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Dissertação

Biotecnologia na aquicultura: efeitos da exposição crônica à temperatura fria sobre o crescimento, expressão de genes relacionados à ingestão de alimentos e imunidade de tilápias-do-Nilo (*Oreochromis niloticus*)

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Para meus pais, com carinho e gratidão.

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“O aprendizado é como o horizonte: não há limites”

Autor desconhecido

Resumo

DELLAGOSTIN, Eduardo. **Biotecnologia na aquicultura: efeitos da exposição crônica à temperatura fria sobre o crescimento, expressão de genes relacionados à ingestão de alimentos e imunidade de tilápias-do-Nilo (*Oreochromis niloticus*).** 2021. 50f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A tilápia-do-Nilo (*Oreochromis niloticus*) é a quarta espécie mais produzida no panorama mundial da aquicultura. Uma particularidade desta espécie é a necessidade de águas com temperatura acima de 16ºC para desenvolvimento e sobrevivência. Pouco se sabe sobre os efeitos da baixa temperatura nos genes relacionados à ingestão alimentar e à resposta inflamatória nesta espécie. O presente estudo traz informações em relação à modulação de genes em tecidos coletados do cérebro, fígado, rim e baço quando tilápias são expostas a baixas temperaturas. Sessenta animais foram divididos em dois grupos, onde um permaneceu na temperatura ótima (24ºC) e o outro m temperatura baixa (15ºC), num período de exposição de 28 dias. Avaliações biométricas foram realizadas antes e depois do período experimental. Amostras de sangue foram utilizadas para a realização de citometria de fluxo enquanto o cérebro, baço, fígado e rim foram coletados, o RNA foi extraído e o qRT-PCR foi realizado. Foi observado menor peso final e taxa de crescimento específico no grupo exposto a baixa temperatura. Houve um aumento de 2 vezes na expressão do gene do *Pyy* e uma redução de 0,5 vezes dos genes do *Npy* e *Cart*, indicando que baixas temperaturas modulam os genes de ingestão de alimentos. Os genes codificantes para citocinas pró-inflamatórias foram moduladas no baço, rim e fígado com maior expressão dos genes da *Il-1b* e do *Tnfa* e redução da expressão dos genes da *Il-8* e do *Nf-kb* no grupo exposto a 15ºC. Análises realizadas com as células sanguíneas revelaram um nível mais baixo de fluidez da membrana, a fragmentação do DNA e a disruptura celular foram maiores no grupo exposto ao frio. Os resultados observados sugerem uma reação causada pelo estresse gerado pelo frio. O presente trabalho se mostra importante, pois traz novas informações sobre o bem-estar em tilápias expostas a baixa temperatura. Este, ao elucidar efeitos moleculares e celulares que ocorrem nesta condição, é um primeiro passo para a melhor compreensão do manejo desta espécie, ajudando a encontrar alternativas para de lidar com os impactos do frio na aquicultura.

Palavras-chave: tilápia-do-Nilo, alimentação, citocinas, expressão gênica, baixa temperatura.

Abstract

DELLAGOSTIN, Eduardo. **Biotechnology in aquaculture: effects of exposure to cold temperature on growth, expression of genes related to food intake and immunity of Nile tilapia (*Oreochromis niloticus*)**. 2021. 52f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Nile tilapia (*Oreochromis niloticus*) is the fourth most produced species in the world aquaculture scene. A particularity of this species is the need for water with a temperature above 16ºC for development and survival. Little is known about the effects of low temperature on genes related to food intake and the inflammatory response in this species. The present study provides information regarding the modulation of genes in tissues collected from the brain, liver, kidney and spleen when tilapia is exposed to low temperatures. Sixty animals were divided into two groups, where one remained at the optimum temperature (24ºC) and the other at a low temperature (15ºC), during an exposure period of 28 days. Biometric evaluations were performed before and after the experimental period. Blood samples were used to perform flow cytometry while brain, spleen, liver and kidney were collected, RNA was extracted, and qRT-PCR was performed. Lower final weight and specific growth rate were observed in the group exposed to low temperature. There was a 2-fold increase in *Pyy* gene expression and a 0.5-fold decrease in *Npy* and *Cart* genes, indicating that low temperatures modulate food intake genes. The coding genes for pro-inflammatory cytokines were modulated in the spleen, kidney and liver with higher expression of *Il-1b* and *Tnfa* genes and reduced expression of *Il-8* and *Nf-kb* genes in the group exposed to 15ºC. Analyzes performed with blood cells revealed a lower level of membrane fluidity, DNA fragmentation and cell disruption were higher in the cold exposed group. The observed results suggest a reaction caused by the stress generated by the cold. The present work is important, as it brings new information about the well-being of tilapia exposed to low temperature. This, by elucidating molecular and cellular effects that occur in this condition, is a first step towards a better understanding of the management of this species, helping to find alternatives to deal with the impacts of cold on aquaculture.

Keywords: Nile tilapia, food intake, cytokines, gene expression, low temperatures.

Sumário

1 INTRODUÇÃO GERAL

A tilápia-do-Nilo (*Oreochromis niloticus*) é uma espécie de peixe da família Cichlidae de grande importância para o comércio mundial, sendo a quarta espécie mais produzida em todo o mundo. No ano de 2020 foram produzidas em todo o mundo mais de 6 milhões de toneladas desta espécie mundialmente, sendo a China o maior produtor de tilápia (FAO, 2020). Seu extenso cultivo se dá pela sua rápida taxa de crescimento, seu baixo custo de produção e sua alta capacidade em tolerar condições adversas de criação. O Brasil está em quarto lugar no ranking de maiores produtores desta espécie com aproximadamente 486 mil toneladas, no entanto ainda há capacidade para aumentar a produção (Lopes et al., 2021). O potencial de crescimento reside principalmente na alta disponibilidade de águas subterrâneas e de superfície, visto que o Brasil é o maior detentor de água doce do planeta (cerca de 12%); devido o amplo território com grande extensão litorânea (cerca 12 de 8,5 mil km); além de condições climáticas favoráveis para criação de ampla variedade de espécies. Além disso, o consumo per capta de pescados aumenta a cada ano no Brasil (ACEB, 2014).

No entanto, o cultivo de tilápia no Brasil acaba por se restringir a regiões tropicais e subtropicais devido ao conforto térmico desta espécie. O conforto térmico, ou seja, a temperatura na qual ocorre a manutenção e as maiores taxas de crescimento da espécie ocorre entre 25° e 28°C (Barcellos and Fagundes, 2012). Estudos utilizando animais desta espécie submetidos a baixas temperaturas já demonstraram, através de análises transcriptômicas, diversos efeitos desta condição em vias metabólicas relacionadas principalmente à imunidade e à adesão celular (Zhou et al., 2019). Ainda, o cultivo desta espécie em temperaturas abaixo de 14ºC é desaconselhado principalmente devido ao fato de ser cessada a sua alimentação nessas condições além de ser letal para os peixes (Barcellos and Fagundes, 2012).

O controle da alimentação é essencial para a manutenção de diversos processos metabólicos. Quando afetada a alimentação pode gerar um déficit energético no animal levando ao desvio de vias metabólicas para suprir ao essencial (Rønnestad et al., 2017; Volkoff and Peter, 2006). O controle da alimentação é extremamente complexo e acaba por depender de diversos fatores que atuam em conjunto regulando a esta necessidade fisiológica. Os principais fatores que regulam o apetite podem ser divididos em duas categorias: os orexigênicos, que induzem a alimentação; e os anorexigênicos que controlam o sentimento de saciedade (Yan et

al., 2017). Dentre estes principais fatores é possível destacar três que acabam tendo destaque nesta regulação: o Neuropeptídeo Y (NPY); o Peptídeo YY (PYY); e o Transcrito regulado por cocaína e anfetamina (CART). A produção destas proteínas se dá principalmente no cérebro, sendo o NPY um fator orexigênico, juntamente com o CART, já o PYY é responsável por sinalizar a saciedade no organismo(Assan et al., 2021; Volkoff and Peter, 2001; Yan et al., 2017). Os mecanismos de alimentação podem ser regulados de diversas formas, no entanto ainda não há informação dos efeitos de baixas temperaturas nestes fatores nas tilápias.

Outro sistema que pode ser afetado sob baixas temperaturas é a imunidade dos animais. Como organismos ectotérmicos, os peixes dependem da temperatura do ambiente para regular seu organismo (Abram et al., 2017). Quando ocorre da temperatura variar a ponto de ultrapassar o ideal para o organismo, o mesmo acaba por se prejudicar. Alguns trabalhos relatam direta associação entre baixas temperaturas e alterações em fatores responsáveis pela defesa dos organismos (Nitzan et al., 2019; Raida and Buchmann, 2007; Zhou et al., 2019). Dentre esses fatores estão as citocinas pró-inflamatórias que exercem um papel fundamental no combate de invasões de microrganismos (Abram et al., 2017). Entretanto, em tilápias, não há informações sobre a capacidade de defesa do organismo e sobre a resposta do mesmo sob baixas temperaturas. O conhecimento sobre os possíveis efeitos das baixas temperatura na expressão de genes pode gerar o conhecimento necessário para a melhora das condições de cultivo de tilápias em regiões subtropicais.

