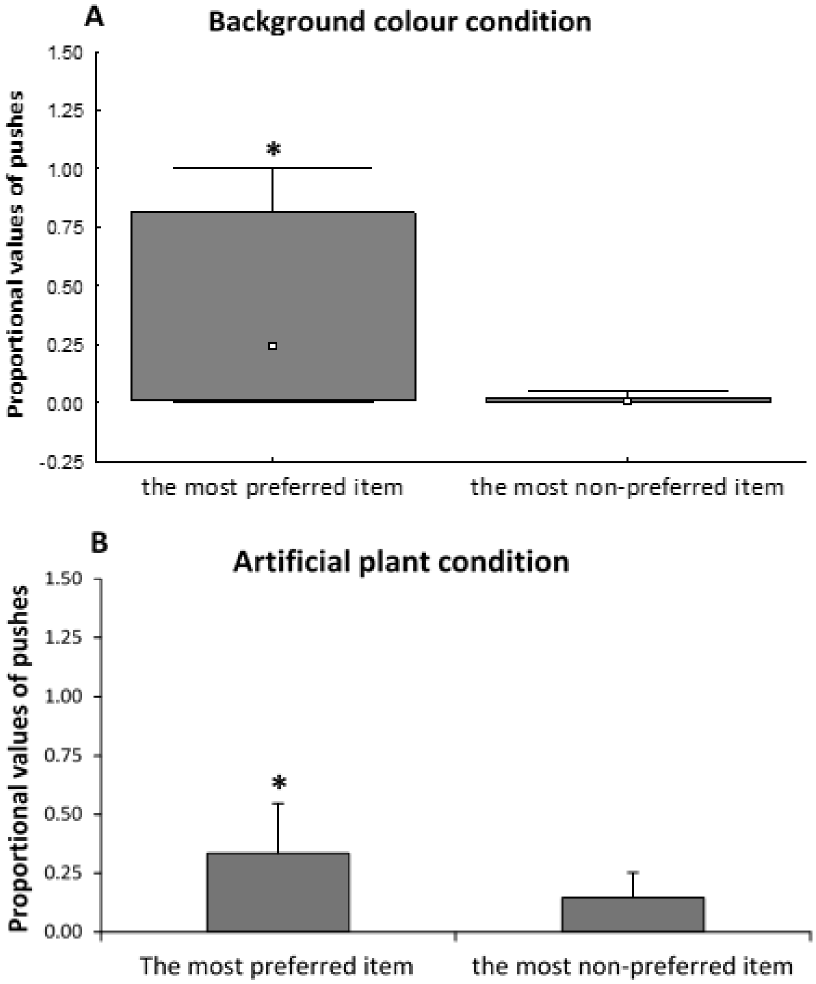

Figure 6: Physical effort test represented by frequency of pushes on hinged doors to reach the choice options in (A) background colour condition and (B) artificial plant condition. * indicates statistical differences in A (Wilcoxon test, W = 41.00, P = 0.04) and B (dependent T-test, t = 2.74, P = 0.02).

In the psychological effort test, all fish crossed over the white and exposed base within the 15-min test period. In both test conditions, a significantly greater number of fish (6 from a total of 10 in background colour and artificial plant conditions) selected to enter their primary preference compartment first compared to fish (1 or 2 from a total of 10 in background colour and artificial plant conditions, respectively) that entered first their most non-preferred compartment (Figure 7). In the background colour condition, 3 individuals entered their secondary preference (or their least non-preferred) compartment first (Figure 7A), and in the artificial plant condition there were only 2 individuals that selected this compartment first (Figure 7B).

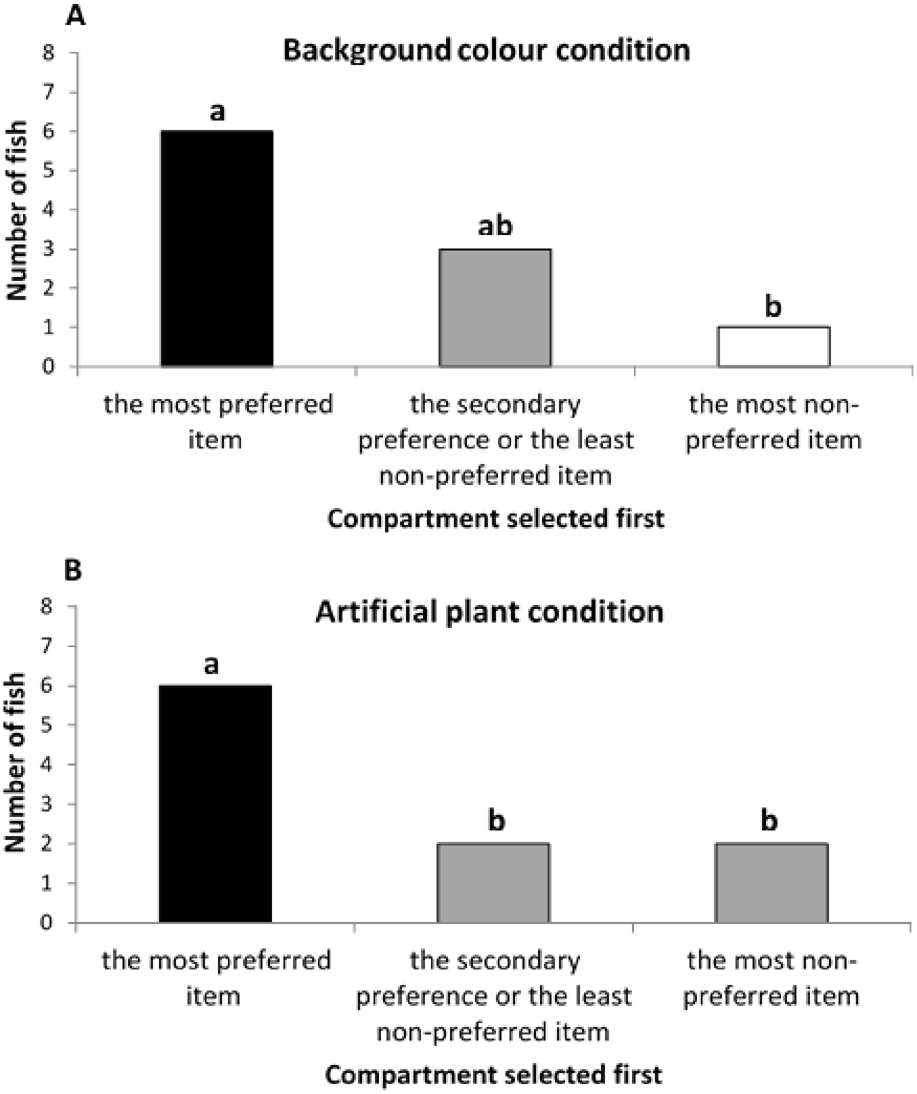

Figure 7: Psychological effort test represent by crossing a long, open and white route to reach the choice options in the opposite site in (A) background colour condition and (B) artificial plant condition. Numbers of fish with different lowercase letters are significantly different (Goodman proportion test, $P > 0.05$).

Discussion

Zebrafish showed significant individual variability in their preference responses both in terms of their preferred option and as in the intensity of their preference responses that were quantified as PIs. There were fish that expressed only one clear preference, while others had more than one; one individual expressed no overall preference across three options that it appeared to use equally. As the preference tests always had four choice options available, a preference for three of these suggests a rejection of the fourth option. In two further tests of motivation, the zebrafish were found to be more motivated, both physically and psychologically, to gain access to compartments with their most preferred items compared to compartments shown to be the most non-preferred or avoided. This study also supports that the PI is expressing motivational forces that drive the animal to express their preferences.

Preference tests have been frequently used to detect conditions that may improve the comfort of captive animals (*e.g.* Bartoshuk *et al.,* 1971; Gonçalves & Oliveira, 2003; Girguis & Lee, 2006; Luchiari *et al.,* 2006; Mendonca *et al.*, 2010; Rioja-Lang *et al.,* 2012; Amcoff *et al.*, 2013; Flamarique *et al.*, 2013; Jung & Kralik, 2013). Typically, such tests infer preference at a group level (*e.g.* Soriguer *et al.,* 2002; Thomas, 2002; Matsumoto *et al.*, 2008; Burfeind *et al.*, 2009; Snowberg & Benkman, 2009; Zizzari *et al.*, 2009), even though some studies have shown significant individual variability in tests applied over a few days (Godin & Dugatkin, 1995; Johnsson *et al.*, 2000; Wolfgang & Birkhead, 2004; Browne *et al.*, 2010). Recently, Maia & Volpato (2016; Attachment) showed that individual preference responses in Nile Tilapia (*Oreochromis niloticus*) can last for at least 10 days, and that not only the qualitative response (there was, or was not a preference) varied among individuals, but also the intensity of the preference responses, measured as PI values, was found to vary (Maia & Volpato, 2016; Attachment). Similarly, in the current study, zebrafish were found to vary in what they prefer, both in terms of what is preferred and how much they preferred it. One individual appeared to reject one option showing no overall preference among the remaining 3 options (fish 4, Figure 4). Therefore, it seems that individuals vary not just in what they prefer, but also what they will avoid. This also demonstrates the utility of calculating the PI values because it can also determine what animals wish to avoid. Given that one of the main focuses of the use of preference tests is to identify conditions that improve the welfare of the animals, our results indicate that future studies of animal preference should take into account the responses made by individuals and not necessarily expect a consistent preference across all members of a population.

A clear observation from the preference tests with the plants was the frequency of preference responses for the empty compartment (8 preferences, out of 21, preferred no plant). Plants are widely used in ornamental aquaria or even in laboratories or other captive environments as a form of environmental enrichment, which is typically considered to be positively promoting fish welfare. However, the results here suggest some caution when using plants in the tank environment, as it seems some individuals prefer to avoid the plants if possible. It is likely that under certain contexts access to plants would be important for zebrafish, perhaps if they perceive an environment to be dangerous and so choose to seek out shelter. Others have noted that the choices a fish makes can depend on previous experience during early development (DePasquale *et al.*, 2016); or on the context, for example, Mendonça *et al.* (2010) found that male Nile-tilapia (*Oreochromis niloticus*) only express a clear choice for a particular substrate when females are present in the environment. Thus, in the case of our study, it may be possible that zebrafish were influenced by their early life experience, or they became more motivated to seek out plants in certain contexts, such as perceiving the environment to be risky or dangerous; however, a direct assessment of this hypothesis remains to be tested.

Considering the motivation shown by the zebrafish, it was apparent that, in both the physical and the psychological tests, fish wanted to access what had previously been shown to be their most preferred option. Therefore, the fish appear to develop preferences and these carry over to different tests. Motivation tests have previously quantified how much an animal is prepared to strive to reach certain environmental resources (*e.g.* Matthews & Ladewig, 1994; Mason *et al*., 2001; Sherwin, 2004; Albentosa & Cooper, 2005; Hovland *et al*., 2006; Kruschwitz *et al.,* 2008; Asher *et al.,* 2009). However, the application of such tests in fish has only been tried recently, and at this stage there is only one published study (Galhardo *et al*., 2011), in which Mozambique tilapia (*Oreochromis mossambicus*) were found to apply more physical effort to gain access to food or a conspecific than they did to reach additional tank space. Here we demonstrate that fish will physically persist at pushing on a hinged door to reach an environment that they have previously shown a strong preference towards. Our results suggest that having access to a specific kind of environment that is strongly preferred is important for the fish. Using the PI (Maia & Volpato, 2016; Attachment) could, therefore, be an important way to quantify what fish want, because it not only detects individual preferences and non-preferences or aversions, but it also describes the intensity with which these occur.

