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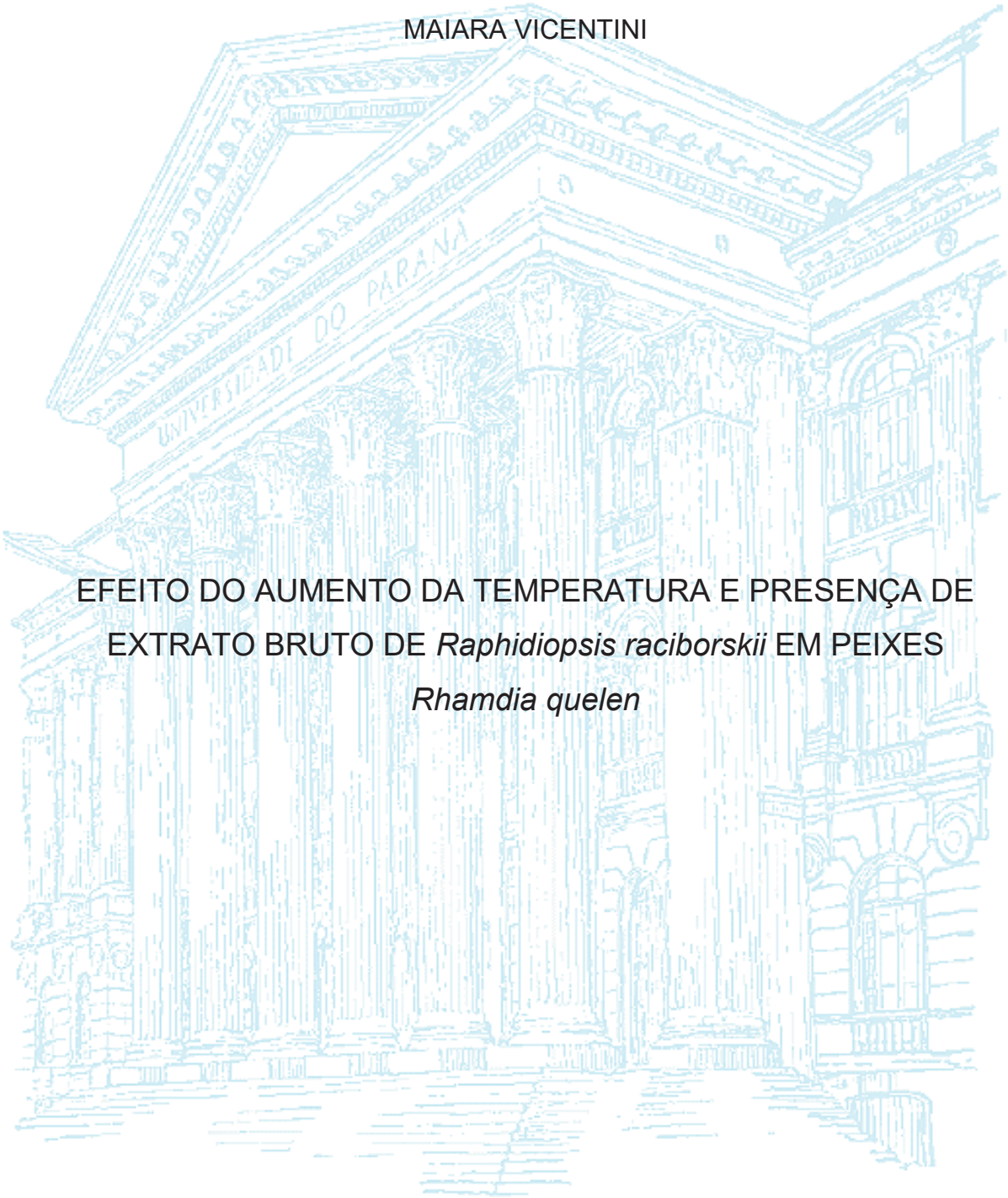
MAIARA VICENTINI



EFEITO DO AUMENTO DA TEMPERATURA E PRESENÇA DE EXTRATO BRUTO DE *Raphidiopsis raciborskii* EM PEIXES *Rhamdia quelen*

CURITIBA

2021

MAIARA VICENTINI

EFEITO DO AUMENTO DA TEMPERATURA E PRESENÇA DE EXTRATO BRUTO DE *Raphidiopsis raciborskii* EM PEIXES *Rhamdia quelen*

Tese apresentada ao Curso de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas, Universidade Federal do Paraná, como parte das exigências para a obtenção do título de Doutora em Ecologia e Conservação.

Orientador: Profª. Drª. Helena Cristina da Silva de Assis

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ECOLOGIA E CONSERVAÇÃO da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MAIARA VICENTINI** intitulada: **Efeito do aumento da temperatura e presença de extrato bruto de *Raphidiopsis raciborskii* em peixes *Rhamdia quelen***, sob orientação da Profa. Dra. HELENA CRISTINA SILVA DE ASSIS, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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“I’m proud of you ...

because regardless of what

you’re going through at this very moment,

you decided not to let it break you.”