2 REVISÃO BIBLIOGRÁFICA

2.1 Piscicultura e tilapicultura

A piscicultura é uma das áreas da produção animal que mais cresceu nos últimos anos. Segundo dados divulgados pela Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) esta área teve um crescimento de 123% entre os anos de 2005 e 2015. A piscicultura pode ser dividida em pesca extrativa a qual trata apenas da retirada de recursos pesqueiros do ambiente natural, e em aquicultura que é o cultivo, geralmente em ambientes controlados, de organismos aquáticos. No Brasil, segundo a Associação Brasileira da Piscicultura (Peixe BR), em 2020 foram produzidas mais de 802 mil toneladas de peixe e, dentre as espécies, a mais produzida é a tilápia (Lopes et al., 2021).

A tilápia-do-Nilo (*Oreochromis niloticus*) é uma espécie de peixe da família Cichlidae, originária da África e introduzida no brasil na década dos anos de 1970. Esta espécie tem grande importância para o comércio mundial de peixes, sendo a quarta mais produzida em todo o mundo devido à sua rápida taxa de crescimento, baixo custo de produção além de sua alta capacidade de tolerar condições adversas de criação (Barcellos and Fagundes, 2012). No ano de 2020 foram produzidas em todo o mundo mais de 6 milhões de toneladas desta espécie, sendo a China o maior produtor deste de peixe no mundo. O Brasil se destaca sendo o quarto maior produtor, com produção aproximada de 486 mil toneladas em 2020, no entanto, ainda há espaço para o aumento da capacidade de produção (FAO, 2020; Lopes et al., 2021). O potencial de crescimento reside na alta disponibilidade de águas subterrâneas e de superfície, visto que o Brasil é o maior detentor de água doce do planeta (cerca de 12%); possui amplo território; possui grande extensão litorânea (cerca de 8,5 mil km); possui condições climáticas favoráveis à criação dessa espécie; além de consumo de pescados per capta que aumenta a cada ano(ACEB, 2014).

No entanto, o cultivo de tilápia no Brasil ainda se restringe a regiões tropicais e subtropicais devido a sensibilidade desta espécie às baixas temperaturas. O conforto térmico, ou seja, a temperatura na qual se dá o crescimento e manutenção da espécie ocorre entre 25° e 28°C (Barcellos and Fagundes, 2012). Além disso, devido as dimensões continentais do país, muitas vezes é necessário realizar o transporte dos animais sob condições de baixa temperatura o que leva o animal a

uma condição de estresse, na qual são observadas a redução do apetite dos animais e o aumento da susceptibilidade desta espécie a diversas doenças. Estudos utilizando animais desta espécie submetidos a baixas temperaturas mostram efeitos desta condição em vias metabólicas relacionadas principalmente à imunidade e à adesão celular (Zhou et al., 2019). Ainda, o cultivo desta espécie em temperaturas abaixo de 14ºC é desaconselhado pois pode ser letal para os peixes (Barcellos and Fagundes, 2012).

Mesmo observando alterações no comportamento animal, ainda pouco se sabe acerca das modulações gênicas causadas pela exposição ao frio na espécie *O. niloticus.*

2.2 Alimentação de tilápias

Segundo descrito por El-Sayet (2006), tilápias tem hábitos alimentares variados. Quando em cultivo, os peixes podem ser alimentados com dietas constituídas majoritariamente de ração peletizada, no entanto em muitos casos é utilizada a adição de fertilizantes de origem orgânica nos tanques de cultivo para a geração de biomassa para o consumo dos animais (El-Sayed, 2006; Perschbacher and Stickney, 2017). Muitos criadores realizam construções de instalações para a criação de suínos à jusante ou até mesmo sobre os tanques de criação de peixes para que os resíduos sejam reutilizados na geração de plâncton e fitoplâncton, os quais são base para a alimentação dos peixes em diferentes estágios de desenvolvimento (Perschbacher and Stickney, 2017).

No entanto, muitas vezes o gasto com alimento para os peixes é em vão durante os períodos em que há a ocorrência de baixas temperaturas, visto que abaixo de 20ºC os hábitos alimentares dos peixes cessam. Entretanto não há informações fisiológicas nem moleculares suficientes para responder o porquê deste comportamento (Volkoff et al., 2010). Uma estratégia para sanar a falta deste conhecimento é a análise de genes relacionados a alimentação destes animais, quando estes estão expostos à tal situação estressante. Existem estudos em outras espécies demonstrando os principais genes relacionados com o controle de ingestão de alimentos, porém em tilápias essa ainda é uma lacuna a ser preenchida (Rønnestad et al., 2017).

A sinalização para o controle da alimentação parte do hipotálamo e é mediada por hormônios de peptídeos (Rønnestad et al., 2017). Além do hipotálamo,

também é possível encontrar sinais que partem diretamente de órgãos periféricos levando à ingestão de comida ou à saciedade (Rønnestad et al., 2017; Volkoff and Peter, 2006). Os mecanismos de sinalização de controle do apetite são relativamente conservados dentre os vertebrados e são divididos em fatores que induzem a alimentação (orexigênicos) e que inibem a alimentação (anorexigênicos). Dentre os principais componentes do sistema de regulação do apetite é possível destacar três, o Neuropeptídeo Y (NPY), o peptídeo YY (PYY) e o transcrito regulado por cocaína e anfetamina (CART) (Assan et al., 2021; Volkoff and Peter, 2001).

O NPY é um dos mais potentes sinalizadores orexigênicos atuantes no organismo. Este peptídeo possui um tamanho de aproximadamente 36 kDa e é pertencente à família do NPY (Assan et al., 2021). O seu efeito é reportado como variável dentre as diferentes espécies de peixes teleósteos. Estudos acerca deste peptídeo mostram que injeções deste componente em peixe dourado, truta arco-íris, carpa e tilápias induzem o comportamento de alimentação (Aldegunde and Mancebo, 2006; Kiris et al., 2007; Narnaware et al., 2000; Zhou et al., 2013). Em algumas espécies como peixe dourado, zebrafish e linguado períodos de jejum acabam por elevar a expressão do NPY, demonstrando um possível padrão de expressão espécie específico (Campos et al., 2012; MacDonald and Volkoff, 2009; Narnaware et al., 2000; Yokobori et al., 2012).

Outra importante molécula que atua sobre a regulação da alimentação em teleósteos, que nos últimos anos tem recebido maior atenção, é o CART. Esta proteína foi primariamente isolada do cérebro de ratos que receberam administração aguda de cocaína ou anfetamina (Rogge et al., 2008). A expressão do CART no cérebro de peixes foi reportada associada ao período pós alimentação em peixes dourado, bagre e salmão do atlântico (Peterson et al., 2012; Valen et al., 2011; Volkoff, 2006). No entanto outros trabalhos citam este gene com expressão variável, podendo atuar tanto como fator orexigênico quanto como anorexigêncio (Zhang et al., 2018).

O PYY é uma molécula produzida principalmente pelo trato gastrointestinal dos vertebrados, no entanto, estudos reportam uma expressão significativa dessa molécula no cérebro (Assan et al., 2021). Este peptídeo tem como principal função atuar na sinalização da saciedade em peixes. Trabalhos nos quais foi realizada a injeção de PYY mostraram que esta proteína acaba tendo um papel anorexigênico

através da inibição da expressão do NPY (Yan et al., 2017). Outros trabalhos também mostraram que o PYY acaba por ser modulado durante períodos pós-prandiais e períodos de jejum (Assan et al., 2021; Gonzalez and Unniappan, 2010).

Sendo assim, o estudo dos mecanismos de regulação do apetite em tilápias e o efeito do frio sobre este sistema regulatório se faz necessário para garantir um menor número de perdas durante a produção desta espécie, visando o bem-estar animal.

2.3 Imunidade em tilápias

Além da redução no hábito de alimentação, as baixas temperaturas também acabam por aumentar a susceptibilidade dos peixes a doenças. Existem trabalhos que associam as baixas temperaturas com uma maior incidência de franciselose causada pela bactéria *Francisella noatunensis,* que acaba por ser letal ao peixe infectado pela mesma (Nguyen et al., 2020). Outro estudo mostra que existe uma regulação negativa em genes do sistema imune de tilápias de Moçambique quando expostas a uma baixa temperatura por um curto período. Porém a realidade se mostra diferente, na região sul do Brasil os períodos de frio podem ser mais extensos podendo causar um dano maior ao organismo destes peixes (Abram et al., 2017; Velmurugan et al., 2019).