To quantify the motivation of the fish to access an option that they had previously selected under a fear-inducing situation, we designed a test where the fish had to swim over an exposed white base. As zebrafish prefer dim and enclosed environments rather than open and exposed ones, swimming across an area that is both bright and exposed was assumed to be aversive and likely induces a degree of fear and anxiety in the fish (Maximino *et al.*, 2010; Blaser & Rosenberg, 2012; Holcombe *et al.*, 2013). Thus, our results demonstrate that fish will strive to overcome psychologically aversive conditions to reach what they have previously been shown to strongly prefer under non-stressful situations. This test helps to reinforce the differentiation between preference and non-preference proposed by Maia & Volpato (2016; Attachment). Three fish in the background colour condition and two fish in the artificial plant condition decided to enter first the compartment that was previously shown to be their second most preferred option (a preference with lower intensity than their primary preference) or their least non-preferred option. What is preferred or non-preferred and the strength with which that response is made is clearly influenced by a number of factors, but the results here indicate that this use of a PI could be useful for creating positive, better welfare for captive fish.

We conclude that there is significant variation between individuals in terms of preferred items, as well as in the intensity of those preferences. Furthermore, considering the high number of zebrafish that preferred to avoid artificial plants, we suggest some caution when deciding on the kind of environmental enrichment to use for this species. In addition, fish were motivated, both physically and psychologically, to access their most preferred options compared to reaching their most non-preferred ones. The PI proposed by Maia & Volpato (2016; Attachment) can therefore be used to identify individual preferences and non-preferences, and can also be used to inform us about the intensity of an individual's response.

Acknowledgments

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Imagem do zebrafish: adaptação de foto retirada do site
https://sites.google.com/a/murraystate.edu/zeruth-lab/home

~ Capítulo 3 ~

A truta arco-íris é física e psicologicamente motivada para acessar cores, tocas ou coespecíficos em opções preferidas, determinadas individualmente por cálculos do IP?

Physical and psychological motivation tests reinforce a preference index to detect preference, non-preference and rejection in trout *Oncorhynchus mykiss*.

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Abstract

Animal preference and motivation have been used to assess different kinds of environmental resources that may help improve welfare conditions. However, preference and motivation are typically tested separately, and these assays disregard the individual nature of responses. Here, in rainbow trout, we evaluated individual variability of preference for different resources, and whether these fish are motivated to access such preferred options. Individual fish were allowed to sample among 4 different compartments. Two different kinds of test were used, one where the compartments varied in background color, the other where the compartments varied in what they contained. Over a series of 10 days, the fish interacted with these different options and during the trials of the last 7 days their visitation frequency to each compartment was registered every 30 s. The data were used to calculate a preference index for each fish. Physical motivation of individual fish to access preferred and non-preferred options was then tested by quantifying the frequency of pushes the fish gave to transparent hinged doors that led to the different compartments. In a separate test, whether fish entered a preferred or a non-preferred option after crossing an aversive open and brightly long area was used as a measure of psychological motivation. Trout showed a strong preference for blue backgrounds, and were both more physically and psychologically motivated to access this color. However, preference and motivation for alternative items such as shelters or conspecifics were more variable. These results show that the physical and psychological motivation of trout depend on the resources

that they are trying to access, but the fish express stronger motivation to gain access to more preferred options.

Introduction

Attempts to detect better environmental conditions for animals have used preference responses, but these are typically inferred from momentary choices in tests performed over a few trials (*e.g.* Bartoshuk *et al*., 1971; Basolo, 1990; Girguis & Lee, 2006; Liste *et al*., 2014). Recently, however, Maia & Volpato (2016; Attachment) have demonstrated that momentary choices do not necessarily represent preferences as consistent responses over time. Their results revealed that there are two biological patterns concerning the choices: preference and non-preference responses, which may represent different meanings for the animals. Based on these measures, a preferred item is expected to be an option that will improve the animal's environmental conditions, because it represents a consistent choice by the animal over time for that item. On the other hand, a non-preferred item should be avoided, because the animal chooses it significantly fewer times. Moreover, still considering the design of preference tests, many studies have ignored individual variation in preference response, even though significant variation of preference for different resources has been widely reported (Godin & Dugatkin, 1995; Johnsson *et al.*, 2000; Wolfgang & Birkhead, 2004; Browne *et al.*, 2010; Maia & Volpato, 2016).

A different approach used to infer animal wants is the motivation test. This is based on the idea that the more the animal is motivated to reach a specific resource, the more important that resource is to the animal. In fact, Duncan (2006) has suggested that to identify what animals want, both preference response for environmental resources, and the importance of these resources should be determined. In this way, many studies of motivation have evaluated the effort that the animals are willing to spend to access different resources (*e.g.* Matthews & Ladewig, 1994; Mason *et al.*, 2001; Hovland *et al.*, 2006; Asher *et al.*, 2009; Houpt, 2012). Such studies usually test the effort spent only on a physical level; for example, by measuring over time the frequency of pushes on weighted doors where the door leads to a specific resource; or by increasing, over time, the frequency of repetition of a specific behavior necessary to access a given resource.

It seems natural to expect that motivation in these kinds of task will be associated with preference responses for resources, as both these approaches appear on Dawkins' proposal (2006, 2008) that animal wants should be known for welfare purposes. However, although there are many papers that have evaluated preference or motivation for different resources, they have usually ignored the relationship between preferred items and the motivational responses to get access to these items. This is even more relevant considering a new proposal by Maia & Volpato (2016; Attachment), that preferred and non-preferred options of the animals are two different biological patterns, that can vary among individuals, which are based on individual choices detected over time, and expressed by a *Preference Index* (PI). If individual preferences and non-preferences (calculated as the PI) represent two distinct processes, they should also evoke different motivational forces, resulting in significant differences in response in motivation tests. It makes sense that an animal will be more motivated to reach its own preferred options than to access their individually non-preferred ones. To date, this new index has been used to identify the individual wants and non-wants for Nile tilapia (*Oreochromis niloticus*); however, it is imperative to evaluate the applicability of this new index as a reliable measure in other species.

Thus, here we ran trials with rainbow trout (*Oncorhynchus mykiss*), another commercially important fish species intensely maintained in captivity, but one with a very different natural history to Nile tilapia. We evaluated whether rainbow trout are more motivated to access their individually preferred options as opposed to reaching their non-preferred ones, detected by PI calculations. For this, we tested trout preferences and motivations for three different kinds of environmental resources: background colors, shelters or conspecifics. Background colors were tested because they affect physiology and behavior of fish species as seen in the stress response (Head & Malison, 2000; Karakatsouli *et al.*, 2008; Barcellos *et al.*, 2009; Maia & Volpato, 2013), growth (Head & Malison, 2000; Ruchin, 2004; Karakatsouli *et al.*, 2008) and reproduction (Volpato *et al.*, 2004). The other tested resources are considered as relevant for rainbow trout; shelter (Conallin *et al.*, 2012) and presence of conspecifics (Dunlop *et al.*, 2006; Øverli *et al.*, 2006). In addition to assessing fish motivation using a physical effort test, we evaluated the psychological motivation of the trout to overcome an aversive stimulus to reach preferred or non-preferred items. Psychological processes have been gradually more considered in the welfare issue in the sense that wellbeing is beyond physical aspects. This approach is more incipient in welfare science, but has received more attention in the last decade (Volpato *et al*., 2007). Moreover, stress has a clear psychological component that cannot be ignored as we try to understand animal welfare needs.

Methodology

Animals and Holding Conditions

We used 36 juvenile rainbow trout, *Oncorhynchus mykiss,* from hatchery centers in Pennsylvania (USA). The fish were kept for at least two weeks in our vivarium, in a low density (1 fish/11 l), before the experiments began. Fish were kept in tanks with stabilized water recirculation system and were fed once daily with appropriate commercial food (Pentair Aquatic Ecosystems - Dense Culture). The water temperature was maintained at around 10^{o}C; pH ~ 6; ammonium < 0.3 ppm; nitrite < 0.5 ppm. Partial water changes were carried out fortnightly and the photoperiod was maintained from 7:00 h a.m. to 6:00 h p.m., with white light.

Experimental Design

Different fish were independently tested in one of three conditions: a series of four compartments of different colors (background color condition; Figure 1A; n = 12); compartments with four options of different numbers of available shelters (shelter condition; Figure 1B; n = 12); or a series of four compartments containing different numbers of conspecifics (conspecific condition; Figure 1C; n = 12). Fish were individually exposed to these conditions for one hour in the morning, at the same time, during 10 consecutive days and were filmed from above in the last 7 days (test days), with the first 3 days considered as an acclimation period. By replaying the films, visitation frequency to each option was registered and used to calculate the PI as described in Maia & Volpato (2016; Attachment). This determined preferences, non-preferences and the intensity of such responses for each individual fish. On day 11, we applied a physical effort test to the same individuals. This was done by registering the number of pushes the fish gave to transparent doors that blocked the compartments. This was used to infer the physical motivation of the fish to access their most preferred and most non-preferred options. On the morning of day 12, we applied a psychological effort test to 10 individuals from each condition (a smaller sample size here owing to the loss of some videos). This assay evaluated how willing the fish were to swim across a long, exposed, white background to access both their most preferred and non-preferred compartments.

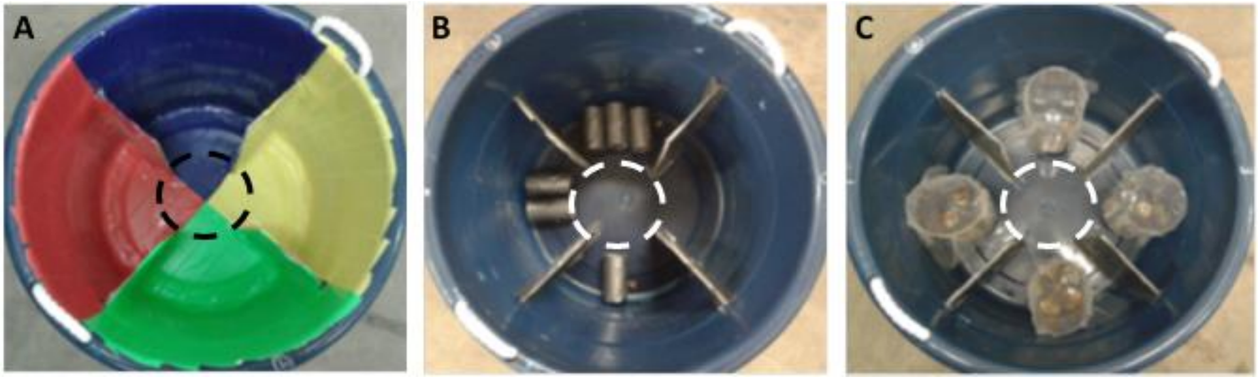

Figure 1: Top view of the test aquaria (~ 50 cm in diameter) used for preference tests in background color (A), shelter (B) and conspecific (C) conditions. The colors (blue, green, red and yellow) in A were obtained by using colored plastic adhesive tape to cover the choice compartments. Shelters in B were made with PVC cylinders (~ 4 cm in diameter and ~ 9 cm long) covered with plastic adhesive tape in a gray color. In C, the transparent perforated containers (~ 10 cm in diameter) with conspecifics were similarly positioned in the center of each choice compartment, with the option of no conspecific being made by the introduction of an empty container in one choice compartment. The dotted circles represent the central compartment, used for daily introduction of the focus fish. The height of the water column was maintained at ~ 15 cm.

Specific Procedures

Throughout the experiment, fish were kept individually in isolation aquaria (35 x 19 x 27 cm; but could see other focal fish in their respective isolation aquaria) after each daily test until their test trial the next day. The isolation aquaria were equipped with aeration and mechanical, chemical and biological filtration, which maintained the water quality similar to that in the group holding conditions. The photoperiod was maintained from 7:00 h a.m. to 6:00 h p.m., with white light, and the room chilling system maintained the water at ~ 10^{o}C throughout the experiment.