Taylor Swift

RESUMO

A água é um dos recursos mais afetados pela intensificação das atividades antrópicas. As consequências dessas atividades, como a eutrofização e o aquecimento global, favorecem as florações de cianobactérias produtoras de cianotoxinas, sendo este um grande problema em reservatórios de abastecimento público de água. O objetivo deste trabalho foi avaliar a resposta de uma espécie nativa de peixe exposta à diferentes temperaturas e às neurotoxinas produzidas pela *Raphidiopsis raciborskii*, utilizando biomarcadores de contaminação ambiental e análise proteômica. Para isso, o extrato bruto foi produzido a partir do cultivo dessas cianobactérias e as cianotoxinas presentes foram quantificadas. Peixes da espécie *Rhamdia quelen*, machos e fêmeas, foram expostos a diferentes tratamentos: controle 25°C, controle 30°C, extrato equivalente a 10^5 células/mL a 25°C e extrato de 10^5 células/mL a 30°C. Após 96 horas, os animais foram anestesiados e o sangue coletado. Após a eutanásia, os arcos brânquiais, rim posterior, cérebro, músculo, fígado e gônada foram coletados para análise de diferentes biomarcadores, enquanto o fígado também foi utilizado para a análise proteômica. A temperatura isoladamente foi capaz de alterar parâmetros sanguíneos, como os níveis de glicose e número de leucócitos, além de causar danos ao DNA. A análise proteômica do fígado mostrou que o aumento da temperatura pode alterar proteínas relacionadas a estruturação e transporte celular, produção de energia, resposta imune e vias metabólicas de diferentes compostos, como os aminoácidos. A quantificação de saxitoxinas, as cianotoxinas produzidas pela *R. raciborskii,* mostrou a estabilidade das mesmas em ambas as condições térmicas, de 25°C e 30°C. Contudo, diferentes efeitos foram observados nos peixes à elas expostos. Na exposição ao extrato da cianobactéria à 25°C, foi possível observar maiores alterações em parâmetros sanguíneos e danos ao DNA em diferentes tecidos, principalmente em machos. Já a exposição ao extrato da cianobactéria à 30°C, foi observado maiores danos bioquímicos em fêmeas, como lipoperoxidação, além de genotoxicidade em ambos os sexos em todos os tecidos analisados. Em ambas as condições térmicas, o extrato da cianobactéria foi capaz de alterar vias proteômicas relacionadas a estrutura celular, produção de energia, processos metabólicos e reprodução, contudo com alterações em proteínas diferentes. Estes resultados demonstram diferença sexual de respostas ao estresse, bem como o agravamento dos efeitos causados por cianotoxinas produzidas por *R. raciborskii* em fêmeas de *Rhamdia quelen*.

Palavras-chave: cianobactérias, peixes, temperatura, saxitoxinas

ABSTRACT

The water is one of the resources most affected by the intensification of the anthropic activities. Consequences of these activities, such as eutrophication and global warming, can cause the cyanobacteria blooms that produce cyanotoxins and be a major problem in public water supply reservoirs. The aim of this study was to evaluate the native fish species response exposed to different temperatures and to neurotoxins produced by *Raphidiopsis raciborskii*, using environmental contamination biomarkers and proteomic analysis. For this, the crude extract was produced from the cultivation of these cyanobacteria and the cyanoxytoxins present were quantified. Male and female *Rhamdia quelen* fish were exposed to different treatments: 25°C control, 30°C control, extract equivalent to 10^5 cells/mL at 25°C and extract of 10^5 cells/mL at 30°C. After 96 hours, the animals were anesthetized and blood collected. After euthanasia, the gill, posterior kidney, brain, muscle, liver and gonad were collected for different biomarkers analysis, while the liver was also used for proteomic analysis. The temperature alone was able to change blood parameters, such as glucose levels and the number of leukocytes, in addition to causing DNA damage. Liver proteomic analysis showed that increasing temperature can alter proteins related to cell structure and transport, energy production, immune response and metabolic pathways of different compounds, such as amino acids. The saxitoxins quantification, the cyanotoxins produced by *R. raciborskii*, showed stability under both thermal conditions of 25°C and 30°C. However, different effects were observed in fish exposed to them. In exposure to cyanobacteria extract at 25°C, it was observed major changes in blood parameters and DNA damage in different tissues, especially in males. The exposure to cyanobacteria extract at 30°C, greater biochemical damage was observed in females than in males, such as lipoperoxidation, in addition to genotoxicity in both sexes in all tissues analyzed. In both thermal conditions, cyanobacteria extract was able to alter proteomic pathways related to cell structure, energy production, metabolic processes and reproduction, however with alterations in different proteins. These results demonstrate sexual differences in stress responses, as well as the effects worsening of cyanotoxins produced by *R. raciborskii* in females of *R. quelen*.

Key words: cyanobacteria, fish, temperature, saxitoxins

APRESENTAÇÃO

Essa tese é composta por uma breve introdução, seguida de revisão de literatura e objetivos. Na sequência estão apresentados quatro capítulos, referentes aos artigos científicos gerados com os resultados da tese, intitulados:

Capítulo I - O aumento da temperatura por curto prazo aumenta os impactos sobre um peixe neotropical.

Capítulo II - Como o aumento da temperatura afeta um peixe de água doce? Uma abordagem proteômica.

Capítulo III - A temperatura pode influenciar os efeitos tóxicos causados pelo extrato bruto de cianobactéria em um peixe neotropical?

Capítulo IV - Abordagem proteômica para avaliar os efeitos extrato bruto de cianobactéria sob diferentes condições de temperatura.

Cada um dos artigos está organizado conforme as revistas as quais serão submetidos, já com suas respectivas referências.
Na sequência há a discussão geral sobre os resultados e as considerações finais da tese, seguida pelas referências utilizadas na introdução, revisão de literatura e discussão geral.

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LISTA DE SIGLAS

µg/L	-	Microgramas por litro
ATP	-	Adenosina trifosfato
CA	-	Anidrase carbônica
CAT	-	Catalase
dcSTX	-	Decarmaboil saxitoxina
DNA	-	Ácido dexorribonucleico
equiSTX	-	Equivalente de saxitoxina
ERO	-	Espécie reativa de oxigênio
ETA	-	Estação de tratamento de água
GPx	-	Glutationa peroxidase
GSH	-	Glutationa reduzida
GST	-	Glutationa S-transferase
GTX	-	Goniautoxina
H_2O_2	-	Peróxido de hidrogênio
LPO	-	Lipoperoxidação
mL	-	Mililitros
neoSTX	-	Neosaxitoxina
$O^{2-\bullet}$	-	Ânion superóxido
PST	-	*Paralytic Shellfish Toxin*
SCGE	-	*Single Cell Gel Electrophoresis*
SOD	-	Superóxido dismutase
STX	-	Saxitoxina

SUMÁRIO

1 INTRODUÇÃO GERAL

Com o aumento da expansão humana, a demanda por recursos naturais e industriais tem aumentado e impactado o meio ambiente, levando a sua degradação. A contaminação de corpos d'água, por exemplo, pode ocorrer tanto por meio de xenobióticos como pelo excesso de nutrientes. Este último é responsável pela eutrofização, um processo de enriquecimento artificial de ambientes aquáticos, causados pela adição de nutrientes, como nitrogênio e fósforo, provenientes de esgoto industrial e doméstico, fertilizantes, entre outros. A eutrofização pode ser agravada pela intensificação das mudanças climáticas, visto que o aumento da temperatura pode acentuar a estratificação térmica e a entrada de nutrientes (FONSECA, 2012).