A imunidade dos animais está intimamente relacionada com as condições ambientais que ele se encontra. Os peixes são organismos ectotérmicos portanto dependem da temperatura ambiental para regular sua temperatura corpórea (Abram et al., 2017; Donaldson et al., 2008). A exposição a temperaturas que fujam do considerado adequado para a espécie pode provocar condições estressantes para os animais influenciando na imunidade e saúde (Abram et al., 2017). Estudos avaliaram o efeito do frio na produção de citocinas pró-inflamatórias em truta arco-íris e mostraram que à medida que se diminui a temperatura, a pressão de IL-1b acaba sendo reduzia concomitantemente até seu bloqueio ocorrer a 4ºC. Este tipo de modulação pode levar a evasão de patógenos e consequentemente uma maior sensibilidade a contrair doenças (Zou et al., 2000). As citocinas são moléculas produzidas por diversos tipos celulares em um organismo, que acabam por mediar a sinalização frente a mudanças metabólicas. As citocinas pró-inflamatórias são produzidas principalmente por macrófagos ativados e estão envolvidas na indução de reações inflamatórias (Zhang and An, 2007).

No entanto até o presente momento não existem estudos sobre a regulação dos genes do sistema imune da espécie *O. niloticus* quando expostas ao estresse térmico crônico. Portanto mostra-se necessária a avaliação da expressão de genes do sistema imune buscando a compreensão dos mesmos e dos mecanismos de regulação que possam estar atuando sobre eles.

2.4 Tolerância ao frio em tilápias

As tilápias como animais ectotérmicos dependem da temperatura ambiental para a regulação de sua temperatura corpórea. Em animais com esta característica, eles adotam comportamentos para evitar que sua temperatura abaixe do conforto térmico da espécie. Uma das estratégias adotadas por peixes é a busca por águas mais quentes para a regulação de sua temperatura corporal (Abram et al., 2017; Donaldson et al., 2008). No entanto, quando em cativeiro, estes peixes acabam ficando suscetíveis a baixa temperatura sem a oportunidade de buscar por uma maior temperatura ambiental. Como consequência da exposição a baixa temperatura os organismos aquáticos acabam buscando outros mecanismos para compensar a situação desfavorável (Soyano and Mushirobira, 2018).

Como forma de manter a sobrevivência do organismo, muitas espécies acabam por reduzir seu metabolismo a níveis basais, quando expostas a temperaturas fora de seu conforto térmico (Soyano and Mushirobira, 2018). Além disso, mudanças neste fator abiótico acabam por estressar o animal gerando mudanças hormonais, e levando a alterações metabólicas importantes (Barton, 2002). A primeira resposta metabólica para a exposição ao frio é a excreção de corticosteroides e catecolaminas via resposta neuroendócrina do sistema nervoso central dos peixes. Em tilápias foi encontrada modulação da liberação de catecolaminas na corrente sanguínea durante a exposição a baixas temperaturas (Chen et al., 2002). Esta liberação ajuda a manter a homeostase através de mudanças fisiológicas no organismo do animal.

Entretanto, ainda não foi elucidado o efeito da exposição prolongada a baixas temperaturas sobre a sobrevivência e crescimento de tilápias, e o efeito na adaptação destes animais a esta condição ambiental.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

A exposição ao frio em tilápias-do-Nilo causa diminuição da alimentação, o que altera a expressão genica de genes relacionados à alimentação, o que consequentemente altera a resposta imune.

3.2 Objetivo Geral

Avaliação e desenvolvimento de um painel de genes relacionados à ingestão de alimentos e resposta imunológica de tilápias-do-Nilo em condições de baixas temperaturas.

3.3 Objetivos Específicos

- Avaliar os efeitos do frio no desenvolvimento e ganho de peso de tilápias-do-Nilo;
- Identificar padrões de expressão dos genes ligados à alimentação em cérebro de tilápias-do-Nilo expostas a estresse térmico crônico;
- Identificar padrões de expressão dos genes ligados à imunidade em rim, fígado e baço de tilápias-do-Nilo expostas a estresse térmico crônico.
- Analisar os possíveis efeitos citotóxicos que o estresse térmico crônico pode gerar em células sanguíneas de tilápias-do-Nilo.

4 CAPÍTULOS

4.1 Manuscrito 1 – Chronic cold exposure modulates feeding and immune related genes in Nile tilapia (*Orechromis niloticus*)

Manuscrito formatado para o periódico *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*

(fator de impacto: 2,32 e qualis B1 na área de Biotecnologia)

Chronic cold exposure modulates genes related to feeding and immune system in Nile Tilapia (*Orechromis niloticus*)

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Highlights

- Chronic cold exposure reduces the specific growth rate of Nile tilapia;
- Food intake genes are modulated by low temperatures inducing fasting;
- Pro-inflammatory cytokine genes had its gene expression altered by cold exposure;
- Cold induces a higher DNA fragmentation and cell disruption.

Abstract

Nile tilapia is the fourth most produced species in the global aquiculture panorama. This species needs water temperatures above 16ºC to grow and survive, and so, little is known about the effects of low temperature on genes related to food intake and inflammatory response. This study brought insights about the modulation of genes in different tissues of Nile tilapia chronically exposed to low temperatures. To do it, sixty animals were divided in two groups, in which one remained at the optimum temperature and the other had the temperature daily decreased until reaching 15ºC. After, those animals were exposed for 28 days. Blood samples were collected for flow cytometry analysis and brain, spleen, liver and kidney were collected for RNA extraction, followed by quantitative PCR (qRT-PCR). There was an upregulation in *pyy* and downregulation of *npy* and *cart* gene expression. Also, pro-inflammatory cytokines genes were modulated in the spleen, kidney and liver with a higher expression of *il-1b* and *tnfa* and a reduction in the *il-8* and *nf-kb* in the group exposed to 15ºC. The blood cell analysis revealed a lower level of membrane fluidity and a higher DNA fragmentation and cell disruption in the group exposed to cold. These findings suggest a possible effect of a stressful situation in the tilapia organism due to cold exposure. This study brings insights on tilapias wellbeing under low temperature stress being a first step to comprehend the best way to cope with cold impacts on aquaculture.

Keywords: Nile tilapia, food intake, cytokines, gene expression, low temperatures.

Introduction

Nile tilapia (*Oreochromis niloticus)* is one of the most produced species in the world. In 2020 the total tilapia production surpassed 4.5 million tons (FAO, 2020). Originated from Africa, this species belongs to the Cichlidae family and is broadly produced due to its fast growth rate, low maintenance cost and high economic value. However, tilapia has its optimum growth temperature between 25 and 28ºC, being capable of surviving in a range between 16 and 38°C without significative losses (T. Zhou et al., 2019). With the upcoming climate change events, this species began to experience more frequently, and with a longer duration exposure, to temperature below 16ºC, leading to losses in the production and prejudice for the productors.

Environmental temperature is one of the most important abiotic factors for the wellbeing of aquatic species (Dominguez et al., 2004). Fishes, as ectotherms, must rely on the environmental to regulate its body temperature and metabolism (Donaldson et al., 2008). Some species have developed mechanisms to survive to low temperatures without further losses. Some reptile species reduce their metabolism during winter times, reducing the energy loss and surviving trough this period (Storey, 2006). Other species of fishes produce anti-freezing proteins that helps the organism to cope with extreme temperatures (Ammar et al., 2018). Temperatures of 10ºC are lethal for Nile tilapia, and temperature below 16ºC can affect some functions of the organism such as food intake and immunity (Wohlfarth and Hulata, 1981).

The process of food intake is a complex mechanism that rely on signals sent from the brain and some peripherical tissues to induce the food consumption or to indicate satiety (Assan et al., 2021). There have been described distinct proteins, peptides and neurotransmitters that participate in the signaling for feeding process. There are three

principal components involved in this communication, the Neuropeptide Y (NPY), the Peptide YY (PYY) and, a less studied due to its recent discovery in comparison to the other two molecules, the Cocaine- and Amphetamine Regulated Transcript (CART) (Volkoff, 2006; Yan et al., 2017). The NPY belong to the NPY family and is a peptide widely distributed in the nervous system and is known by the orexigenic effect (Assan et al., 2021). The PYY belongs to the same NPY family, is widely produced in the foregut of teleost fishes but have been reported production of this peptide in some brain regions and this peptide have similar function of NPY (Assan et al., 2021). On the other hand, CART has been described recently in aquatic species, but is known to take part in the regulation of satiety, studies with the injection of this peptide reduced the food ingestion in goldfish (Volkoff and Peter, 2001; X. Zhang et al., 2018). As mentioned, tilapias change its feeding habits when exposed to low temperatures, however there is a lack of information about the molecular mechanisms involved on this behavior.

Immunity processes are essential for the survival of an organism. The defense system must be in homeostasis to protect an organism from the invaders attack such as bacteria, parasites, fungi and viruses (Velmurugan et al., 2019). It is known that environmental conditions such as salinity, temperature, pH and dissolved oxygen can influence the immunity system of aquatic organisms (El-Leithy et al., 2019; Sood et al., 2021; Velmurugan et al., 2019; C.-N. Zhang et al., 2018). Studies have demonstrated this association with diverse organisms (Dominguez et al., 2004). One of the principal mechanisms of the immunity process is the pro-inflammatory response mediated by cytokines (Cook et al., 2018, p. 1). Those are molecules produced by immunity cells that serve as signaling for actuation of the innate immune system (Li et al., 2021). Although there is a lack of knowledge about the response of the inflammatory mechanisms under low temperature conditions in Nile tilapia.