Thus, in test aquaria, the temperature (mean ± sd) did not vary among the test conditions during the preference tests (10.08 ± 0.19^{o}C, 10.03 ± 0.24^{o}C and 10.00 ± 0.26^{o}C for background color, shelter and conspecific conditions, respectively; repeated measures ANOVA, $P > 0.05$). Moreover, no significant variation of temperature among test conditions was shown in physical effort test (9.86 ± 0.55^{o}C, 9.88 ± 0.50^{o}C and 9.81 ± 0.57^{o}C for background color, shelter and conspecific conditions, respectively; one-way ANOVA, $P > 0.05$), nor in the psychological effort test (9.59 ± 0.54^{o}C, 9.72 ± 0.44^{o}C and 9.73 ± 0.51^{o}C for background color, shelters and conspecific conditions, respectively; one-way ANOVA, $P > 0.05$).

Preference tests

Fish were individually isolated for 24 h. Then, we selected focal fish that were swimming naturally in the presence of the observer, without constantly swimming around the bottom of the tank, which indicates fish with little fear or anxiety (Levin *et al*., 2007; Wong *et al*., 2010). Next, fish were individually placed in the central compartment of test aquaria (Figure 1), inside transparent plastic cylinders (~ 12 cm in diameter) for 5 min. After that, we carefully removed the cylinders, allowing free access of fish to the choice compartments. For background color the 4 choice compartments were blue, green, red or yellow; for shelter condition the choice compartments contained 0, 1, 2 or 3 shelters; and for conspecific condition the choice compartments contained 0, 1, 2 or 3 conspecifics. Test aquaria were distributed alternately among the test conditions in the laboratory, to decrease any possible effect of differences in light intensity on the surface of such aquaria. Fish were filmed from above for 1 h. After that, they were returned to their isolation aquaria, where they were fed. From the films, we recorded the visitation frequency of fish to each choice compartment every 30 s, totalizing 120 observations per hour. We repeated these procedures for 10 consecutive days, but fish were filmed just for the last 7 days (test days).

Before starting the shelter preference tests, the position of the shelters was adjusted, so that they stayed in a similar position throughout the trials. In the conspecific condition, we used perforated and transparent plastic containers to house conspecifics inside the compartments of test aquaria, allowing both visual and chemical communication among conspecifics and focus fish. After each daily test, we returned conspecific groups (groups of 1, 2 or 3 conspecifics) to their respective isolation aquaria, which were enriched in order to decrease aggression between individuals, and the fish were fed. We used three replicates for each choice option of conspecifics (*e.g.* 3 groups of 1, 2 and 3 conspecifics). Each replicate fish group was randomly distributed over all tests for each focal fish, thus avoiding pseudoreplication.

Physical and psychological effort tests

On day 11 we applied the physical effort test. Fish were individually placed inside a dark chamber with transparent hinged and locked doors (Figure 2). The chamber was placed in the central compartment of a test aquarium. Each door of the chamber faced a different choice compartment. Then, we filmed the test aquaria from above for 5 min, a time period shown to be sufficient to fish to be motivate to push on the doors in previous pilot tests. After that, we returned fish to their isolation aquaria, where they were fed. The films were used to score the frequency of pushes made to each door.

Figure 2: Dark chamber (16 x 16 x 32 cm) used in the physical effort tests. Each one of the four transparent doors was made of thin and malleable plastic pasted on the chamber walls, which could be moved but not fully opened by the fish.

On the following morning, day 12, we applied the psychological effort test. This was performed in a long test aquarium containing a dark chamber (start point) with a single exit door at one end, and two compartments of the same size containing the most preferred or the most non-preferred choice options at the other end (Figure 3). The position of such options in the two choice compartments was alternated among fish. The dark chamber and the choice compartments were connected by a long (~ 51 cm), white and exposed route in a "V" shape, which was used to create an aversive area across which the fish had to swim (Brown *et al.*, 2005). Fish were individually placed in the dark chamber, which was blocked by a removable transparent, acrylic door. After 5 min, we carefully removed the door and the fish had free access to the aversive route. The test aquaria were filmed from above for 30 min. From the films, we recorded the first choice compartment where fish entered after crossing the aversive route. After filming, we returned fish to their respective isolation aquaria, where they were fed.

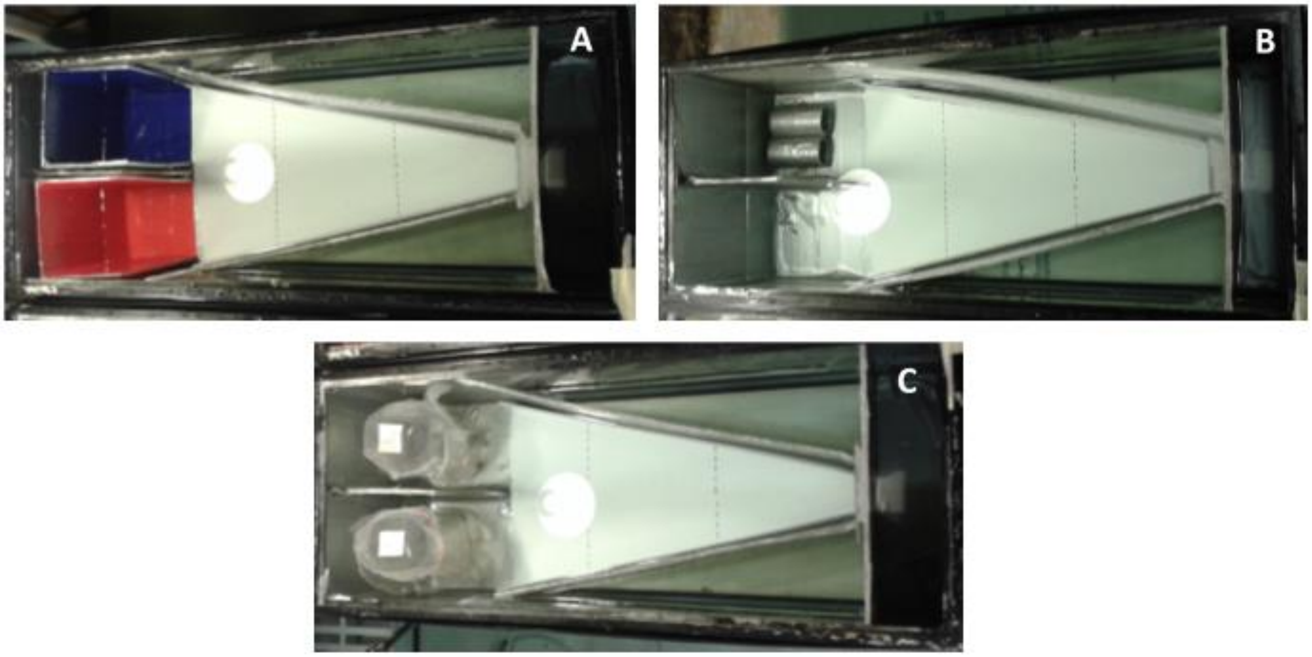

Figure 3: Top view of the long test aquaria (76 x 30 x 33 cm) used in the psychological effort test in background color (A), shelter (B) and conspecific (C) conditions. The dark chamber (start point; 7.5 x 30 cm) is on the right side of each aquarium. On the left side, there are the two choice compartments (13 x 14 x 33 cm each). We added a white light (~ 800 lumens) over the route in each test aquarium (directed to the exit door of the dark chamber) to ensure that crossing it would be aversive for fish. The height of the water column was ~ 10 cm.

On day 13, we measured weight (g) and total length (cm) of all focal individuals and stimulus fish used as conspecific choice options. Weight and total length (mean ± sd) of the focal fish did not vary among the test conditions (total length: 8.69 ± 0.73 cm, 8.44 ± 0.80 cm and 8.26

± 1.08 cm; weight: 6.35 ± 1.84 g, 5.87 ± 1.38 g and 5.42 ± 2.35 g, for background color, shelter and conspecific conditions, respectively; one-way ANOVA, P > 0.05). Moreover, individuals used as conspecifics in different choice options (groups of 1, 2 or 3 individuals) also were of a similar size (total length and weight; mean ± sd) to each other (groups of 3 conspecifics: 8.44 ± 0.96 cm and 6.03 ± 1.96 g; groups of 2 conspecifics: 8.33 ± 0.69 cm and 6.25 ± 1.61 g; groups of 1 conspecific: 9.10 ± 1.40 cm and 7.50 ± 2.65 g; one-way ANOVA, P > 0.05).

Preference Index (PI) Calculations

PI was calculated independently for each choice option and for each individual fish as described in Maia & Volpato (2016; Attachment). Accordingly, the visitation frequencies for each option are summed. Next, the areas above the line of cumulative-frequency (daily visitation frequency summed over time) are determined, so that these calculated areas increase as the trials progress. This is done because the most recent choices may have a higher impact for the calculations of preference responses, as these may vary over time and the more recent ones may better represent the animal wants considering the history of its choices over time. Then, all the calculated areas are summed over time, resulting in cumulative areas. Preference responses are detected as positive PI values, while non-preference responses are detected as negative PI values. Moreover, each calculated value of PI represents the intensity of preference/non-preference responses. Thus, the most preferred and the most non-preferred items per fish are identified as the highest and the lowest PI values per individual, respectively. As some videos from preference tests were lost, data from some test days were missing, thus resulting in gaps in the graphs of figures representing individual profiles of preference and non-preference response.

Statistical Analyses

To compare the number of pushes on the doors in the physical effort test, first we transformed data into proportions of the total number of pushes per fish. This was done because the fish expressed high individual variation in the total number of pushes. When these transformed data were normally distributed and homoscedastic (Kolmogorov-Smirnov and

Levene tests, P > 0.05; respectively), we compared the proportional frequencies between the most preferred and the most non-preferred items by dependent T-test. Otherwise, when data did not reach the assumptions for parametric analysis, we used Wilcoxon test.

A Goodman's proportion test (1964, comparisons among multinomials) was used to analyze data from the psychological effort test. We compared the frequency of fish that first entered their most preferred compartment with the frequency of fish that first entered their most non-preferred compartment. As only frequencies of fish were compared, there was no need to test these data for normality or homoscedasticity. For all analyzes, we fixed $\alpha = 0.05$.

Results

All fish preferred blue (positive PIs) in the background color condition, although the intensity of such responses indicated by PI values varied among individuals (Figure 4). From these, just two fish also preferred another color (fish 2 and 11 preferred green and yellow besides blue, respectively; Figure 4). For shelter and conspecific conditions, preferences varied among individuals, both in terms of preferences (positive PIs), non-preferences (negative PIs) and of intensity of such responses (Figures 5 and 6). In the shelter condition, 4 fish preferred no shelters; 5 fish preferred 1; 9 fish preferred 2; and 5 fish preferred 3 (Figure 5; positive PIs). In the conspecific condition, 1 fish preferred no conspecific; 5 fish preferred 1; 4 fish preferred 2; and 11 fish preferred 3 (Figure 6; positive PIs). In all tested conditions, there were fish with more than one preference (Figures 4, 5 and 6; graphs with more than one positive PI value). Moreover, 1 fish in shelter condition and 2 fish in conspecific condition expressed 3 preferences in relation to the 4 available options in the tests (fish 4 in Figure 5 and fish 7 and 8 in Figure 6), a response not detected in background color condition (Figure 4). The intensity of preference response was much higher in background color (PIs ranged from 1,302.00 to 5,403.00; Figure 4) than in shelter and conspecific conditions (PIs ranged from 221.25 to 1,942.86 in Figure 5 and from 270.00 to 1,992.63 in Figure 6, respectively). Individual profiles of preference and non-preference responses over the days of test are shown in Figures 4, 5 and 6.