O aquecimento global por si só, causado pelas mudanças climáticas, pode levar a diferentes alterações na biota local, visto que essa estratificação pode reduzir os níveis de oxigênio da água (COLLINS et al., 2021). Se as espécies não forem capazes de tolerar, se adaptar ou fugir dessas mudanças, principalmente de temperatura e oxigênio, as mesmas irão se extinguir (PECL et al., 2017).

Além de todos esses problemas, ao agravar a eutrofização, o aquecimento global pode favorecer o aumento da ocorrência e da intensidade de florações de cianobactérias potencialmente tóxicas (FONSECA, 2012). Este vem se tornando um grave problema em reservatórios de água destinados ao abastecimento humano, como nos reservatórios situados no Paraná. Isto porque estas cianobactérias podem ser capazes de produzir toxinas, chamadas de cianotoxinas. Uma das cianotoxinas que pode ser produzida por esses organismos é a saxitoxina (STX), uma neurotoxina extremamente tóxica que pode se acumular no músculo de peixes, tornando este um problema de saúde pública, além de um problema ambiental (CALADO et al., 2017).

Sabendo que as mudanças climáticas estão se agravando cada vez mais, como que o aquecimento das águas pode afetar, subletalmente, uma espécie neotropical de peixe com interesse econômico em curto período de exposição? E visto que a poluição e as mudanças climáticas podem intensificar as florações de cianobactérias potencialmente tóxicas, como que essas toxinas irão afetar uma espécie de peixe neotropical sob essa nova condição térmica?

2 REVISÃO DE LITERATURA

2.1 DEGRADAÇÃO DE AMBIENTES AQUÁTICOS

A globalização e a expansão humana têm como consequência o aumento do consumo e da demanda por diferentes recursos, sejam eles de origem natural ou industrial (SOUZA; OLIVEIRA, 2016). O aumento populacional maior do que a capacidade suporte da natureza pode levar à escassez de recursos, bem como a degradação dos ambientes (MORAES; JORDÃO, 2002).

A degradação ambiental, seja por alteração das paisagens ou poluição dos recursos naturais, têm graves consequências ambientais, econômicas e de saúde pública. Ela pode trazer muitos custos ao homem, seja pela perda econômica pela falta de água, aumento de gastos para filtração de ar e tratamento de água e, principalmente, danos à saúde humana (MA et al., 2020).

O ambiente aquático é um dos que mais sofrem com o aumento excessivo de atividades antrópicas. Esse tipo de ecossistema cobre mais de dois terços do planeta e, portanto, desempenha um papel fundamental para a estabilização do planeta. No entanto, esse ambiente sofre fortemente com a atividade antrópica, principalmente com a poluição, sendo que esta pode ocorrer, por exemplo, por meio derrames de petróleo, descarte incorreto de materiais fonte de metais, descarte de incorreto de lixo plástico, uso de agrotóxico que alcança corpos hídricos e despejo incorreto de esgoto (HADËR et al., 2020). Algumas dessas fontes de poluição podem levar a eutrofização dos ambientes aquáticos, que é o enriquecimento artificial de ambientes aquáticos, causados pela adição de nutrientes, como nitrogênio e fósforo, provenientes de esgoto industrial e doméstico, fertilizantes, entre outros (FONSECA, 2012).

O processo de eutrofização de ambientes aquáticos pode acarretar em diferentes problemas como o aumento da biomassa de fitoplâncton, reduzindo assim o oxigênio e a transparência da água, levando a morte de peixes (SMITH; SCHINDLER, 2009). A eutrofização pode ser agravada pela intensificação das mudanças climáticas, visto que o aumento da temperatura pode diminuir a densidade da água, acentuar a estratificação térmica, diminuir a mistura na coluna d’água, alterando o regime hidráulico e a ciclagem/entrada de nutrientes no ambiente (FONSECA, 2012).

2.2 MUDANÇAS CLIMÁTICAS E AQUECIMENTO DAS ÁGUAS

As mudanças climáticas são outra consequência das atividades antrópicas exacerbadas. Um dos parâmetros mais avaliados desse processo em ambientes aquáticos é a alteração da temperatura, visto que essas mudanças aumentaram a frequência, duração e intensidade das ondas de calor (BAKER et al., 2020). No entanto também ser encontradas, em decorrência das mudanças climáticas, as alterações de pH, salinidade e nível de oxigênio (BRAGA et al., 2018).

A temperatura do planeta é controlada por gases atmosféricos, como o gás carbônico e metano, que absorvem parte da energia solar e retém seu calor. Esse fenômeno é algo natural, pelo qual o planeta se mantém aquecido e se dá o nome de Efeito Estufa (SIVARAMANAN, 2015). Contudo, a intensa atividade industrial, a queima de combustíveis fósseis e o desmatamento tem intensificado esse processo, o que gerou alteração de clima mundial (SHAHZAD, 2015). Esse aumento da média global da temperatura do planeta dá-se o nome de aquecimento global (LE TREUT et al., 2007).

O Acordo de Paris, de 2015, declarou 1,5 °C como limite de aumento da temperatura global, valor que corresponde à média global combinada das temperaturas do ar e da superfície do mar. O objetivo foi que os países assinantes de tal acordo se comprometessem a reduzir suas emissões de gases do efeito estufa para que esse limite não seja ultrapassado (ALLEN et al., 2018). No entanto, no Brasil, esse valor corresponde ao aumento da temperatura entre 1850 a 2010 (ARTAXO, 2014). Essas mudanças de temperatura do ar influenciam na temperatura da água, que também têm aumentado nas últimas décadas.