Being aware of the potential effects of the low temperature on the immunity system and the food intake signaling, altogether with the little knowledge about the effects of those mechanisms in wellbeing of tilapia. The present study aims to elucidate the role of low temperatures on gene expression related to food intake and inflammatory response on Nile tilapia, together with the effects on its biometric measures and blood cells parameters.

Materials and methods

Experimental animals

Nile tilapias (*Oreochromis niloticus*) were obtained from the Laboratory of Pisciculture from the Barragem of Chasqueiro Fish Farming (Arroio Grande, Brazil, 32°14′15″S/53°05′13″W). These specimens were previously acquired from a commercial producer and reared until suitable body weight of 120±20g. Fish were maintained for four weeks in a freshwater recirculating system made of 1000L plastic tanks with 650 L of operating volume. During this period, fish were fed three times a day until satiety with commercial feed (Supra, 38 % crude protein) and acclimated to water temperature of 24±1.5 ºC under a 12L:12D photoperiod. Water was renewed at a rate of two thirds once in two days. The use of animals and all handling practices were approved by the Ethics Committee on Animal Experimentation of Federal University of Pelotas (Process no. 23110.014105/2020–56).

Experimental design and sample collection

After 4 weeks of acclimation, sixty fishes were divided in two experimental groups, with three 1000 L tanks each with a final number of 10 fish per tank. In one of the experimental groups the water temperature was maintained at 24ºC and on the other experimental group the temperature started a ramp until 15ºC. The temperature decreasing rate was 0.5 ºC/day and the experimental period started when the cold group achieved the

temperature of 15 ºC. Temperature was measured and registered daily. Fish were kept in these conditions for 4 weeks. Feeding was adjusted after two weeks for food loss reduction. At the end of the experiment, tilapias were caught by hand net and transposed to a 10 L container with tricaine 400 $mg.L^{-1}$ and maintained until loss of equilibrium. Blood samples were collected from the branchial branch artery using a 26G heparinized syringes. 20uL of each blood sample was added to 1 mL of fetal bovine serum (FBS) and stored at 4°C in the dark until use for flow cytometry analysis. Fish were euthanized and the brain, kidney, spleen and liver tissues were collected and preserved in liquid nitrogen until further use. Fish were weighed and measured individually on day 0 and 28, and specific growth rate (SGR) and weight gain were calculated (Kang'ombe and Brown, 2008).

RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples using TRIZOL Reagent (Thermo Fisher Scientific, USA), following manufacturer's instructions. RNA was then treated with DNase using DNAse-free kit (Ambion, USA) to remove genomic DNA contamination. Subsequently, RNA concentration and purity were measured using a NanoVue Plus spectrophotometer (GE Healthcare Life Science, USA) and after the samples were stored at − 80 °C. Complementary DNA (cDNA) was synthetized using High Capacity cDNA Reverse Transcription (Applied Biosystems, USA) according to manufacturer's recommendation at a final concentration of 500 ng. Finally, cDNA was stored at − 20 °C until further use.

Gene expression analysis by qRT-PCR

The primers used in this study are presented in Table 1. The technique of RT-qPCR was performed using GoTaq® qPCR Master Mix (Promega Corporation, USA), and carried out in the Bio-Rad CFX96 Real-Time qPCR Detection System (Bio-Rad, USA). This study used primers that have been previously validated in tilapia. Were used specific primers for

food ingestion regulation genes: neuropeptide Y (*npy*), Cocaine- and Amphetamine-regulated transcript (*cart*) and peptideYY (*pyy*); for immune related genes: Interleukin 1β (*il-1b*), Interleukin 8 (*il-8*), Nuclear factor kappa beta (*nf-kb*) and Tumoral necrosis factor alpha (*tnfa*); and for normalization: β-actin. All reactions were performed in duplicates. The $2^{-\Delta\Delta CT}$ method was used to normalize the fold change in gene expression (Livak and Schmittgen, 2001) using *β-actin* as the reference gene.

Flow Cytometry analysis

Flow cytometry analysis was performed using Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems, USA) to evaluate the effect of chronic low temperature exposure in tilapia physiology by analyzing the erythrocytes. Erythrocytes were assayed for cell disruption, lipid peroxidation, membrane fluidity and DNA fragmentation using a flow cytometer previously mentioned, equipped with violet laser (UV 405 nm-450/40, VL-1). Erythrocytes were stained with Hoechst 33342 (16.2 mM) (Martinez-Alborcia et al., 2012). Cell debris were excluded based on forward scatter × side scatter plot and negative fluorescence of Hoechst 33342. To read all parameters, the fluorophore-stained cells were added into calcium- and magnesium-free phosphate-buffered saline (80 g L^{-1} of NaCl, 11.5 g L^{-1} of KCl, 24 g L^{-1} of Na_2HPO_4, and 2 g L^{-1} of KH_2PO_4 dissolved in deionized water). A total of 20,000 erythrocytes were analyzed during each analysis.

The erythrocyte lipid peroxidation (LPO) was quantified using the final concentration of 1 μM of the lipid peroxidation sensor 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY) in 10 μL of the sample, which was incubated for 2 h at room temperature (20°C). The rate of lipoperoxidation was calculated as the median intensity of green fluorescence (peroxidised lipid) / median green fluorescence intensity + median red fluorescence (non-peroxidised lipid) × 100 (Hagedorn et al., 2012).

To evaluate the DNA damage in erythrocytes, 100 μL of blood sample was added to 5 μL of 0.01 M Tris-HCl, 0.15 M NaCl, and 0.001 M EDTA (pH 7.2), followed by the addition of 10 μL of Triton 1X (Triton X-100, 1%, v/v) 30 s later. Then, 50 μL of acridine orange dye (2 mg mL^{-1}, #A6014, Sigma-Aldrich, USA) was added to the sample, followed by incubation from 30 s up to 2 min before each reading. The DNA of erythrocytes was classified as righteous (green fluorescence emission) or damaged (orange/red fluorescence emission). The percentage of DNA fragmentation index (DFI) was calculated as the median of red fluorescence intensity / (median of the red + green fluorescence intensities) × 100.

Erythrocyte's membrane fluidity was analyzed by hydrophobic merocyanine 540 dye (M540) at final concentration of 2.7 M (Sigma-Aldrich, USA) and YO-PRO, which fluoresces green, at final concentration of 0.1 M (Invitrogen, USA). Only live cells (YO-PRO negative) were selected and classified into high fluidity cells (high M540 concentration) and low fluidity cells (low M540 concentration) (Gillan et al., 2005).

Cell disruption was evaluated using a combination of fluorescent dyes, namely, Annexin V-FITC conjugate (AnV, Sigma A9210) and propidium iodide (PI) (7.3 μM). To detect the disruption, phosphatidylserine that translocate to the outside of the cell membrane was stained. The erythrocytes were classified as live (AnV− and PI−), necrotic (AnV+ and PI+), or disrupted (AnV+ and PI−). The results are expressed as the total number of disrupted cells / total number of erythrocytes × 100.

Statistical analysis

The statistical analyses of weight gain, specific growth rate, gene expression and flow cytometry parameters were realized using the software Statistix version 10.0. Data normality and homogeneity of variances were previously verified. Graphs were generated using

GraphPad Prism 8.0. The data generated were evaluated by Student´s t-test and the differences were considered significant when $P<0.05$.

Results

Growth in low temperature

The growth performance parameters of the studied animals are shown in Table 2. The initial weight did not differ among the experimental groups. Specific growth rate was significantly lower in the group exposed to cold (SGR 1.81 ± 0.16% per day) compared to the control group (SGR 2.92 ± 0.18% per day). Also, the final weight and the weight gain during the experimental period was significantly lower in the group exposed to low temperature (230.41 ± 13.6g and 91.95 ± 13.65g respectively) when compared to the control group after the same period of experiment (334.55 ± 7.09g and 186.91 ± 9.45g respectively).

Feeding-related gene expression in tilapia

The results of the assessment of feeding-related gene expression in the brain of tilapias exposed to a low temperature are represented in Figure 1 A – C. The *cart* expression in the group exposed to cold was lower compared to the control group ($p < 0.05$) (Fig 1A). An increase was observed in the expression of the gene *pyy* in fishes submitted to cold water when compared to the unexposed group ($p < 0.05$) (Fig 1B). The expression of the *npy* gene was lower in the brain of fishes of the cold group ($p < 0.05$) (Fig 1C).

Immune related gene expression in tilapia

The modulation of gene expression of immune related genes in the spleen, kidney and liver are shown in Figure 2 A-D, Figure 3 A-D and Figure 4 A-D respectively. In the spleen, a significant reduction was observed in the expression of *nf-kb* and *il-8* in the group exposed to low temperature (Fig 2A and 2C). At the same time, a significant increase was observed in the

expression of *il-1b* and *tnfa* in the spleen of the same group when compared to the control group (Fig 2B and 2D). In the kidney, no difference between groups was observed in the expression of *nf-kb* (Fig 3A). Also in the kidney, *il-1b*, *il-8* and *tnfa* mRNA expression increased in the cold group compared to the control group (Fig 3B – D) ($p < 0.05$). In the liver, *nf-kb* expression decreased significantly in the exposed group compared to the control group (Fig 4A) whilst *il-1b* showed a significant increase in expression in the group exposed to cold (Fig 4B). No statistical significance was observed in the expression of *il-8* and *tnfa* in the liver of the analyzed groups (Fig 4C and 4D).