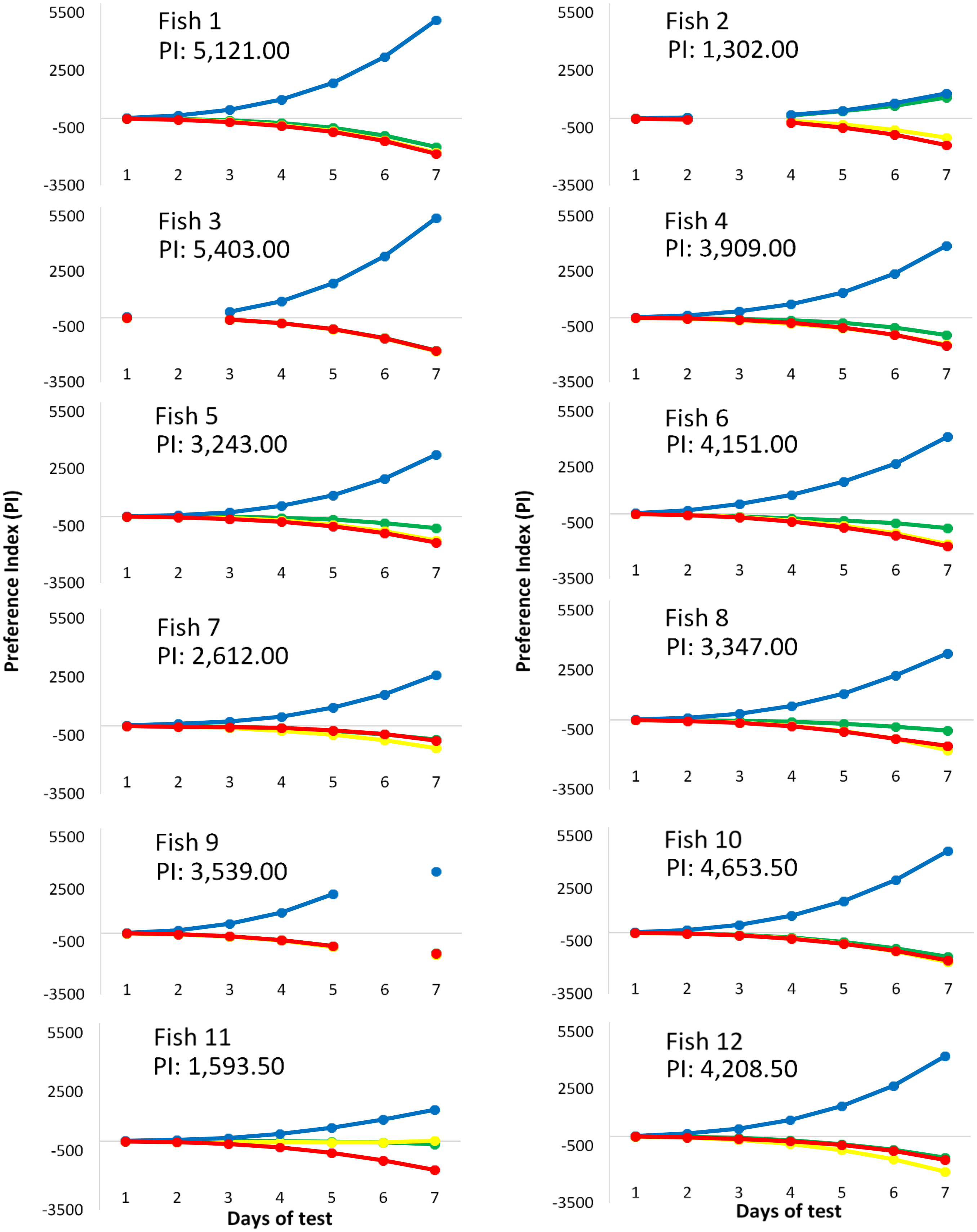

Figure 4: Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses in background color condition. PI values for the most preferred option for the last test are indicated above lines for each fish. Absent data in the graphs were derived from data missing from the videos.

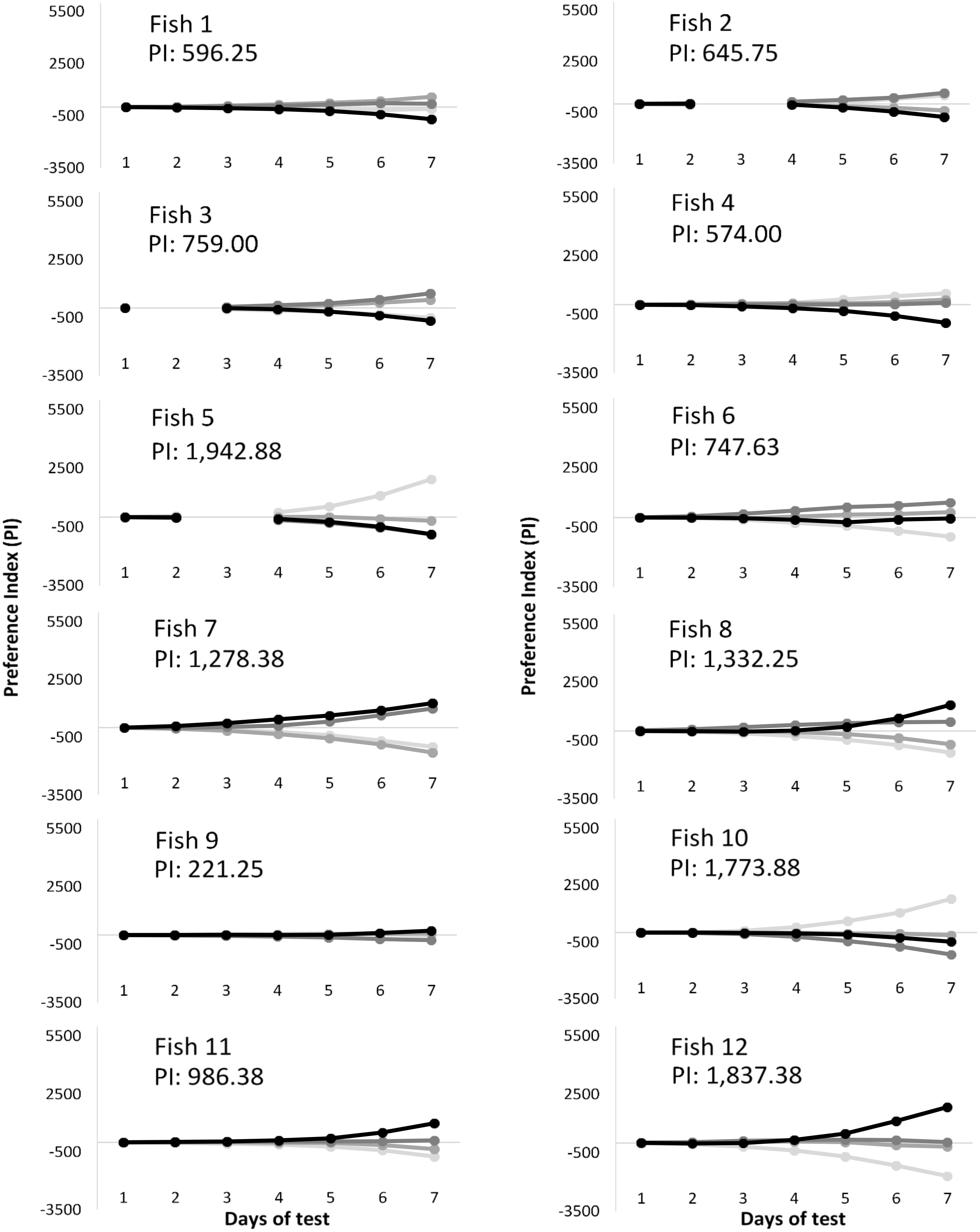

Figure 5: Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses in shelter condition. PI values for the most preferred option for the last test are indicated above lines for each fish. Absent data in the graphs were derived from data missing from the videos. —●— = no shelter; —●— = 1 shelter; —●— = 2 shelters; —●— = 3 shelters

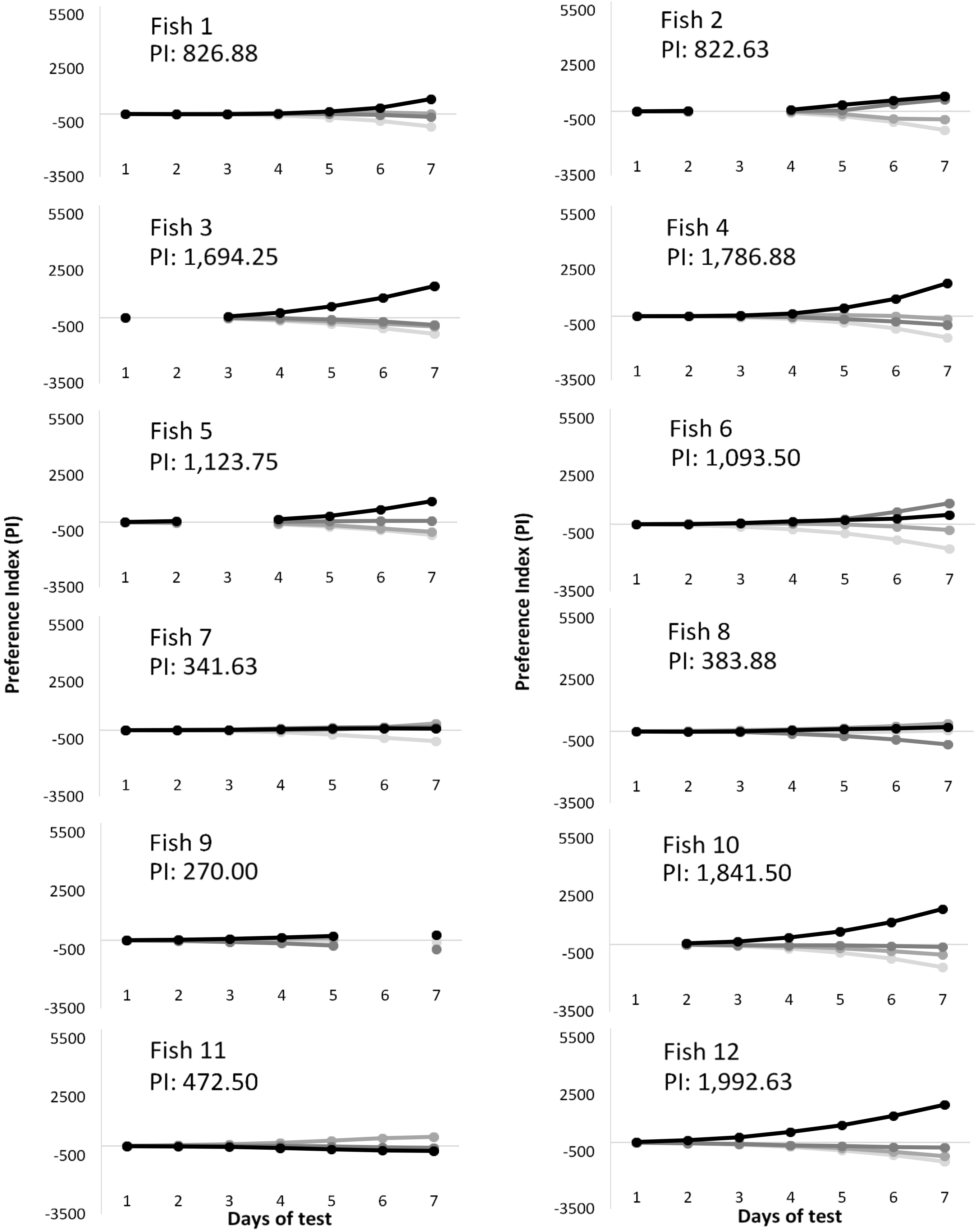

Figure 6: Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses in conspecific condition. PI values for the most preferred option for the last test are indicated above lines for each fish. Absent data in the graphs were derived from data missing from the videos. —●— = no conspecific; —●— = 1 conspecific; —●— = 2 conspecifics; —●— = 3 conspecifics

With regard to the physical effort test, all fish pushed on the doors to access the options during the 5 min of test period. Fish pushed significantly more to access their most preferred color and also their most preferred number of conspecifics than to access their most non-preferred options in these test conditions (Figure 7 A and C, respectively). However, there was no more physical effort spent to access the most preferred number of shelters compared to the most non-preferred number of these (Figure 7 B).