No ambiente aquático, o aquecimento das águas pode causar diversos efeitos negativos. As espécies precisam aprender a tolerar a mudança, se mover ou se adaptar para evitar a extinção, no entanto nem sempre isso acontece (PECL et al., 2017). No indivíduo, esse aquecimento pode alterar os desempenhos respiratório e metabólico e reduzir o tempo de sobrevivência (COLLINS et al., 2021). Esses efeitos individuais podem acarretar em efeitos em níveis biológicos superiores, desregulando processos ecológicos, acelerando a taxa de perda de espécies e alterando a composição e estrutura funcional das comunidades, seja de peixes ou fitoplâncton, por exemplo (LOGEZ; PONT, 2012; FERREIRA et al., 2018). Dessa forma, estudos moleculares e fisiológicos podem ajudar a prever os efeitos das

alterações de temperatura. Como as espécies exibem diferenças na tolerância térmica, as teias alimentares podem ser afetadas por causa por essa diferença da aptidão entre as espécies (SOMERO, 2010). E o aumento da temperatura acima do considerado crítico pela espécie pode levar ao aumento do consumo de energia e funções mitocondriais, além do aumento de mecanismos protetivos, como o aumento de proteínas de choque térmico e a indução do sistema antioxidante (PÖRTNER; PECK, 2010).

2.3 CIANOBACTÉRIAS

As cianobactérias são microorganismos fotossintéticos, também conhecidas como algas azuis, que podem ser encontradas em diversas formas, sejam unicelulares ou coloniais (SOULE; GARCIA-PICHEL, 2019). São um grupo diverso, que podem se proliferar em diferentes ambientes, normalmente induzidas por excesso de nutrientes, baixa circulação da água e altas temperaturas (MALIK et al., 2020). São importantes produtoras primárias, sendo responsáveis pelo oxigênio nos primórdios do planeta, antes da vida complexa surgir. Constituem até 70% da biomassa fitoplanctônica total, produzindo mais de 30% do oxigênio livre total (ARAOZ; MOLGÓ; MARSAC, 2010). Seus registros fósseis datam de 3.500 milhões de anos e a base do seu metabolismo é a conversão da energia de radiação em energia química (ATP), além de conseguir fazer a redução de gás carbônico em oxigênio molecular com auxílio da molécula de água (SOULE; GARCIA-PICHEL, 2019; SÁNCHEZ-BARACALDO et al., 2021).

A importância deste grupo para a vida do planeta é inegável, no entanto estes organismos não são somente capazes de produzir oxigênio, como também alguns compostos tóxicos: as cianotoxinas. Essas toxinas são compostos secundários, classificados em três principais grupos, de acordo com seu órgão alvo de ação: hepatotoxinas, dermatotoxinas e neurotoxinas (FIGURA 1). Dentro da última categoria se encontram as anatoxinas, anatoxinas-a, beta-N-metilamino-L-alanina (BMAA) e saxitoxinas (CHRISTENSEN; KHAN, 2020).

As saxitoxinas são um grupo de toxinas alcalóides também conhecidas como toxinas paralisantes de mariscos (*Paralytic Shellfish Toxin*, PST) por estarem associadas com frutos do mar (FIGURA 2). Elas são produzidas por diferentes gêneros de cianobactérias e dinoflagelados, como *Alexandrium*, *Anabaena*,

Aphanizomenon, *Lyngbya*, *Gymnodinium* e *Raphidiopsis* (WIESE et al., 2010). Sua estrutura foi descrita há 64 anos e existem mais de 50 análogos de saxitoxinas que se diferem pela presença de sulfatos e toxicidade, sendo a saxitoxina em si o mais tóxico (WIESE et al., 2010). Esses diferentes gêneros podem produzir diferentes análogos ou esses análogos podem sofrer interconversão e se transformar em outros, sob determinadas condições (FIGURA 3, CALADO et al., 2019). Normalmente as saxitoxinas são apresentadas como equivalentes de saxitoxinas (equiSTX), valor que fornece uma medida agregada dos análogos considerando a toxicidade relativa de cada um (HE et al., 2016).

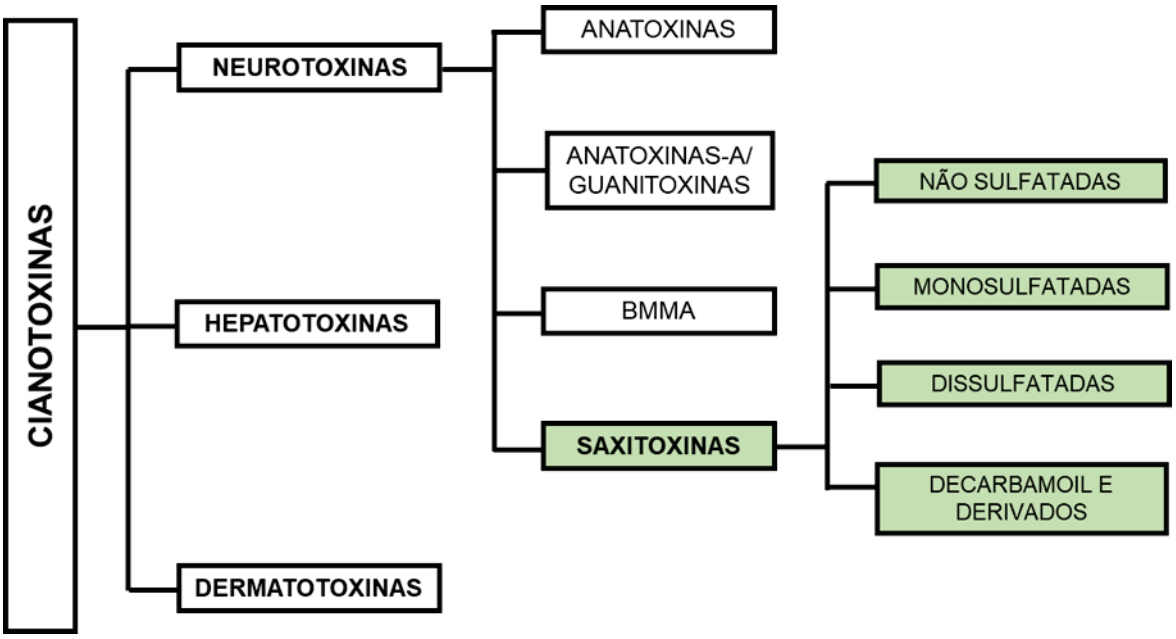


FIGURA 1: Classificação de cianotoxinas, com ênfase em saxitoxinas. Adaptado de: CHRISTENSEN; KHAN, 2020

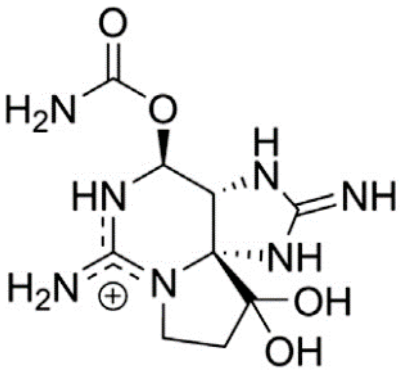


FIGURA 2: Estrutura química da saxitoxina. Fonte: KAUR (2019).