Erythrocytes flow cytometry analysis

DNA fragmentation increased in the erythrocytes of the cold exposed group (Fig 5A) ($p < 0.05$). A reduction in the red blood cell membrane fluidity was observed in fishes exposed to low temperatures compared to the non-exposed fishes (Fig 5C and 5D) ($p < 0.05$). Also, there was an increase in the number of disrupted cells in the group exposed to low temperature compared to the control group (Fig 5E) ($p < 0.05$). No difference was observed in the lipid peroxidation among the groups analyzed (Fig 5B) ($p > 0.05$).

Discussion

Our study was the first to analyze the long-term exposure to a stressful low temperature to Nile tilapia owing to the economic losses occurred on similar events. The temperature of 15ºC showed effect on weight gain and specific growth rate after rearing the animals for 28 days in stressful conditions compared to control group. Similar effects were observed in European seabass (*Dicentrarchus labrax*) exposed to extreme winter conditions, showing a decrease on weight gain similar as observed in our study (Islam et al., 2021). Along with the reduction on the weight gain, the SGR also showed a decrease compared to control group, Pang et al. (2016) had observed a similar effect on SGR on common carp

(*Cyprinus carpio*) when acclimated to low temperatures. The SGR showed a strong correlation between feeding rate and feeding efficiency demonstrating that the cold temperatures could have effects on the feeding axis diminishing the feeding (Pang et al., 2016).

We found that the *cart* expression was reduced in the cold exposed group, Zhang et al. (2019) showed cart expression having its peak at 15 days of fasting with a reduction in the next days, this reduction may indicate a stimulus for the fish to search for food in the environment. Vertebrate animals show a specific regulation of food intake, commanded specifically by central effectors within the brain (Volkoff, 2006). Cocaine- and amphetamine-regulated transcript (*cart)* plays a bidirectional role acting as an orexigenic and anorexigenic molecule having its expression regulated on fasting periods and shortly after refeeding (X. Zhang et al., 2018). The *cart* has been related as a satiety signal having its expression elevated after feeding and decreased during fasting in gibel carp (*Carassius auratus gibelio*) (C. Zhou et al., 2019), Siberian sturgeon (*Acipenser baerii*) (X. Zhang et al., 2018) and goldfish (*Carassius auratus*) (Volkoff and Peter, 2001). Other studies have reported that *cart* may act as a fasting indicator, having its expression elevated after 15 days of food abstinence (X. Zhang et al., 2018; C. Zhou et al., 2019). Although we have observed a reduction on the feeding habit of those fish exposed to 15 ºC for such a long period, indicating that this stimulus may be overlapped by other feeding control mechanism.

In our study was observed a reduction on *npy* and an increase on *pyy* expression, revealing that this mechanism was inducing a fasting state in the fish organism. Another important family of effectors of the food intake in fish is the NPY family, which the most important members are the neuropeptide Y (*npy*) and the peptide YY (*pyy*) (Volkoff, 2006). While *npy* stimulates food ingestion, *pyy* act on the other had signaling satiety (Volkoff and Peter, 2006; Yan et al., 2017). Other studies have reported an association between *npy* levels

and insulin showing that the injection of insulin modulates negatively the *npy* expression (Mitchell and Begg, 2021). This type of regulation can be associated with our study once cold temperatures cause a stressful condition for tilapia leading to an increase in the glucose blood level and consequently an increase in the insulin levels and a decrease in the expression levels of *npy* (Mitchell and Begg, 2021; Yilmaz et al., 2021). On the other hand, the enhance in the expression of *pyy* can be associated with anorectic effects leading to the loss of appetite and reduction on the weight gain. It has been described an enhance in *pyy* levels in teleosts during fasting periods, as observed in yellowtail (*Seriola quinqueradiata*) (Murashita et al., 2006), although *pyy* has been suggested as a gene that has a species-specific mechanism during fasting/refeeding periods (Assan et al., 2021). Understanding the *npy* and *pyy* regulation in fish exposed to low temperatures helps elucidating the effect of this environmental condition on the feeding habits and could possibly cope to solve deficits on the production of tilapia and to improve the wellbeing if the species.

Another important factor that influences the tilapia rearing in low temperatures is the increased susceptibility for diseases (Dominguez et al., 2004). The response to pathogens in cold temperatures is essential due to the growth of specific microorganisms that can infect fishes, e.g., *Vibrio anguillarum* and *Aeromonas hydrophila* (Elgendy et al., 2015). Inflammation is one of the first and most important response to pathogens infection in general and cytokines play an important role in the inflammation signaling (C.-N. Zhang et al., 2018).

In our study was observed an up-regulation of *il-1b* in the spleen, kidney and liver. This cytokine is one of the most studied since present multiple functions within the inflammatory process (Dinarello, 2009). The increased expression of the *il-1b* have already been related to different temperature exposure in rainbow trout (*Oncorhynchus mykiss*) vaccinated against *Yersinia ruckeri*, the fish showed a delayed expression of *il-1b* at lower temperatures compared to higher temperatures (Raida and Buchmann, 2007).

In the tilapia exposed to low temperatures *tnfa* had its expression enhanced in the spleen and kidney of the cold exposed group, however no significant difference was observed in the liver. The higher expression of the cytokine implies that an inflammatory reaction is occurring in the organism, possibly due to the stress caused by the cold water. the expression of the pro-inflammatory mediator *tnfa* is closely related to *il-1b* (Dinarello, 2009). The *tnfa* is responsible for the triggering of cellular responses leading for both cellular proliferation, by the activation of NF-Kβ pathway, and apoptosis (Cook et al., 2018; Li et al., 2014). In studies with tilapia, the increase of *il-1b* along with *tnfα* have been reported caused by the administration of a *Bacillus spp.* in diet to promote a resistance against *Streptococcus agalactiae* (Van Doan et al., 2021), however Van Doan et al. (2021) mentioned that this modulation could be caused by environmental factors, dietary regimes, sampling strategies and host genetic variations (Tarnecki et al., 2017).

In this study the *il-8* expression was variable among the evaluated tissues, with a lower expression in the spleen, a higher expression in the kidney and no difference in the liver. Added to the inflammatory response, the *il-8*, also known as CXCL8, is a chemokine responsible primarily for mediating the singling for immune cell migration (van der Aa et al., 2010). Along with this signalization, the *il-8* is related to inducing the respiration burst, exocytosis, degranulation of storage proteins and production of lipid mediators (Wang et al., 2019). The specific expression found in this study could be explained by the activation of anti-inflammatory mechanisms, such as interleukin 10, to prevent damage to the specific tissues during inflammation process (Sabat et al., 2010). This pattern has already been observed in black rockfish stimulated with *Streptococcus iniae* and poly I:C after 24h post-immunization, with a higher expression in the head-kidney and liver and lower expression in the spleen and blood cells (Herath et al., 2016). The anti-inflammatory mechanism is important to maintain the tissue integrity by protecting from apoptosis events, our study

showed a modulation of pro-inflammatory cytokines during the 28 days exposure to cold temperature. Actions derived from the anti-inflammatory system may be the key for the tilapia surviving during this experimental period.

In our study the *nf-kb* was downregulated in the spleen and liver, and no difference was observed in the kidney of the cold group fish. An important pathway also of the inflammatory process is the NF-Kβ pathway. It has multiple possible regulators, being the *il-1b* and the *tnfa* the principal activators of this pathway (Li et al., 2021). Along with the results observed in the *il-8* expression, the down regulation of this pathway could be explained by the actuation of anti-inflammatory mechanisms in those tissues (Li and Verma, 2002; Sabat et al., 2010). The downregulation of *nf-kb* was also observed in liver juvenile black seabream (*Acanthopragus schlegelii*) fed with betaine, a natural by-product in sugar beets (*Beta vulgaris*) showing the anti-inflammatory potential of this compound and demonstrating the effects of this mechanism in the *nf-kb* gene (Jin et al., 2021). Further studies may be necessary to understand profoundly the modulation of the immune system in Nile tilapia exposed to low temperatures.