In the psychological effort test, all fish crossed over the long, white and exposed route within the 15-min test period. In background color condition, a significantly greater number of fish (9 out of 10) crossed the aversive route and entered their most preferred color compartment first. (Figure 8 A). However, both for shelter and conspecific conditions, there were no differences between number of fish that entered their most-preferred compartments first (5 and 6 fish from a total of 10, respectively) or their most non-preferred compartment (5 and 4 fish from a total of 10, respectively) (Figure 8 B and C, respectively).

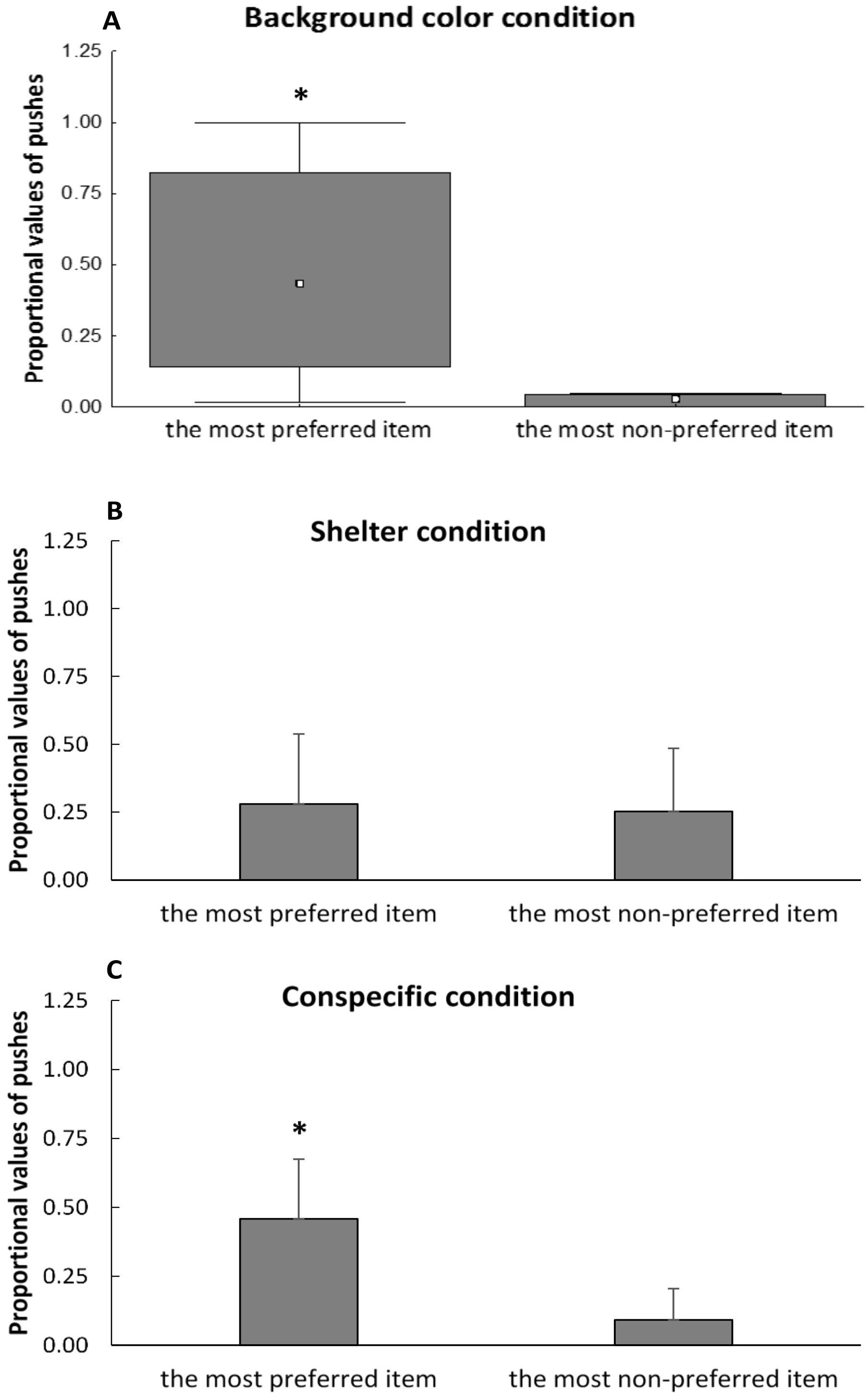

Figure 7: Physical effort test represented by frequency of pushes on hinged doors to reach the choice options in (A) background color, (B) shelter and (C) conspecific conditions. * indicates statistical differences in A (Wilcoxon test, W = 66.00, P = 0.00) and C (Dependent-T test, t = 4.93, P = 0.00). There was no significant difference in B (Dependent-T test, t = 0.29, P = 0.78).

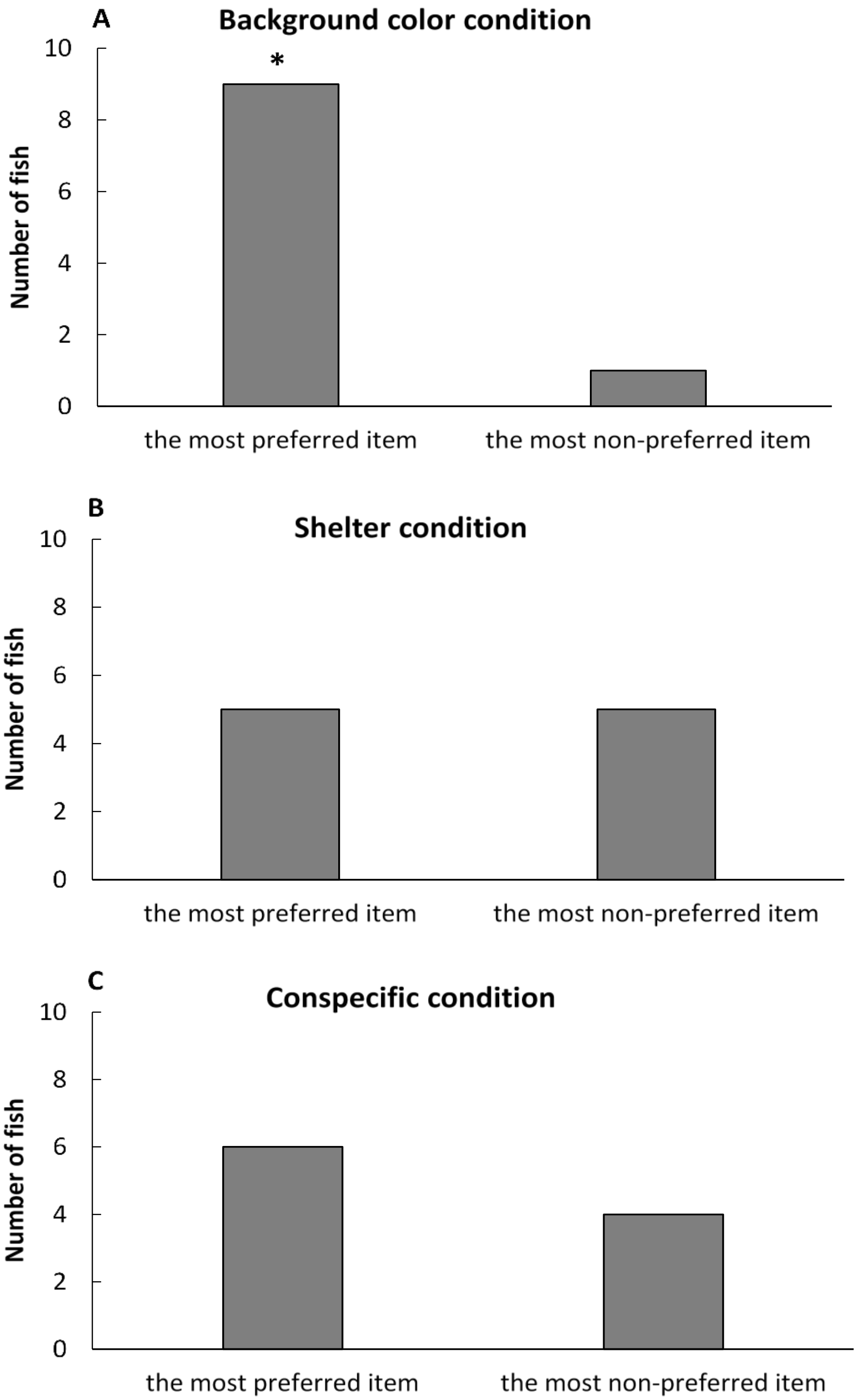

Figure 8: Psychological effort test represented by crossing a long, open and white route to reach the choice options in the opposite site in (A) background color, (B) shelter and (C) conspecific conditions. * indicates statistical differences in A (Goodman proportion test, $P < 0.05$). There was no significant difference in B or in C (Goodman proportion test, $P > 0.05$).

Discussion

Here we demonstrate that rainbow trout prefer a blue background color, but their preference for the number of shelters or conspecifics are individually more variable and less intense, as demonstrated by individual PI values. Three fish selecting shelters or conspecifics preferred three options. As the preference tests always had four available options here, a preference for three of these suggests a rejection of the fourth option. Moreover, rainbow trout are both physically and psychologically motivated to access their preferred blue color, but not necessarily to access preferred shelters or conspecifics. These findings highlight that preference responses, including rejection of options, and physical and psychological motivation of trout may depend on the resources being evaluated. In this case, stronger motivation to access preferred options may be expected when stronger preferences are expressed, thus supporting PI as a reliable tool to indicate individual preference and non-preference responses of the animals.

Taking into account background color condition, rainbow trout expressed a consistent inter-individual preference for blue, as all tested fish preferred this color while just two of them also preferred another color, although with a less intense response (Figure 4). This is in line with the demonstrations that blue improves reproduction (Volpato *et al.*, 2004), decreases stress response (Karakatsouli *et al.*, 2008; Barcellos *et al.*, 2009; Volpato & Barreto, 2001; Maia & Volpato, 2013) and improves growth (Ruchin, 2004) in some fish species. In rainbow trout, this dark-blue preference (as blue was the darkest color tested here) may be related to the fact that this fish species, in natural conditions, are found in darker areas of water bodies, where the contrast of their bodies against the background is less obvious. Thus, the dark-blue color may have been more appealing for these reasons.

On the other hand, considering shelter and conspecific conditions, much more variable and less intense preferences were observed (Figures 5 and 6). While PI values in background color condition varied from 1,302.00 to 5,403.00, the PI range in shelter and conspecific conditions were from 221.25 to 1,942.86 and from 270.00 to 1,992.63, respectively. Thus, although shelters and conspecifics have been demonstrated to be important resources for rainbow trout (shelter: Conallin *et al.*, 2012; conspecifics: Dunlop *et al.*, 2006; Øverli *et al.*, 2006), we suggest that a blue, or a darker background, maybe more relevant for this fish, at least when compared to numbers of shelters or conspecifics. This is reinforced by the fact that 4 fish preferred no shelter and 1 fish preferred no conspecific in our tests (Figure 5 and 6). An explanation for these results is based on the context of our tests. For example, if fish were exposed to a context of predatory threat, it is possible that shelters and grouping with conspecifics would have been more important to the trout, which would have been seen as more positive PI values. Something like this would be consistent with observations made by Mendonça *et al.* (2010), who demonstrated that preference for substrate in Nile tilapia fish depends on the context of the test. However, as preferences of fish may also depend on previous experience during early development (DePasquale *et al.*, 2016), this needs further investigation.