O principal mecanismo de ação desta toxina é a sua capacidade de se ligar nos canais de sódio das membranas dos axônios e bloquear a permeabilidade do sódio por ligação reversível (MANTOVANI; MOSER; FAVERO, 2011). Esse bloqueio impede a correta geração de potencial de ação, levando a paralisia neuromuscular, morte e tornando esse grupo de toxinas um dos compostos mais tóxicos conhecidos (ARAOZ; MOLGÓ; MARSAC, 2010). As saxitoxinas também podem se ligar a canais de cálcio e potássio, além da óxido nítrico sintase neuronal (LLEWELLYN, 2006). São solúveis em água e estáveis ao calor, além de permanecer em ambientes ligeiramente ácidos (MALIK et al., 2020). Já foram descritos seus efeitos tóxicos em diferentes animais como peixes de água doce (SILVA et al., 2011), peixes marinhos (BAKKE; HUSTOFT; HOSBERG, 2010; COSTA et al., 2011), invertebrados de água doce (ABI-KHALIL ET AL., 2017), invertebrados marinhos (BORCIER et al., 2017; BRAGA et al., 2018) e mamíferos (RAMOS et al., 2014).

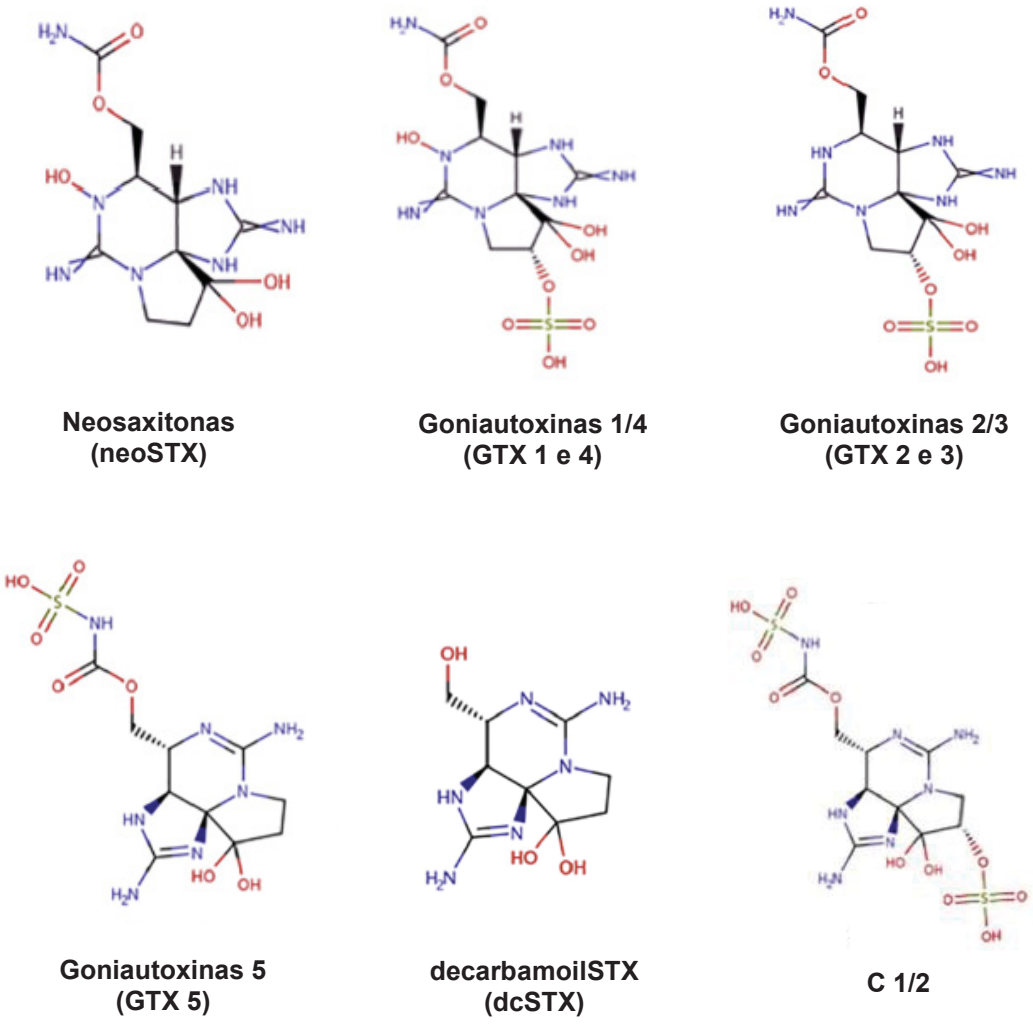


FIGURA 3: Estrutura química de alguns análogos de saxitoxinas. Fonte: CALADO et al. (2019).

Saxitoxinas já foram detectadas em reservatórios de abastecimento público de água, com florações de *Raphidiopsis raciborskii* (antigamente chamada de *Cylindrospermopsis raciborskii*) (CLEMENTE et al., 2010; CALADO et al., 2017; CALADO et al., 2020). Essa espécie é considerada um cianobactéria dominante, com produção de acinetos e outras características que a tornam uma boa competidora. Ela tem uma ampla faixa de temperatura de crescimento, que vai de 20 até 35°C (O'NEILL et al., 2012).

No Brasil, há uma legislação para cianobactérias e cianotoxinas. De acordo com a portaria 888 de 2021 do Ministério da Saúde, a análise de cianotoxinas (microcistinas, saxitoxinas e cilindrospermopsinas) deve ser feita semanalmente no ponto de captação de água caso a contagem de cianobactérias ultrapasse o limite de 20.000 células/mL. Como forma alternativa, deve-se medir a concentração de cianotoxinas na água bruta, mais precisamente na entrada da estação de tratamento (ETA). Para STX, o valor máximo permitido é de 3 µg/L de equiSTX. Caso ocorra a detecção de cianotoxinas na água após o tratamento, as autoridades de saúde pública devem ser imediatamente comunicadas para evitar a contaminação humana.