Along with the results obtained with the gene expression, flow cytometry showed insights on the effects of cold water in blood cells parameters such as membrane fluidity, DNA fragmentation and cell disruption. Membrane fluidity is related primarily with the membrane fatty acids propriety of fluidity and dynamicity (Saita et al., 2016). There is known that environmental conditions could affect the conformation of those molecules affecting the dynamics and fluidity. The bilayer changes it fluidity to protect the cell from cold, ranging from fluid in optimal temperature conditions to an anionic fluid (ordered and thick) array (Shen et al., 2021). Other blood cell parameter affected by the low temperature is the DNA fragmentation that enhanced. The DNA fragmentation is correlated with the oxidation caused principally by reactive oxygen species acting at the DNA level. In *Channa punctatus* there

was observed an increase in the DNA fragmentation index because of Cadmium induced reactive oxygen species production (Choudhury et al., 2021). Another possible cause of the enhanced DNA fragmentation along with the higher number of cell disruption, as observed in pufferfish (*Takifugu obscurus*) exposed to cold stress, is the lower number of blood cells, leading to a cell toxicity (Cheng et al., 2017). This was also observed in other aquatic organisms exposed to low temperatures (Hagedorn et al., 2012; Qi et al., 2011; Qiu et al., 2011). Further analysis is necessary to complement and better understand the effects of cold environmental condition in tilapia's blood cells parameters.

In this study we have demonstrated the effects of a chronic exposure to suboptimal temperature on the growth, expression of genes related to food ingestion and immunity, and parameters of blood cells of Nile tilapia (*O. niloticus*). The results showed a decreased specific growth rate along with the modulation of specific genes responsible for the food ingestion and inflammation processes. The knowledge acquired on this study can help to elucidate the effects of cold temperature on Nile tilapia and to cope with the production of this fish in view of the climate changes that is a near reality for the planet.

Ethics Statement

The methodology used in this study was approved by the Ethics Committee of the Federal University of Pelotas/RS, Brazil (Process no. 23110.014105/2020–56)

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Table 1 – List of primer sequences used for real-time quantitative PCR (q-PCR) analysis in this study

Gene name	Gene symbol	Function	Primer sequences (5' → 3')	Genbank accession no	Reference
Neuropeptide Y	*npy*	Orexigenic neurotransmitter	F: ACAAGACAGAGGTATGGGAAGA R: GGCAGCATCACCACATTG	KJ778894.1	(Yan et al., 2017)
Cocaine- and Amphetamine-Regulated transcript	*cart*	Anorexigenic neuropeptide	F: TGCTGACATCACTCTGTCAAGG R: AGCCAGCTCACTGGTTGTG	MW556307.1	*
Peptide YY	*pyy*	Anorexigenic hormone	F: AACACTGGCTGATGCCTACC R: TTCCATACCTCTGCCTGGTG	MW556314.1	*
Interleukin 1 beta	*Il-1b*	Inflammatory response	F: GACAGCCAAAAGAGGAGC R: TATCAGCGATGGGTGTAG	DQ061114.1	
Interleukin 8	*Il-8*	Inflammatory response	F: GCACTGCCGCTGCATTAAG R: GCAGTGGGAGTTGGGAAGAA	XM_003455949.2	(Elbahnaswy and
Nuclear factor kappa beta	*nf-kb*	Transcription factor	F: TCACGAGTGCCATCCTTCTG R: TCGTCTCGATCCTACGGGAA	XM_019363515.2	Elshopakey, 2020)
Tumour necrosis factor alpha	*tnfa*	Inflammatory response	F: TAGAAGGCAGCGACTCAA R: CCTGGCTGTAGACGAAGT	AY428948.1	
β-actin	*actb*	Reference gene	F: TGGTGGGTATGGGTCAGAAAG R: CTGTTGGCTTTGGGGTTCA	XM_003455949	(Yang et al., 2013)

Table 2 - Effect of low temperature on weight gain and specific growth rate (SGR) of Nile tilapia (mean ± SD) for 28 days. Asterisks depict statistically significant changes identified by Student´s t-test ($p < 0.05$).

Groups	**Initial Weight (g)**	**Final Weight (g)**	**Weight gain (g)**	**SGR (%/day)**
Control	147.64 ± 10.35	334.55 ± 7.09	186.91 ± 9.45	2.92 ± 0.18
Cold	138.45 ± 2.58	230.41 ± 13.6*	91.95 ± 13.65*	1.81 ± 0.16*

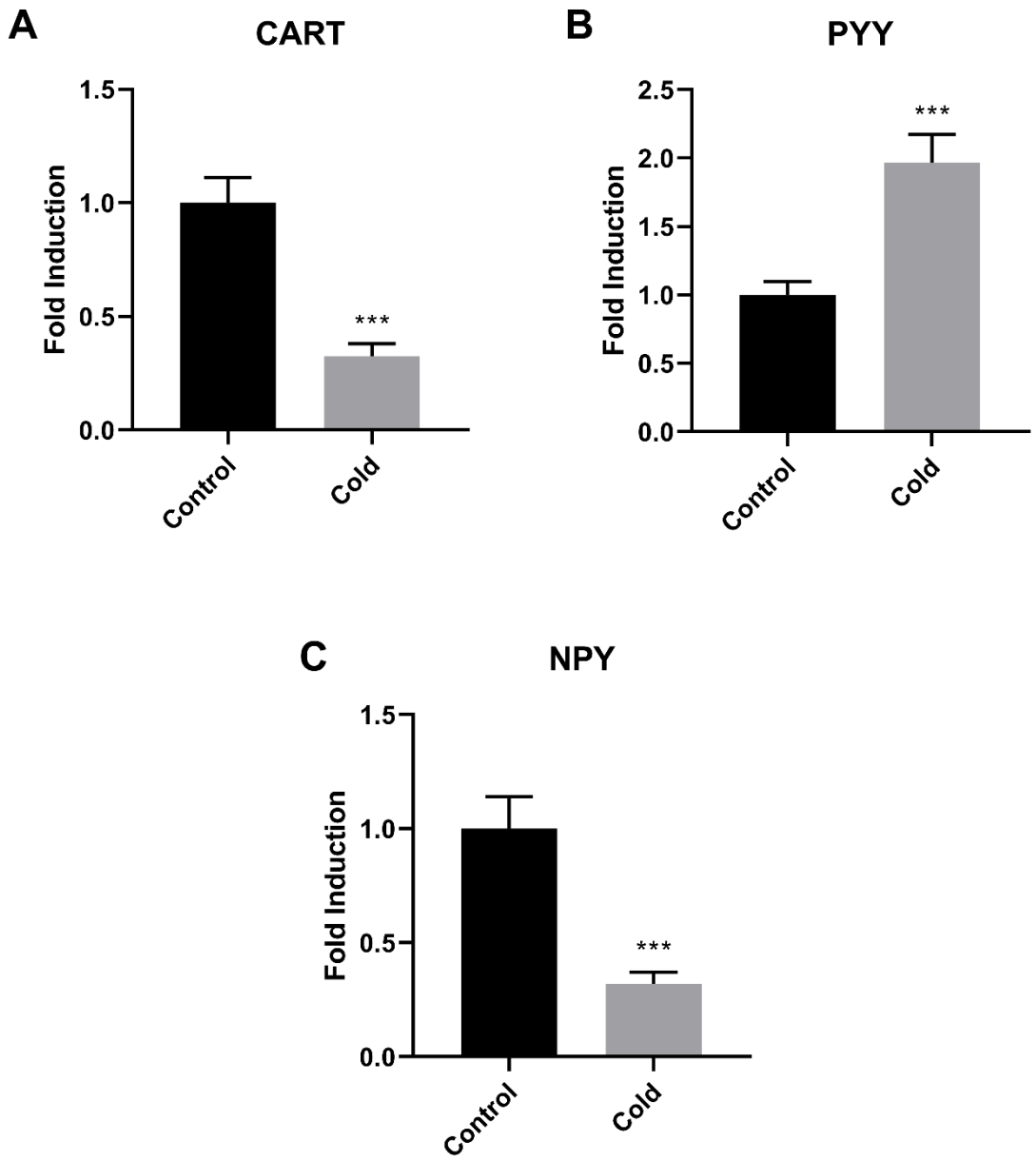

Figure 1 - Gene expression in the brain of Nile tilapias in the control group and those exposed to a low temperature during 28 days. The mRNA relative expression of *cart* (**A**), *pyy* (**B**) and *npy* (**C**) was evaluated by quantitative reverse transcription-polymerase chain reaction and normalized using the β-actin gene. The values are expressed as mean ± standard error of the mean. Different symbols indicate significant differences between the experimental groups (Student's t-test; $n = 6$, $p < 0.05$).