Considering shelter and conspecific conditions, we could identify rejection responses in preference tests. These were characterized when fish preferred 3 out from the 4 available options, seen in one fish in the shelter condition (Fish 4, Figure 5) and two fish in the conspecific condition (Fish 7 and 8, Figure 6). As preference responses are relative to the available options in the tests, rejection responses are also relative, such that an option is rejected when all the other available options in the tests are preferred. Thus, this indicates that the single non-preferred option probably represents an avoided item by the animal in relation to the other options. Interestingly, this rejection response was not observed in the background color condition (Figure 4). In this way, it seems that individuals vary not just in terms of what they prefer, but also in terms of what they will avoid. Moreover, taking into account that both preference and rejection responses vary not only among individuals, but also among different resources (*e.g.* background colors, shelters or conspecifics), here we show that PI calculations are a reliable measure of what individuals prefer, do not prefer or reject, regardless of the resources being tested.

Blue was not only the most unequivocally preferred color, but it also represents an environmental color option that rainbow trout were both physically and psychologically more motivated to reach (Figures 7 and 8). On the other hand, although trout were physically motivated to reach their individually preferred number of conspecifics (Figure 7), there was no more psychological motivation to access these (Figure 8). Moreover, there was no more physical nor psychological motivation of trout to reach preferred options with shelters (Figures 7 and 8).

Therefore, motivational responses indicated preferred options for background color conditions, but not necessarily for conspecific or shelter conditions. As motivational responses have been inferred to detect the importance of resources (*e.g.* Matthews & Ladewig, 1994; Asher *et al.*, 2009; Houpt, 2012), in rainbow trout background color is more important than specific number of conspecifics, which is more important than specific number of shelters. Moreover, we suggest that the association between preference and motivation responses may depend on the nature of the resources being tested. Stronger motivation may be expected when stronger preferences are observed, (*e.g.* background color condition; Figure 4). These findings support the PI proposed by Maia & Volpato (2016; Attachment) to calculate preference responses of the animals in the light of the welfare issue, as the highest values of PI in background color condition also represented the most motivated responses of trout. Furthermore, our results also add to the literature concerning motivation tests. As far as we know, there is only one physical (Galhardo *et al*., 2011) and one psychological (Chapter 2) motivation study with fish, which demonstrates that fish are more motivated to reach what they prefer, thus reinforcing our results here.

In conclusion, rainbow trout have a general and more intense preference for dark-blue backgrounds, but express individual variation and lower responses of preference, including rejection of some options, for the number of shelters or conspecifics available to interact with. Furthermore, individual fish are not just physically, but also psychologically, more motivated to access their preferred color, a response not necessarily detected in shelter or conspecific conditions. Together, these findings support that motivational responses, both in a physical or in a psychological level, are associated with preferences, where more intense responses are expected when more intense and clear preferences are observed. This illustrates that PI calculations are a useful tool to determine individual preference, non-preference and rejection responses of the animals, regardless the environmental resources being tested. Thus, PI is also supported as an easy and reliable way to know what animals want to better fit their captivity environments for a better welfare condition.

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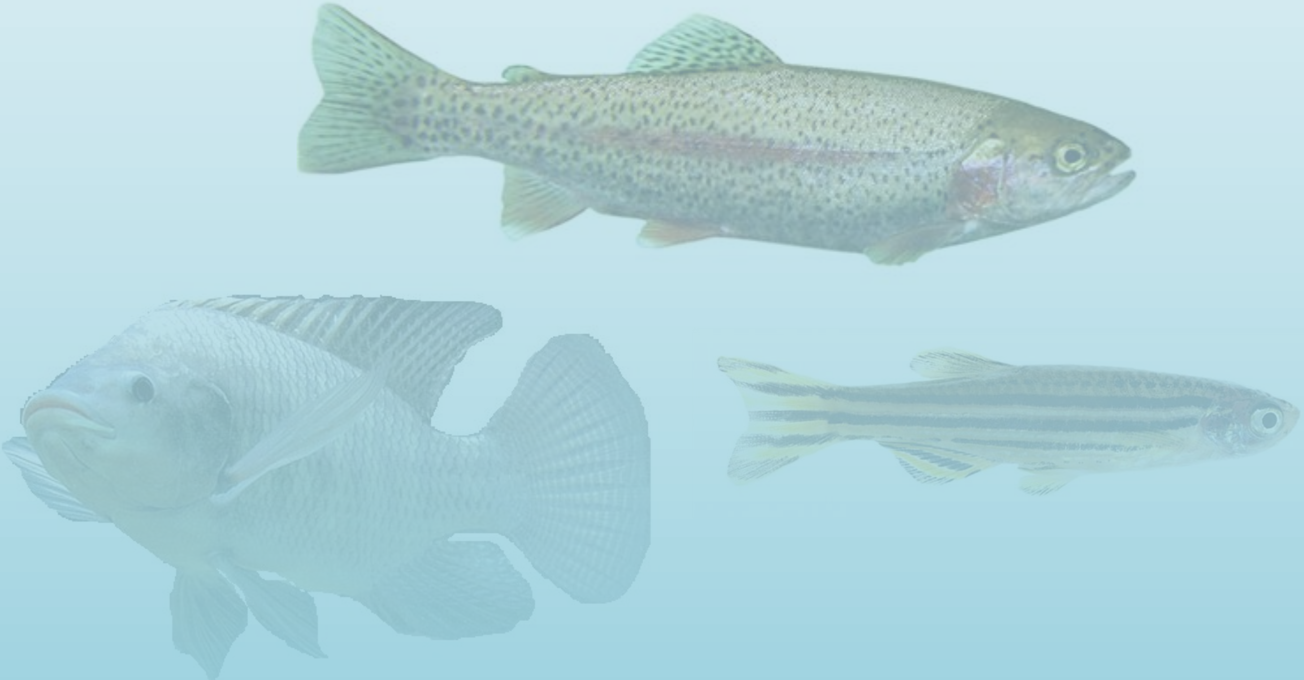
- Conclusões -



Concluímos que o *Índice de Preferência* (IP) é uma ferramenta que reflete as vontades dos animais por itens de recursos ambientais, uma vez que os animais exibem maior motivação física e psicológica para acessar itens preferidos do que aqueles não preferidos com base nesse índice. Tais achados apoiam a proposta de Maia & Volpato (2016; Anexo) de que existem dois padrões de resposta com diferentes relevâncias biológicas em relação às escolhas dos animais: as preferências e as não-preferências. Dessa forma, ressaltamos que as preferências individuais são relevantes para os animais e, portanto, devem ser consideradas a fim de melhorar suas condições de bem-estar, enquanto as não preferências devem ser evitadas. Além disso, respostas individuais de motivação indicaram itens individualmente preferidos de diferentes recursos ambientais e em 3 espécies de peixe pouco aparentadas (tilápia-do-Nilo – Capítulo 1; zebrafish – Capítulo 2; e truta arco-íris – Capítulo 3). Assim, fica evidente que a aplicabilidade do IP não é restrita a uma única espécie e nem dependente do tipo de recurso ambiental.

- Anexo -

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A history-based method to estimate animal preference

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Giving animals their preferred items (e.g., environmental enrichment) has been suggested as a method to improve animal welfare, thus raising the question of how to determine what animals want. Most studies have employed choice tests for detecting animal preferences. However, whether choice tests represent animal preferences remains a matter of controversy. Here, we present a history-based method to analyse data from individual choice tests to discriminate between preferred and non-preferred items. This method differentially weighs choices from older and recent tests performed over time. Accordingly, we provide both a preference index that identifies preferred items contrasted with non-preferred items in successive multiple-choice tests and methods to detect the strength of animal preferences for each item. We achieved this goal by investigating colour choices in the Nile tilapia fish species.

A crucial challenge in the field of animal care is how to identify animal welfare along a continuum from poor to good[1,2]. Although identification of health or disease states is undoubtedly necessary for this purpose, this issue requires deeper analyses. A healthy state does not assure good welfare, as even a stressed animal might be in good health[3,4]. This problem has spurred philosophical controversies over whether animals are aware of suffering or discomfort[5–9]. In this debate, Marian Dawkins[8,10] has proposed that we should provide the animals what they want. This obvious and parsimonious proposal compels us to understand what animals prefer, which is not an easy task[11].

Most studies have evaluated animal preferences based on a few choice tests *e.g.*[12–17], thus providing similar considerations of choices and preferences. Moreover, most researchers have used the frequency/time spent in the available options of just one trial or summed/averaged data from few trials to determine what animals prefer. However, animal choices may differ in subsequent tests[18,19]. Thus, in such few days of testing, a spurious choice response from one specific test could significantly impact the overall preference response when equally summing or averaging data over tests. This implies momentary choices may bias the overall preference response of the animal. Furthermore, most studies have evaluated the preferences at a group level, regardless of the individual variations in such responses *e.g.*[20–25]. However, various studies have demonstrated significant individual variability even when preference responses are analysed from a few tests *e.g.*[26–29].

In this context, we propose a history-based method to determine individual preferences from multiple-choice tests. Because internal motivation and development might change over time, we assumed that in the context of animal welfare, animals have variable wants in addition to their innate needs. Thus, detecting preferences to give to these animals a better environment should assume that animal preferences might change over tests. For these reasons, animal preferences are best determined by a cumulative set of tests. Thus, our history-based method focuses on a consecutive set of tests that incorporates the more recent decisions of the animals. This method was decided after considering that the more recent the choice is made, the more weight it has on the preference response. This method was conceptually developed and applied over a 10-day period of successive daily tests. We used the fish Nile tilapia, *Oreochromis niloticus* (L.), in background colour choice tests. This species has been described to prefer yellow light (2-day test[30]), and some colours have been demonstrated to affect its behaviour[31–34] and physiology[32,34,35].

Materials and Methods

The procedures of this study were in accordance with the guidelines stated by the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Bioscience Institute/UNESP Ethical Committee for Animal Research (CEEA), protocol # 220. Our research procedures were carried out strictly in accordance with these approved guidelines.

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Animals and Holding Conditions. We used juvenile Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1759), with no sex determination, provided by commercial hatcheries. The fish were maintained in indoor tanks (2 fish/10 L in 1000–1700 L tanks) for at least for 30 days prior to experimentation. In this period, we fed the fish commercial tropical fish pellets once per day (5% of biomass/day). The water temperature was approximately 26 °C; pH $\cong$ 6.8; ammonium $<$0.3 ppm; and nitrite $<$0.05 ppm. The aeration was constant, and the photophase (white light) was set from 07:00 h to 19:00 h.

Theoretical Assumptions.

1. Because animals might vary their preferences over time[18,19], we considered each animal's history of successive choice tests instead of only the last few tests.
2. However, due to practical purposes and because an animal's preference might change over time, we required the most recent data related to an animal's preferences to provide better environments and conditions. The most recent choices should have a higher impact on the calculations of the animal's preferences. Focusing on current preference of an animal, successive choice tests provide results of a time sequence of momentary choices. However, the main focus is to know what is the preference of an animal in a specific moment, but considering its whole history. A choice test run at that moment is based on a very specific condition of the animal, which is more susceptible to be influenced by several momentary factors. If this result is considered to provide the animals with a better environment, then a response at a time would be the base for longer decisions about animal welfare conditions. To counteract this, we added to the result at a time (present time) other animal choices from previous tests. This balances our decision for more reasonable information about animal's wants. The results of recent tests overestimate momentary factors influencing choices and results of older tests overestimate the previous choices of that animal. At each new choice test, older tests become more frequent, so that overweighing the recent tests could better balance this profile.
3. The effect of any spurious high choice in one or a few tests should be minimized for calculations of an animal's preferences to avoid bias.
4. An animal might prefer more than one choice item. The lower the number of preferred items, the stronger the preference should be. Thus, different calculations for animals with only one preference item and more than one preferred item should be considered.
5. The technical approach to obtain data should be feasible in practical terms for zookeepers dealing with captive animals. Thus, we employed an easy procedure for registration of animal choices: the number of visitations in an instantaneous sampling scheme during a fixed observation interval[36].
6. We also considered the need to know which item(s) an animal preferred and which animal had stronger preferences when compared with conspecifics. Moreover, the method to detect an animal's preference should consider individual variations among the preferred items, once various studies have demonstrated significant individual variability[26–29].