No entanto, além da contaminação por via hídrica ou por via hospitalar, como foi o caso de Caruaru com as microcistinas (AZEVEDO et al., 2002), a contaminação por cianotoxinas também pode ocorrer por alimentos. Análogos de STX produzidas pela *R. raciborskii* já foram encontrados acumulados em peixes de diferentes espécies, incluindo no músculo desses animais, tornando as florações de cianobactérias potencialmente tóxicas um problema de saúde ambiental e pública (GALVÃO et al., 2009; CALADO et al., 2017).

Além da possível produção de saxitoxinas, para cepa T3 da *R. raciborskii*, isolada do reservatório de Billings em São Paulo, já foram detectados as microgininas (SILVA-STENICO et al., 2011). Essas microgininas são cianopeptídeos lineares não ribossomais, capazes de inibir metaloproteases de zinco e enzimas de conversão da angiotensina (LENZ; MILLER; MA et al., 2019), logo também são de grande importância para a saúde humana.

2.4 PEIXES COMO ORGANISMOS TESTES

O ambiente aquático, como mencionado, é um dos que mais sofre com as consequências do aumento das atividades antrópicas, seja por meio da poluição ou

das mudanças climáticas. Dessa forma, peixes são bons modelos para estudos desse tipo de ambiente. Esses animais são euritérmicos e estão sempre em contato direto com a água, sendo então mais sensíveis as alterações ambientais (PÖRTNER; PECK, 2010).

Nesses organismos é possível avaliar a influência da temperatura em diferentes níveis biológicos, como molecular, celular e sistêmico (PORTNER; KNUST, 2007; ASHAF-UD-DOULAH et al., 2019). O efeito de diferentes cianotoxinas também pode ser avaliado (SILVA et al., 2011; TIAN et al., 2014; CALADO et al., 2018). No caso das STX, o maior foco é em ambientes marinhos, onde o maior interesse para saúde pública são os moluscos. Estudos em água doce são menos comuns, mas são extremamente relevantes (CHRISTENSEN; KHAN, 2013; CALADO et al., 2017; CALADO et al., 2020).

Dentre as espécies de peixe dulcícolas que podem ser utilizadas está a *Rhamdia quelen* (Quoy & Gaimard, 1824). É um bagre, tipo de peixe que está entre os preferidos para a aquicultura pela preferência do consumidor e pelo seu valor comercial (FATMA; AHMED, 2020). É um peixe dulcícola, também conhecido como jundiá ou bagre prateado (FIGURA 4), amplamente distribuído em rios e lagos brasileiros, com distribuição do sul da América do Sul até o sul do México. É uma espécie onívora, ovulípara e que habita lugares calmos e mais profundos (GOMES et al., 2000). Estudos com aumento da temperatura mostraram que larvas dessa espécie podem sobreviver na faixa de 15 a 27,8 °C (CHIPPARI-GOMES; GOMES; BALDISSEROTTO, 2000). Já alevinos podem suportar 33,5°C se aclimatados por 22 dias à 16 °C, mas com melhor desempenho, isto é, maior crescimento em 23,7 °C (CHIPPARI-GOMES, GOMES; BALDISSEROTTO, 1999; PIEDRAS; MORAES; POUEY, 2004). Com relação ao seu desenvolvimento embrionário, jundiás são capazes de se desenvolver em diferentes temperaturas, desde 21 até 30 °C, mas com malformações (edema cardíaco) quando incubadas em temperaturas mais altas, como 30 °C (RODRIGUES-GALDINO et al., 2009).

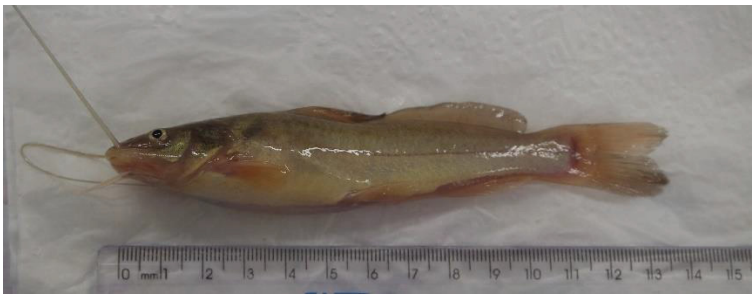


FIGURA 4: Espécime juvenil de *Rhamdia quelen*. Fonte: a autora.

2.5 BIOMARCADORES DE CONTAMINAÇÃO AMBIENTAL

Biomarcadores são respostas biológicas mensuráveis dos organismos como consequência de contaminação ambiental, que possibilitam a avaliação de efeitos subletais (LAM; GRAY, 2003). Contudo, além de apresentarem alterações perante a exposição de contaminantes ambientais, esses biomarcadores também podem ser influenciados por variáveis abióticas, como a temperatura (SOUZA-BASTOS et al., 2017). Diversos parâmetros podem ser considerados biomarcadores, como os hematológicos, bioquímicos, de genotoxicidade, histopatológicos e análises ômicas.

Os biomarcadores hematológicos são muito utilizados para monitoramento da saúde de peixes por ser um biomarcador menos invasivo e em que não é necessário o sacrifício do animal. Com a contagem do número dos eritrócitos, leucócitos e trombócitos, é possível verificar processos inflamatórios, anemia, leucocitose, trombocitopenia e outros distúrbios sanguíneos (CLAUSS; DOVE; ARNOLD, 2008). Além desses, alguns biomarcadores bioquímicos também podem ser avaliados no sangue, como é o caso dos níveis de glicose e lactato, que estão relacionados ao tipo de metabolismo, aeróbio ou anaeróbio, e, portanto, indicar se o organismo pode estar em condições de hipóxia ou privação de alimento, por exemplo (COATES; SODERHALL, 2020).