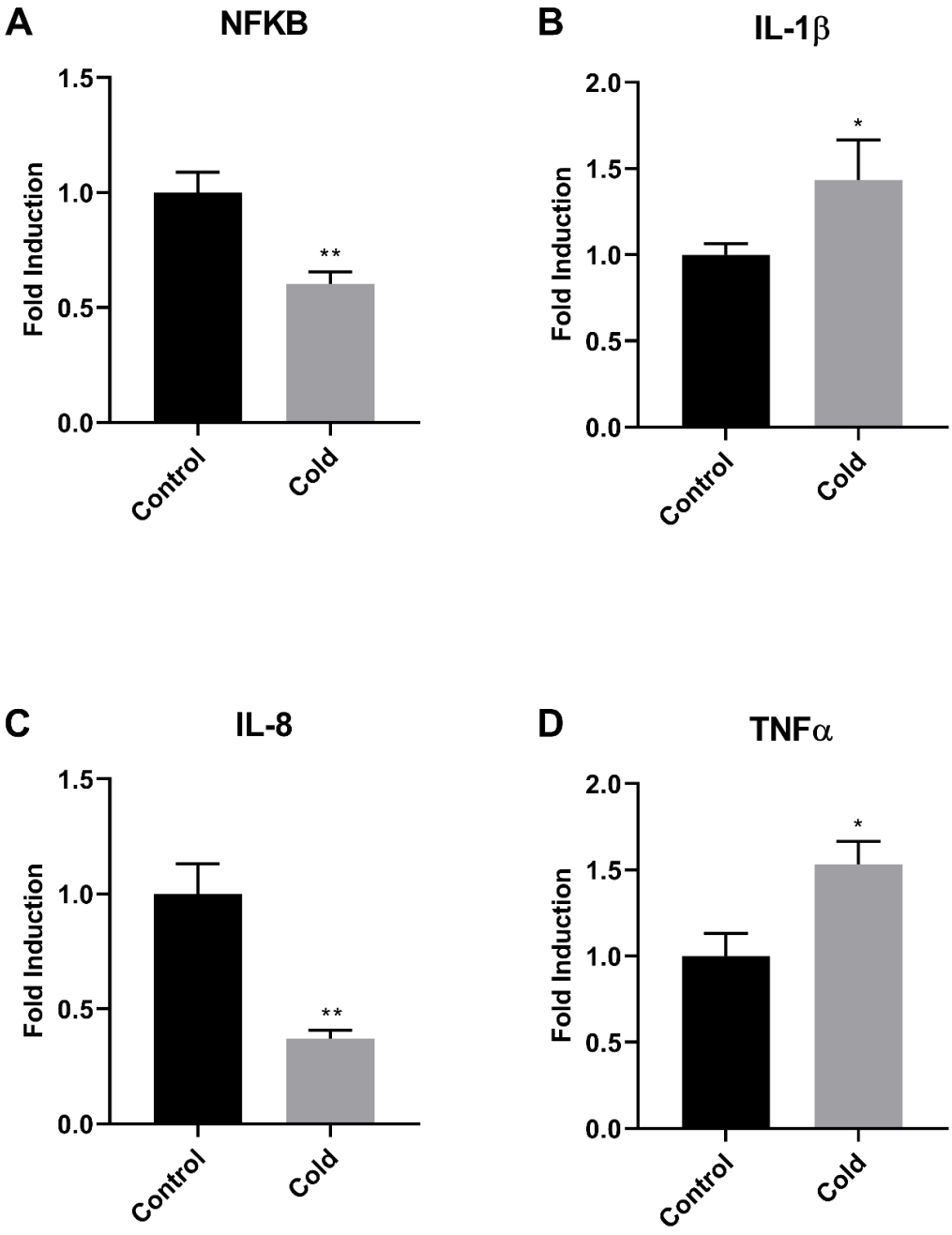

Figure 2 - Gene expression in the spleen of Nile tilapias in the control group and those exposed to a low temperature during 28 days. The mRNA relative expression of *nf-kb* (**A**), *il-1b* (**B**), *il-8* (**C**) and *tnfa* (**D**) was evaluated by quantitative reverse transcription-polymerase chain reaction and normalized using the β-actin gene. The values are expressed as mean ± standard error of the mean. Different symbols indicate significant differences between the experimental groups (Student's t-test; $n = 6$, $p < 0.05$).

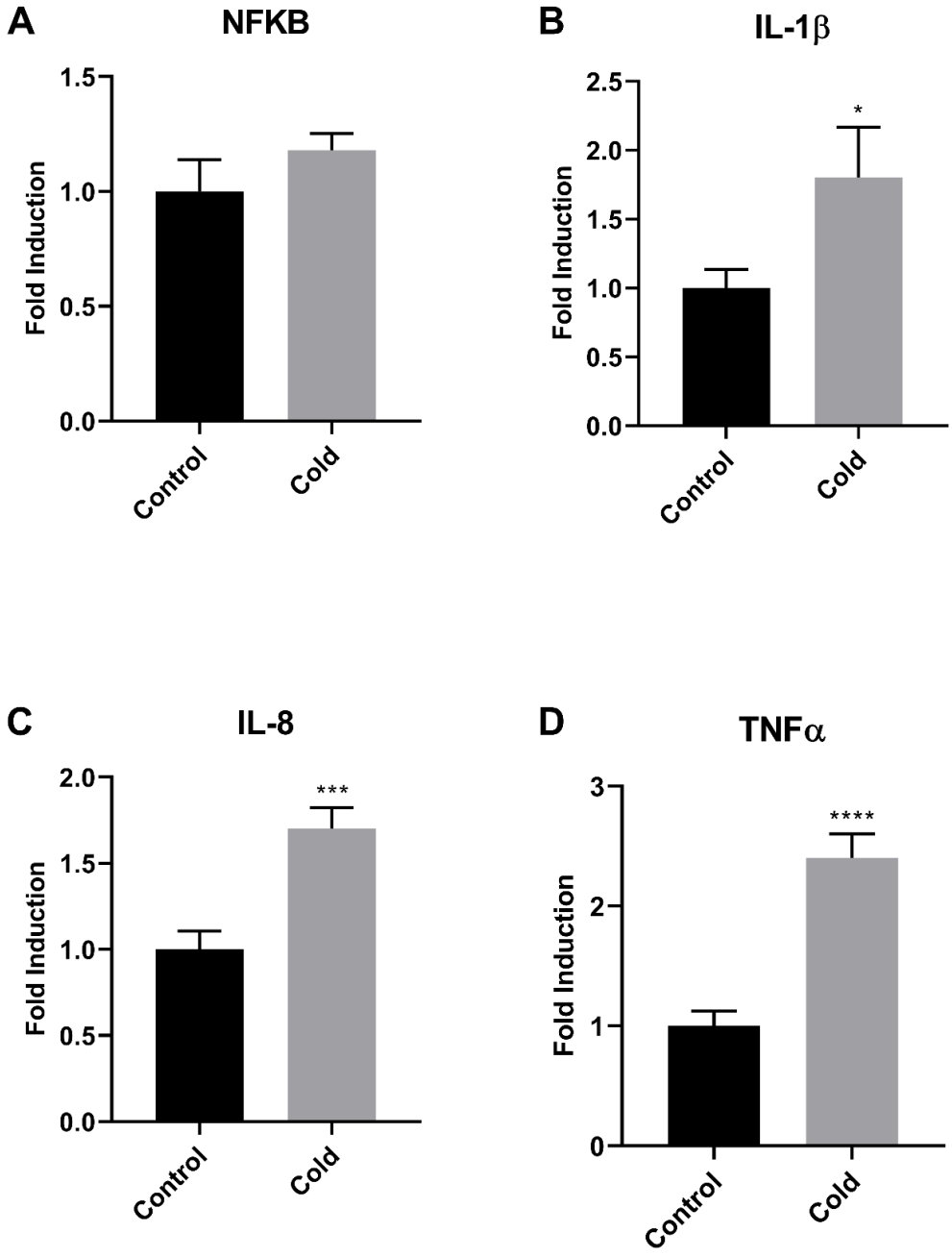

Figure 3 - Gene expression in the kidney of Nile tilapias in the control group and those exposed to a low temperature during 28 days. The mRNA relative expression of *nf-kb* (**A**), *il-1b* (**B**), *il-8* (**C**) and *tnfa* (**D**) was evaluated by quantitative reverse transcription-polymerase chain reaction and normalized using the β-actin gene. The values are expressed as mean ± standard error of the mean. Different symbols indicate significant differences between the experimental groups (Student's t-test; $n = 6$, $p < 0.05$).