General Design. Each fish was carefully caught in the stock tanks and individually isolated in glass aquaria (40 × 20 × 25 cm) and monitored for 24 h before the choice tests began. Only fish that appeared to be unstressed after isolation (paler eyes[37]) were used. The fish sample consisted of 24 juveniles (mean total length = 6.91 cm ± 0.41 cm; mean body weight = 5.51 g ± 0.96 g), with no sex determination. After the fish were transferred to a test apparatus, we monitored each fish's choices for four compartments of different background colour (yellow, green, red or blue). Over 10 consecutive days, we measured animal choices every 30 s for 1 h each day and recorded the frequencies that the fish visited each compartment (instantaneous sampling[36]). In each test day, fish were video recorded between ~08:30 h to 09:30 h or ~09:45 h to 10:45 h. In the first period, we filmed 4 fish simultaneously from above, each fish placed in individual test apparatus. In the next period, we filmed more 4 fish in the same conditions. Thus, we repeated all these procedures more two times to reach 24 tested fish. The position of the test apparatus in the lab was constant during each successive test for each fish tested. From these data, we developed a method to separate preferred backgrounds from non-preferred backgrounds and calculate a preference index (PI) and a preference rate (PR), thus separating individuals based on the intensities of their preference responses.

Apparatus and Procedures. The fish were introduced into the central compartment in a transparent 8-cm-diameter cylinder of the respective test apparatus, a cylindrical aquarium of 40-cm diameter with a water column of 15-cm height (~6.3 L; see Fig. 1). After a 5-min acclimation period, we gently removed the cylinder and video-recorded the fish from above for 1 h each day. Despite this 5 min of space restriction, the fish was able to freely move and, in a previous study[38], 5 min of a stronger confinement was not stressful for this species. Every 30 s the fish position was recorded during the 1 h period resulting in 120 measurements per daily test. The fish was considered to be in a compartment when its eyes were inside the compartment. When the eyes were between two compartments, the chosen compartment was the one containing the majority of the fish body. After each test, we returned the fish to the respective isolation aquarium until testing the following day. We repeated these procedures each morning over 10 consecutive days.

In the isolation holding aquaria, aeration was constant, and 30% water changes were performed 1 to 3 times per week by siphoning water out and replacing this volume when the fish were in the test apparatus to maintain good water quality. The photophase was maintained from 08:00 h to 18:00 h and consisted of white light. For the test apparatus, aeration ceased during video recordings; the intensity of the light (white) at the water surface was

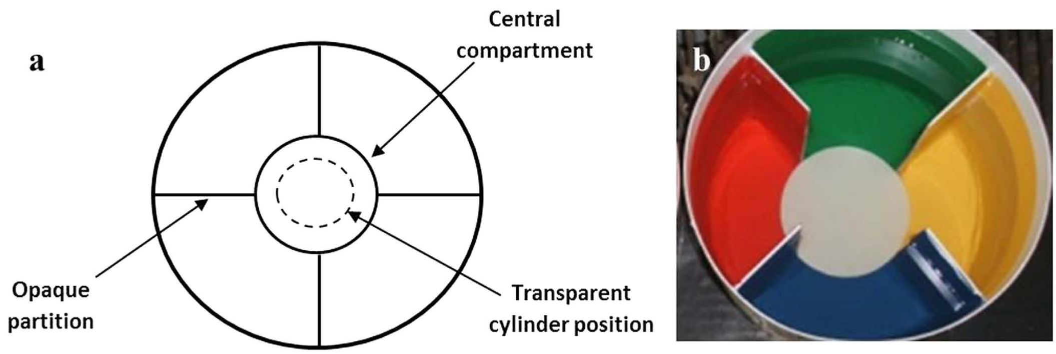

Figure 1. Upper view of test apparatus. (**a**) Scheme indicating four compartments with the same volume and area. Aquarium: 40-cm diameter; ~15-cm height of water column; opaque internal and external walls. (**b**)Photo depicting background colours offered as choice items. Compartment colours were obtained by addition of coloured adhesive plastic for the fourth peripheral compartments and by a circular white perplex in the central compartment.

	STEP 1	STEP 2	STEP 3	STEP 4	STEP 5	STEP 6	STEP 7
Test Number (tn)	Raw Frequency (RF)[1]	Cumulative frequency[2]	Area[3]	Cumulative area[4]	Expected Area[5] (ExA)	Variation of cumulative area from the ExA[6]	Preference Index (PI)[7]
1	9	9	4.5	4.5	12	−7.5	−7.5
2	57	66	85.5	90.0	48	42.0	34.5
3	28	94	70.0	160.0	108	52.0	86.5
4	56	150	196.0	356.0	192	164.0	250.5
5	72	222	324.0	680.0	300	380.0	630.5
6	59	281	324.5	1004.5	432	572.5	1203.0
7	77	358	500.5	1505.0	588	917.0	2120.0
8	79	437	592.5	2097.5	768	1329.5	3449.5
9	99	536	841.5	2939.0	972	1967.0	5416.5
10	99	635	940.5	3879.5	1200	2679.5	8096.0

Table 1. Preference Index calculations. This procedure is carried out for each colour. Example extracted from fish 1, yellow colour. [1]Frequencies obtained in each test. [2]$\sum_{t1}^{tn} RF$. [3]Areas calculated as shown in Fig. 2. [4]$\sum_{t1}^{tn} Area$. [5]Mean of cumulative areas from each colour in the respective test number and fish. In this table, we show data only for the yellow colour; the ExA is calculated from data of all tested colours at tn for fish 1. [6]Data from Step 4–Step 5. [7]Cumulative data of Step 6.

~150 Lux, and the water temperature was 25.8 ± 0.87 °C. Feeding was provided once a day in the isolation aquarium after each choice test.

Calculations. The steps for calculations of choice and/or preference profiles are exemplified in Table 1, based on Fig. 2 reasoning and with data for fish 1 (Fig. 3).

The daily frequencies of visitation to each background colour (step 1) were summed per item (cumulative frequency; step 2). In step 3, we calculated the area above the cumulative-frequency line (as shown in Fig. 2). This area differentially weighs each frequency of choice in subsequent tests and increases the value of frequencies obtained in the most recent tests. Step 4 calculated the cumulative areas obtained in step 3. In step 5, we calculated an expected area (ExA); that is, we calculated the mean of the cumulative areas obtained between colours for the same fish on the same day of testing. This mean area was used as a reference to indicate how areas for each colour behave in relation to this mean area. This information was presented in step 6 (cumulative area–ExA). For each test day, we could detect the position of the cumulative area of each colour respective to a mean value expected if the area was completely random (similar to what is used in the chi-square test and also used by Larrinaga[39], in a study of feed consumption). The preference index was assumed as the cumulative values obtained in step 6. The definition of the PI is obtained in each test (each day in this study), and we considered the final PI obtained in the last test performed. This rationale was assumed because the last test summarizes a given animal's interaction history with the item of choice.

The preference rate (PR) was calculated for fish that exhibited more than one preference item (that is, more than one positive PI). First, we calculated the percentage of PI (each preferred colour) in relation to the sum of all positive PIs detected. For two preference items, the PR was then calculated as the lowest %PI subtracted from the highest %PI. For more than 2 preferences, the PR was calculated as the highest %PI minus [(100–% highest PI)*

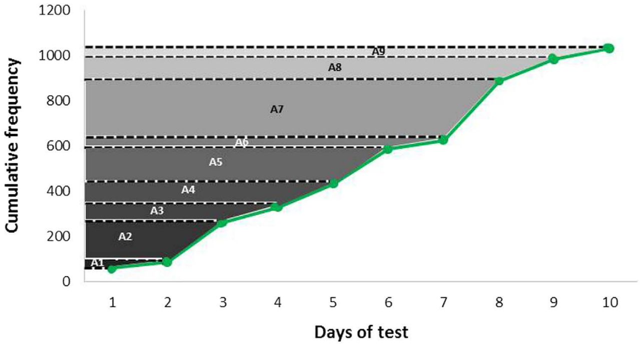

Figure 2. Schematic view of area calculations. See impacts on the most recent choices in a series of consecutive choice tests. Data obtained from fish 6 to visualize calculations of the areas (A1 to A9) above the line of cumulative frequencies. This assumption implies that unit 1 will always result in a higher area in subsequent tests.

(Number of Preferences−1)]. This equation subtracts from the highest %PI a higher value as the fish demonstrates a greater number of preferred items.

Results

The frequencies of individual fish visitation to each compartment, their daily PI values over time and their PR values are displayed in Figs 3–5. For all fish, we considered positive PI values as preferences and negative PI values as non-preferred items in the last choice test (10th day). The one-preference fish are ranked in descending order according to their PI (Fig. 3) values in the last choice test. The more than one-preference fish are ranked in descending order according to their PR values in the last choice test, indicating 2 (Fig. 4) or 3 (Fig. 5) preference responses. For example, fish 2 (Fig. 4) exhibited a preference for the yellow colour only on the 9th and 10th days, but this colour was assumed as a preferred colour based on the whole 10-day evaluation period. The PI value is used to contrast interindividual profiles when fish expressed only one preference response. In fish with more than one preference, the PR value indicates the intensity of the difference between the first and other preferences in a same individual, thus contrasting interindividual profiles.

From the 4 colors for choice, 11 fish (45.8%) preferred only one color. From fish that preferred 2 colors, 8 fish (33.3%) clearly preferred only one color and 3 fish (12.5%) similarly preferred 2 colors. Two fish (8.3%) similarly preferred 3 colors. That is, in 19 fish (79.2%) out of 24 we could clearly identify one preferred color. Moreover, we did not detect one colour clear-cut preference from all fish. From the 11 one-preference fish, blue was preferred once (9.1%), yellow twice (18.2%), green 3 times (27.3%) and red 5 times (45.4%). From the 11 two-preference fish, yellow was preferred 3 times (13.6%), green 4 times (18.2%), red 6 (27.3%) and blue 9 (40.1%) times. The three-preference fish preferred yellow and blue 2 times each (33.3% each), green and red once each (16.7% each).

Discussion

To offer better conditions to confined animals, we must determine what these animals want[10]. In this issue, most studies have evaluated animal preferences considering choice and preference as synonymous terms *e.g.*[12–17]. However, to improve welfare, the animals should want these conditions not only momentarily, but over days because choices might differ during subsequent tests[18,19]. Here, we presented a history-based method to identify animal preferences from a set of successive choice tests. A PI and a PR are provided, for which data from successive choice tests can be added at any time, thus gradually providing appropriate weight to the results of successive tests for the overall inference of preference. This information allows us to separate fish by number of preferred items. Among fish that expressed preference for only one item (one-preference fish) (Fig. 3), the PI values differentiated individuals according to the intensity of the preferred item (in sequence, from fish 18 to 11 in Fig. 3). Among fish that expressed preference for two or more items (more than one-preference fish), the PR value indicates how strongly the most preferred item is relative to the other preferred items for the same fish. Therefore, PR differentiates individuals based on the intensity of differences between their strongest preference and the others. Thus, the fish in Fig. 4 preferred fewer items than did fish in Fig. 5.

Although we used frequencies of visitation instead of time spent in each compartment, we used an instantaneous sampling method, which is sensitive to variations in time spent and visitation frequency in each available item when the interval period is short and the observed behaviour is frequent[36]. In this study, fish position was registered every 30 s during 1 h per test, providing a scanning measurement of 120 observations that could detect a reliable picture of the fish behaviour in the test apparatus. Moreover, in practical terms, inspections in captive conditions (*e.g.,* zoos) are more easily performed when quantifying frequencies than time spent in items in choice tests (assumption #5 in Methods).