Outros biomarcadores bioquímicos podem ser analisados, em diferentes tecidos. No sistema nervoso, por exemplo, um composto ou um fato abiótico podem alterar a enzima acetilcolinesterase (AChE). Essa é responsável por hidrolisar o neurotransmissor acetilcolina em colina e ácido acético, sendo essa então responsável por controlar o impulso nervoso (MOTA et al., 2012). Além disso, ao entrar em contato com algum xenobiótico, o organismo pode ativar seu sistema de biotransformação, para tentar eliminar o mesmo ou transformá-lo em algum

composto mais hidrossolúvel e menos tóxico. No caso das saxitoxinas, a principal via de eliminação que o organismo utiliza é por meio das glutationas (GUBBINS et al., 2000). Na fase II da biotransformação ocorrem reações de glucuronidação, metilação e conjugação. A glutationa S-transferase (GST) é uma enzima que participa da fase II da biotransformação e que se conjuga a saxitoxina, e a outros xenobióticos, com o auxílio do co-fator glutationa reduzida (GSH) para que a excreção da mesma seja facilitada (OGA; CAMARGO; BATISTUZZO, 2008).

O metabolismo celular pode gerar gás carbônico, catalisado pela anidrase carbônica (CA). Essa enzima, de extrema importância para o tecido branquial, atua na captação de íons, regulação ácido-base e respiração de peixes. Alteração em sua atividade pode indicar uma resposta compensatória do animal frente a algum distúrbio (EVANS; CLAIBORNE, 2009; LIONETTO et al., 2012).

Esse metabolismo de substâncias exógenas, ou as próprias substâncias em si, podem levar a formação de espécies reativas de oxigênio (ERO) (LUSHCHAK, 2011). Como demonstrado por Madeira et al. (2013), biomarcadores relacionados ao estresse oxidativo também podem ser alterados pelo estresse térmico. Existem enzimas e outros fatores não enzimáticos que atuam como substâncias antioxidantes e combatem as EROs, como a superóxido dismutase (SOD, EQUAÇÃO 1), a catalase (CAT, EQUAÇÃO 2), a glutationa peroxidase (GPx, EQUAÇÃO 3) e a própria GSH. A SOD é responsável por dismutar o ânion superóxido ($O_2^{\bullet -}$) em peróxido de hidrogênio (H_2O_2). Este peróxido ainda é tóxico para a célula e degradado pelas enzimas GPx e CAT (VAN DER OOST; BEYER; VERMEULEN, 2003). A GSH também é considerada um antioxidante endógeno, além de estar envolvida nas reações da GST e GPx (CHOI et al., 2006).

$$2\ O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad \text{(EQUAÇÃO 1)}$$

$$2\ H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(EQUAÇÃO 2)}$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O \quad \text{(EQUAÇÃO 3)}$$

Contudo, quando há um desequilíbrio e o sistema antioxidante não é capaz de degradar essas substâncias pró-oxidantes, o organismo pode entrar em um quadro de estresse oxidativo, no qual diferentes efeitos em macromoléculas podem ser observados (LUSHCHAK, 2011). A presença de proteínas carboniladas é um exemplo de dano irreversível, que ocorre pela introdução de grupos carbonilas

(cetonas e aldeídos reativos) às proteínas. Essas macromoléculas se tornam não funcionais e podem ser degradadas pela célula (MØLLER; ROGOWSKA-WRZESINSKA; RAO, 2011). Efeitos em lipídios também podem ser observados por meio da lipoperoxidação ou peroxidação lipídica (LPO). Radicais e hidroperóxidos são formados durante a reação em cadeia da lipoperoxidação, o que pode levar a desestabilização de membranas celulares, por exemplo (VAN DER OOST; BEYER; VERMEULEN, 2003). Essa desestabilização pode facilitar a entrada de substâncias exógenas ao núcleo celular e ocasionar danos ao material genético.

Para mensurar danos no nível biológico de DNA, se utilizam os biomarcadores de genotoxicidade, que visam a detecção de agentes genotóxicos no ambiente por diferentes metodologias, como o teste de anormalidade nucleares (e micronúcleo) e o ensaio cometa (FRENZILLI; NIGRO; LYONS, 2009). O teste do micronúlceo písceo, bem como a análise das demais alterações nucleares em eritrócitos, podem indicar danos cromossômicos. Os micronúcleos representam uma parte do material genético que se desprendeu do restante e podem ser formados por meio de rupturas cromossômicas ou interrupção do processo mitótico (SAMANTA; DEY, 2012). Além do micronúcleo, outras alterações nucleares podem ser encontradas (Figura 5), como pequenas evaginações (*bebbled*), evaginações mais largas (*lobed*), vacúolos no interior do núcleo (*vacuolated*) e fenda bem definida e profunda (*notched*). Por meio do ensaio cometa é possível avaliar essas rupturas no DNA. Utilizando a técnica de ensaio de eletroforese em gel de célula única (SCGE - *Single Cell Gel Electrophoresis*) é possível visualizar as quebras da cadeia de DNA, por meio da migração do material em um gel de eletroforese (LEE et al., 1996). Os níveis de danos são classificados de 0 a 4, de acordo com a quantidade de DNA disperso, ou seja, “a cauda do cometa, conforme mostrado na figura 6 (LEE et al., 1996). Estudos mostraram que biomarcadores de genotoxicidade são sensíveis às variações de temperatura e em exposição à saxitoxinas, separadamente (FRENZILLI; NIGRO; LYONS, 2009; CHEN et al., 2020).

Todos esses processos podem levar à danos teciduais nos organismos. Por meio da histopatologia é possível avaliar danos estruturais, indicando injúrias em diferentes tecidos como brânquias, fígado e rins, induzidos por agentes externos (YANCHEVA et al., 2015). Com o índice de lesão proposto por Bernet et al. (1999), é possível classificar diferentes tipos de lesões, como necrose e infiltração leucocitária, em diferentes tecidos, por meio de pesos relativos a cada um conforme

a gravidade que o mesmo representa, resultando em informações valiosas a respeito das condições do tecido.