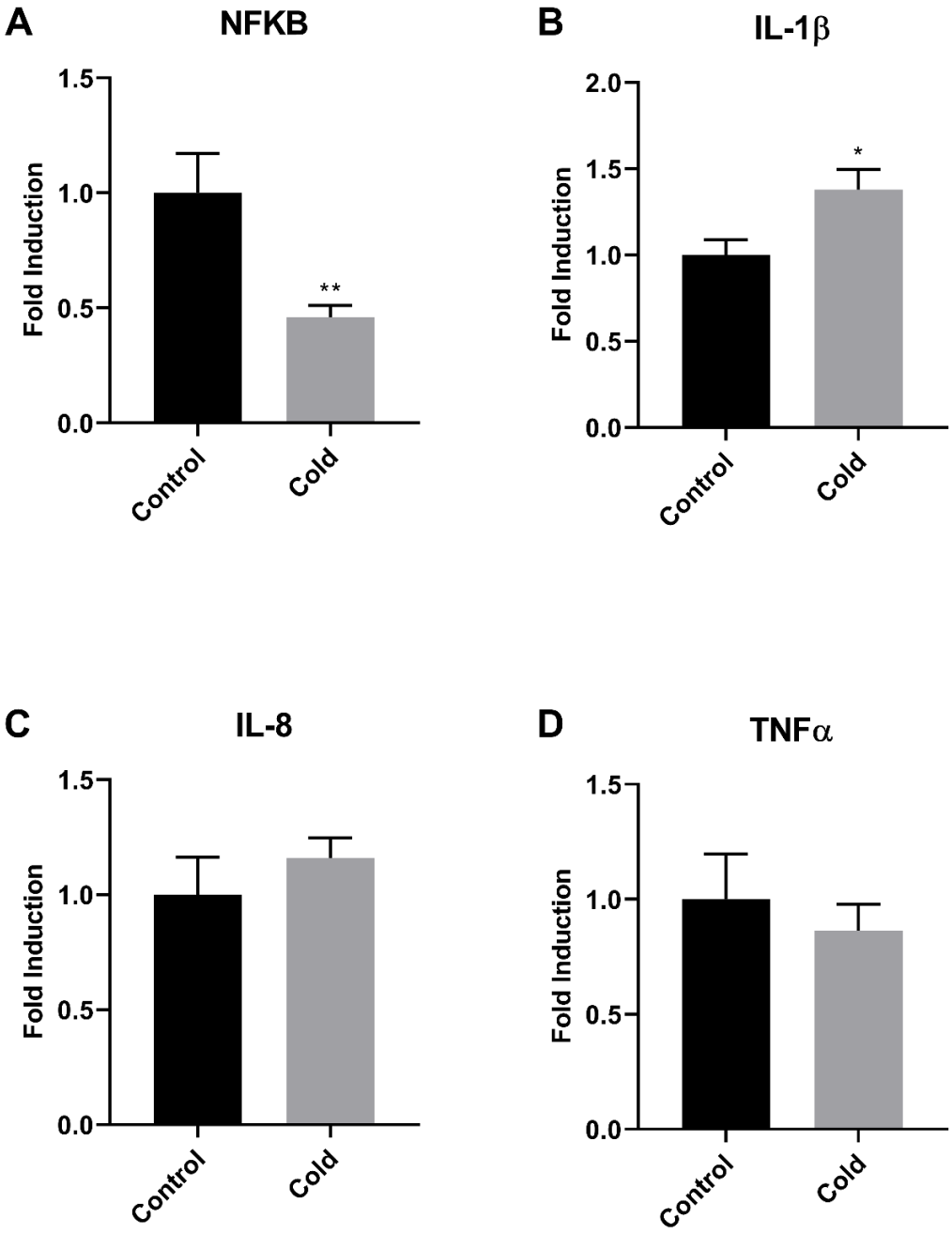

Figure 4 - Gene expression in the liver of Nile tilapias in the control group and those exposed to a low temperature during 28 days. The mRNA relative expression of *nf-kb* (**A**), *il-1b* (**B**), *il-8* (**C**) and *tnfa* (**D**) was evaluated by quantitative reverse transcription-polymerase chain reaction and normalized using the β-actin gene. The values are expressed as mean ± standard error of the mean. Different symbols indicate significant differences between the experimental groups (Student's t-test; $n = 6$, $p < 0.05$).

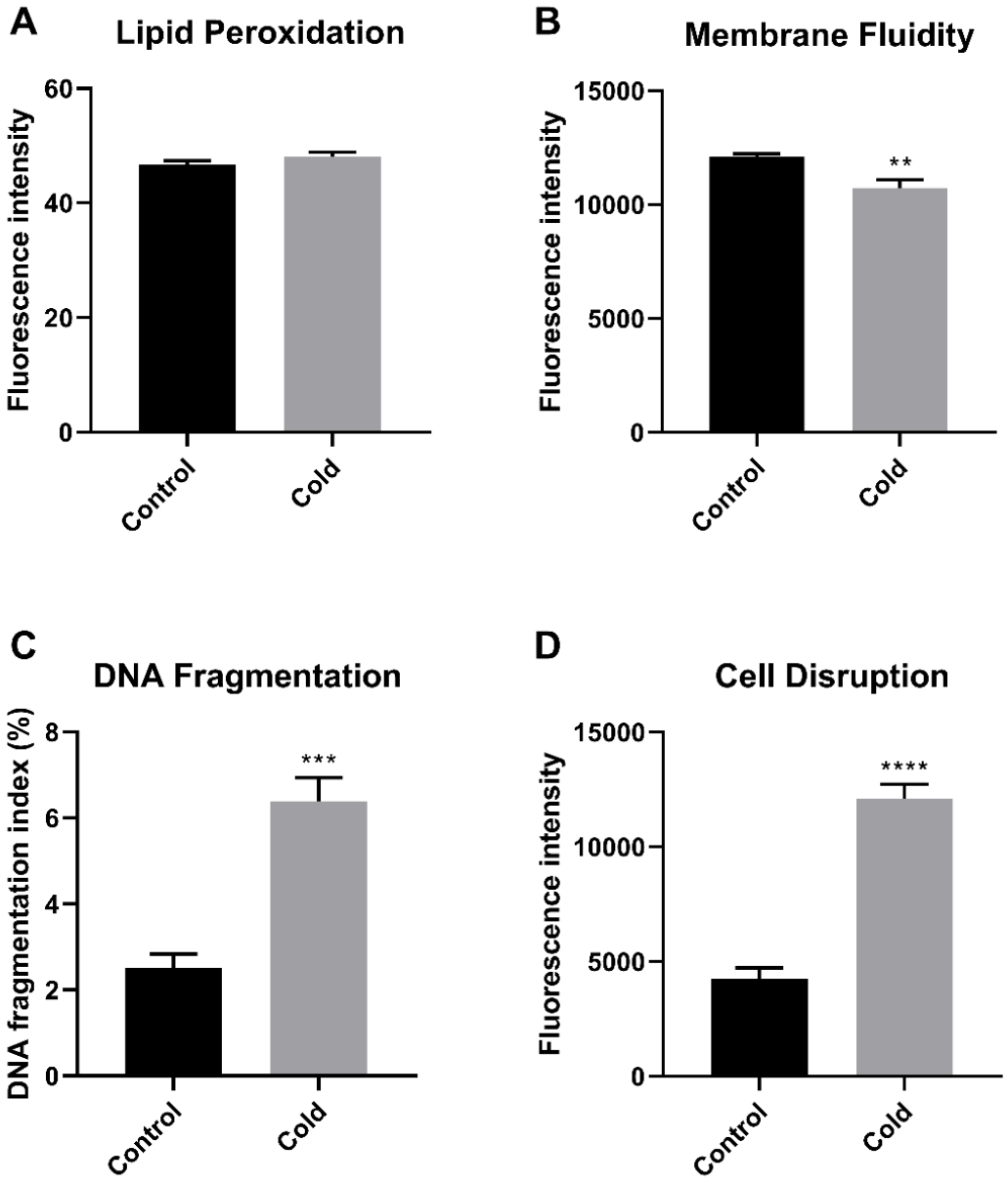

Figure 5 - The effects in terms of the lipid peroxidation (fluorescence intensity) (**A**), membrane fluidity (fluorescence intensity) (**B**), DNA fragmentation index (%) (**C**) and cell disruption (fluorescence intensity) (**D**) in the erythrocytes of Nile tilapia in the control group and those exposed to a low temperature of 15 ºC for 28 days, as evaluated by flow cytometry. The values are expressed as mean ± standard error of the mean. Different symbols indicate significant differences between the experimental groups (Student's t-test; $n = 9$; $p < 0.05$)

5 CONCLUSÃO GERAL

De acordo com os resultados obtidos, o frio acaba por afetar as tilápias levando-as demonstrarem um menor peso final e menor taxa de crescimento específico. Além disso o frio causa alterações metabólicas que acabam por gerar uma situação de estresse no animal.

A modulação nos genes responsáveis pela alimentação mostrou a atuação de sinalização anorexigênica quando os peixes foram expostos a baixas temperaturas. Foi observada a redução da expressão do *cart* e *npy*, e o aumento na expressão do *pyy* nos animais após 28 dias de exposição à temperatura de 15ºC. Esta alteração na expressão gênica reforça e consolida os motivos pelos quais os animais acabam por cessar a sua alimentação em temperaturas amenas. A utilização destas informações para a geração de uma tecnologia que possa induzir a alimentação em animais submetidos a baixas temperaturas pode ser uma alternativa para contornar possíveis perdas devido a estas condições.

A maior expressão de algumas citocinas pró-inflamatórias demonstra que o frio também atua levando o peixe a gerar processos inflamatórios. Também é possível observar através da redução na expressão do gene *nf-kb* que o organismo tenta compensar através da produção de fatores anti-inflamatórios para evitar um possível dano no tecido alvo. Uma forma de ajudar o peixe a sobreviver seria utilização de tecnologias para modulação e reforço da imunidade podendo servir como aliado para combater o enfraquecimento do sistema imunológico durante períodos de estresse causados pelas baixas temperaturas.

Os resultados obtidos através deste estudo forneceram importantes dados acerca da fisiologia de tilápias (*O. niloticus*) expostas à baixas temperaturas que não haviam sido descritas até o presente momento. Além disso, este estudo gerou conhecimento necessário para o desenvolvimento de biotecnologias que auxiliem na melhora da produção de peixes de cultivo economicamente importantes.

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7 ANEXOS

Anexo A – Parecer do comitê de ética em experimentação animal