Colour preference for fish has been demonstrated by several studies; however, most studies demonstrate one preference for the entire sample of fish[30,40–42]. These studies, however, used a small number of daily tests (usually 1 to 4 tests), where as we used 10 successive days of testing. Moreover, the decision to identify a preferred item in these cited studies was based on the mean and total or cumulative frequencies/time spent; that is, data from the first day of testing have the same weight as a frequency obtained in a test performed several days later. In our method, the determination of the areas corrected this by assigning greater value to each frequency obtained in

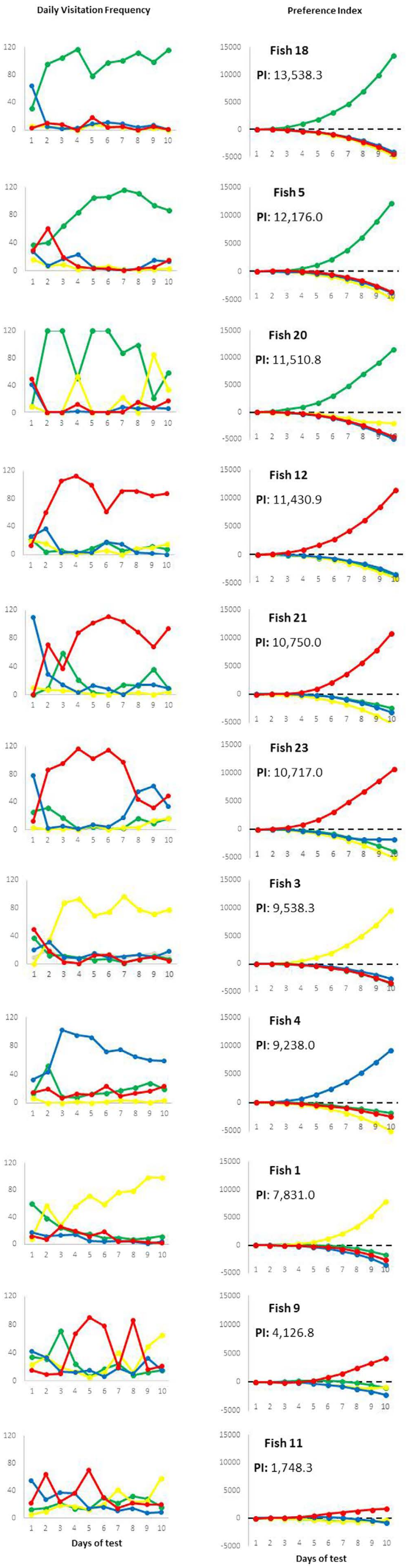

Figure 3. One-preference fish. Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses. PI values for the last test are indicated for each fish.

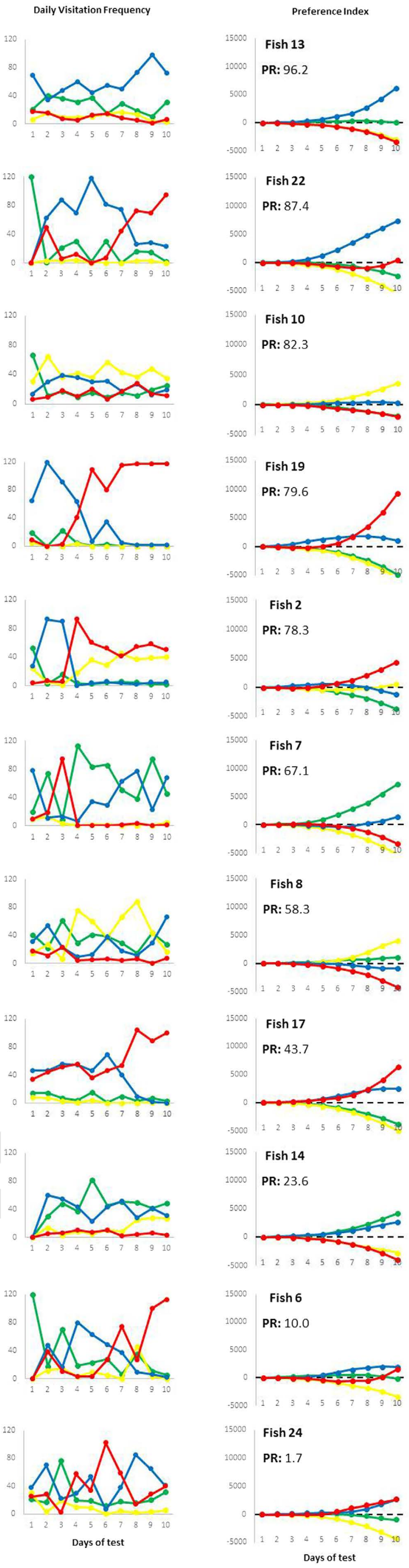

Figure 4. Two-preference fish. Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses. PR values for the last test are indicated for each fish.

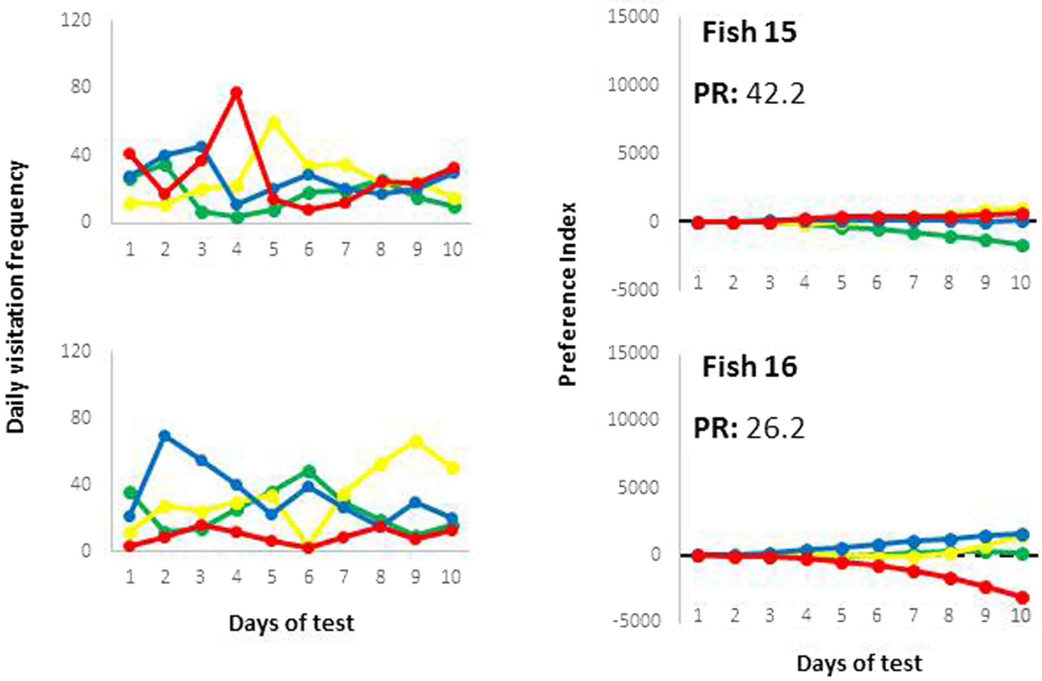

Figure 5. Three-preference fish. Individual profiles of preference (positive PIs) and non-preference (negative PIs) responses. PR values for the last test are indicated for each fish.

more recent tests (see Fig. 2 and fish 19 in Fig. 4). This assumption was made (assumption #2 in Methods) because recent tests reflect the most recent state of the individual and preferences can change over time. However, we did not consider only the last test because those data may contain a spurious decision by the animal. Thus, as we have incorporated the entire history of frequencies in successive tests and given more weight to the most recent tests, we implied that our results are a balance between the most consistent response of the animal over time and its momentary state in the last test, thus supposedly reflecting a more natural response. In practical terms, an animal keeper might be interested in knowing what the animal wants to adjust its holding condition. If he/she uses the PI concept described here, the result of the choice test is balanced by the whole set of previous tests, thus reflecting the actual choice, but does not ignore the previous choices of that animal. Therefore, with only one test each time (*e.g.*, once a day or a week) the animal keeper gets a better impression about the wants of that animal. Moreover, it is more reliable to include more short-term choice tests than long-term tests. This emphasizes the need for knowing individual animal wants as much as possible.

The assumption that positive PIs indicate preference items, whereas negative values are non-preferred items, is not a cabalistic one. A positive PI indicates the animal chose that item more frequently above the expected mean (ExA) relative to the other items. This information represents a relative response and a differentiated performance. Of course, this procedure will always result in animals showing at least one preferred and one non-preferred item. This assumption makes sense and is reasonable, as a preference response in a multiple choice test should always be relative to the other available items in that specific test. For example, a preference response for blue colour may supposedly appear in a choice test including blue, green, yellow and red colours but may not appear when another colour, *e.g.*, purple, is also offered as an available item and is preferred instead of blue. Moreover, the responses we identified here through negative PI values as non-preferred items may be similar to the "dispreferred" options of food colour as described by Larrinaga[39].

Once an animal expresses a preference for only one item, the magnitude of its preference can be easily inferred from the magnitude of PI (fish in Fig. 3). However, this index cannot be contrasted with animals that chose more than one item because to choose only one item is qualitatively very different from choosing two or more items (assumption #4 in Methods). Thus, for the other animals, PR is provided to indicate a response relative to the other preferred items (Figs 4 and 5). More specifically, PR indicates how strongly the animal prefers the most preferred item compared with the other preferred items. We also assume that preference for only two items gives the animal a more clear decision-making process than when more preferred items are detected, which was also included in the PR calculations (by including number of preferences minus 1 in the calculations, as specified in the Methods).

To our knowledge, only one published study[43] used a PI to identify the intensity of choices. This index was based on the difference of time spent in each available item. However, only two trials were examined, and preference was investigated at a group not individual level. Based on the same principle of choices, our method examines this issue more deeply by clearly separating preferences from non-preferences and by individually identifying the intensity of such responses.

The use of choice tests to infer preferences requires caution. For example, the chosen item might draw the animal's attention–the choice is only a perceptual consequence[44]. Moreover, choice tests repeated over time (necessarily involving previous experience) and the nature of the stimulus are also intrinsic factors in preference tests that might change animal's preference. Part of these limitations can be minimized by increasing the number of successive choice tests. The PI solved this problem in our study as variations of fish choices over time (graphs on the left in Figs 3–5) were transformed into more consistent profiles of PI overtime (gradually increasing PI). Only one fish (Fig. 4, fish #19) exhibited inversion of the most preferred item during observations. This finding is expected (assumption #2 in Methods) and was clearly detected from PI. Note that PI detected this inversion 3 tests later in relation to the raw frequency data, a result from the high impact of the first days when fish chose strongly the blue environment. This phenomenon occurs because PI change more slowly than do direct observations of frequency of choices. Thus, PI is more robust and less sensitive to immediate changes, as we should assume when searching for preferences instead of choices (non-preferences). Because of this property of PI, very confusing results are obtained with raw frequencies (*e.g.,* fish 2, 8, 10, and 24 in Fig. 4) and can be clarified using PI calculations. The limitation related to the previous experience in a serial set of tests cannot be abolished, but the history-based approach adopted here assumes that preference, except in strongly genetic preferences, is the result of different intrinsic (learning and physiological needs) and extrinsic forces (nature of the stimulus and environmental context). That is, PI does not reveal preference causes, but the preference in a history of animal experiences.

According to Kirkden & Pajor[45], choice tests also indicate animal's motivation to get a resource. Our individual data show that animals usually inspected all the colour items and stayed more frequently in one or few of them in each daily test. That is, higher frequencies in one item should mean more motivation for that item. The PI described here should thus indicate such a motivation, but in a history-based approach. The consumer surplus concept applied to motivational strength[46] is an interesting approach for which the present PI could be helpful because this index somehow incorporate motivational forces. Motivation for a resource depends on the resource type, once the animal's choices might be influenced by its satiation to the resource[46]. Thus, caution is required to compare PI values among resources.

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Author Contributions

C.M.M. and G.L.V. conceptualized the study; C.M.M. collected the data; C.M.M. and G.L.V. analysed the data and wrote the manuscript.

Additional Information

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