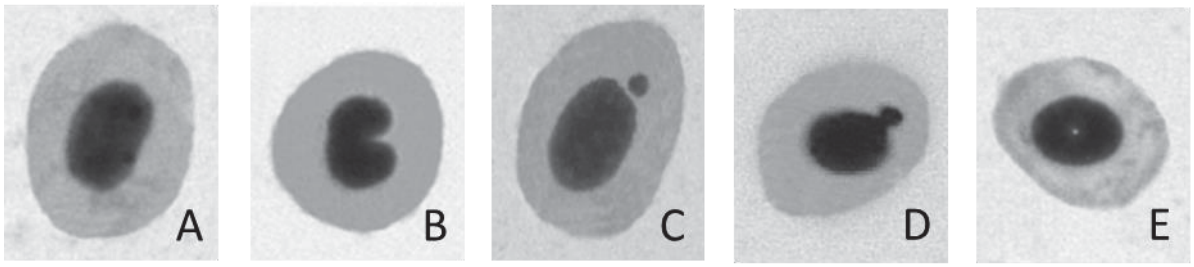


Figura 5: Diferentes tipos de anormalidades nucleares. A: Eritrócito normal; B: *Notched*; C: Micronúcleo; D: *Blebbed*; E: *Vacuolated*. Adaptado de SANTOS (2010).

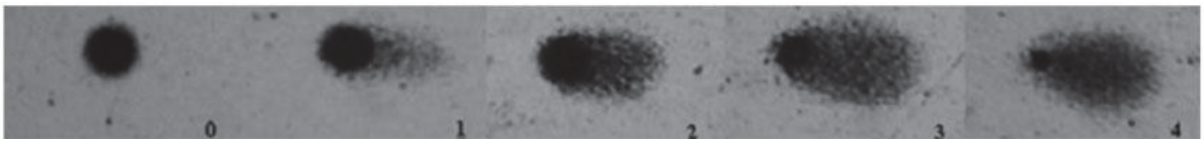


FIGURA 6: Classificação de danos ao DNA por meio de ensaio cometa, sendo 0 o núcleo mais íntegro e 4 com maiores níveis de quebras do DNA. Fonte: GÜEZ et al. (2012).

Além dos biomarcadores já citados, as ciências ômicas têm ganhado muita atenção dentro de estudos ecofisiológicos e ecotoxicológicos, tanto para estudos com cianotoxinas (KARIM; PUISEUX-DAO; EFERY, 2011), quanto para estudos envolvendo o aquecimento global (YANG et al., 2020). Por meio dessas técnicas é possível investigar minuciosamente sistemas biológicos complexos, investigando simultaneamente muitas respostas moleculares ao mesmo tempo (MARIE, 2020). A proteômica é uma dessas ferramentas, que mensura o complemento total de proteínas do tecido ou célula em determinada condição, fornecendo informações a nível mecanístico e captura modificações pós-tradução de proteínas (RODRIGUEZ et al., 2012). Com esta técnica também é possível identificar candidatos a possíveis biomarcadores (LÓPEZ-PEDROUSO et al., 2020). Em peixes, a mesma já foi utilizada para responder diferentes questões biológicas envolvendo toxicologia, reprodução, segurança alimentar e questões ambientais, por exemplo (FORNÉ; ABIÁ; CERDA, 2010).

Com estes biomarcadores se faz uso da abordagem *bottom-up,* sendo possível, dessa forma, inferir sobre os efeitos de substâncias tóxicas em níveis biológicos superiores a partir de alterações em níveis biológicos inferiores (FIGURA 7; SNAPE et al., 2004; PETITJEAN et al., 2019).

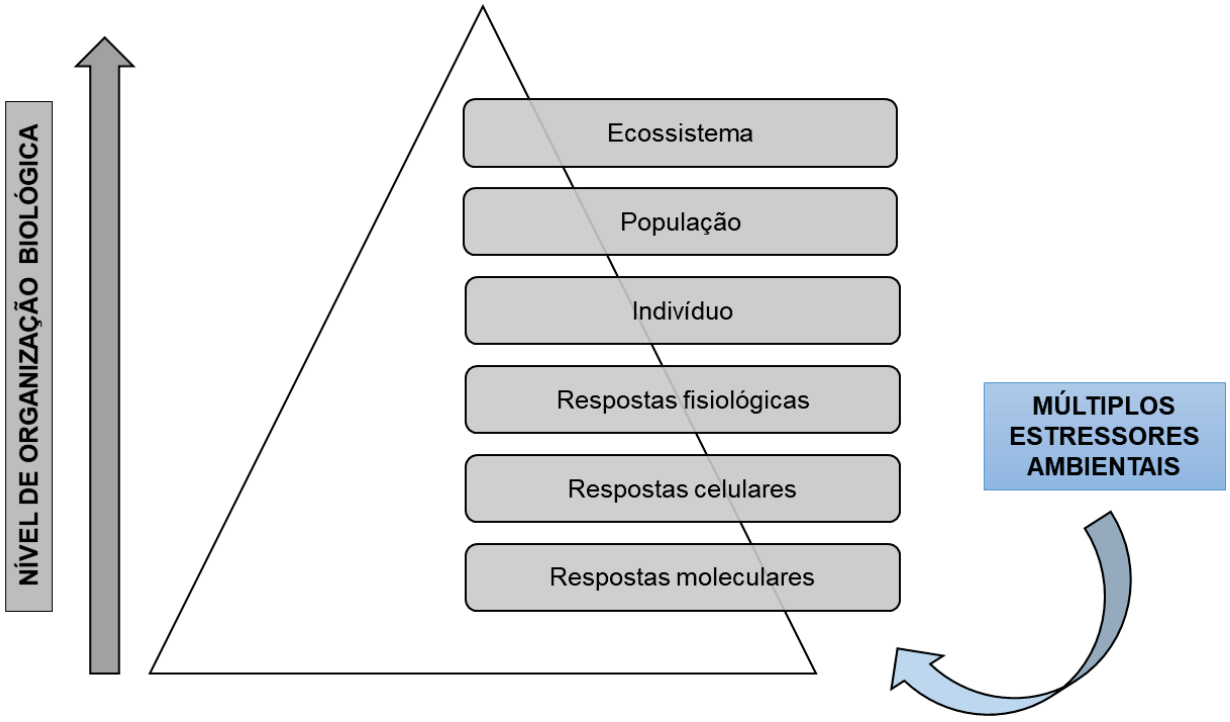


FIGURA 7: Níveis de respostas biológicas aos múltiplos estressores ambientais.

3 OBJETIVOS

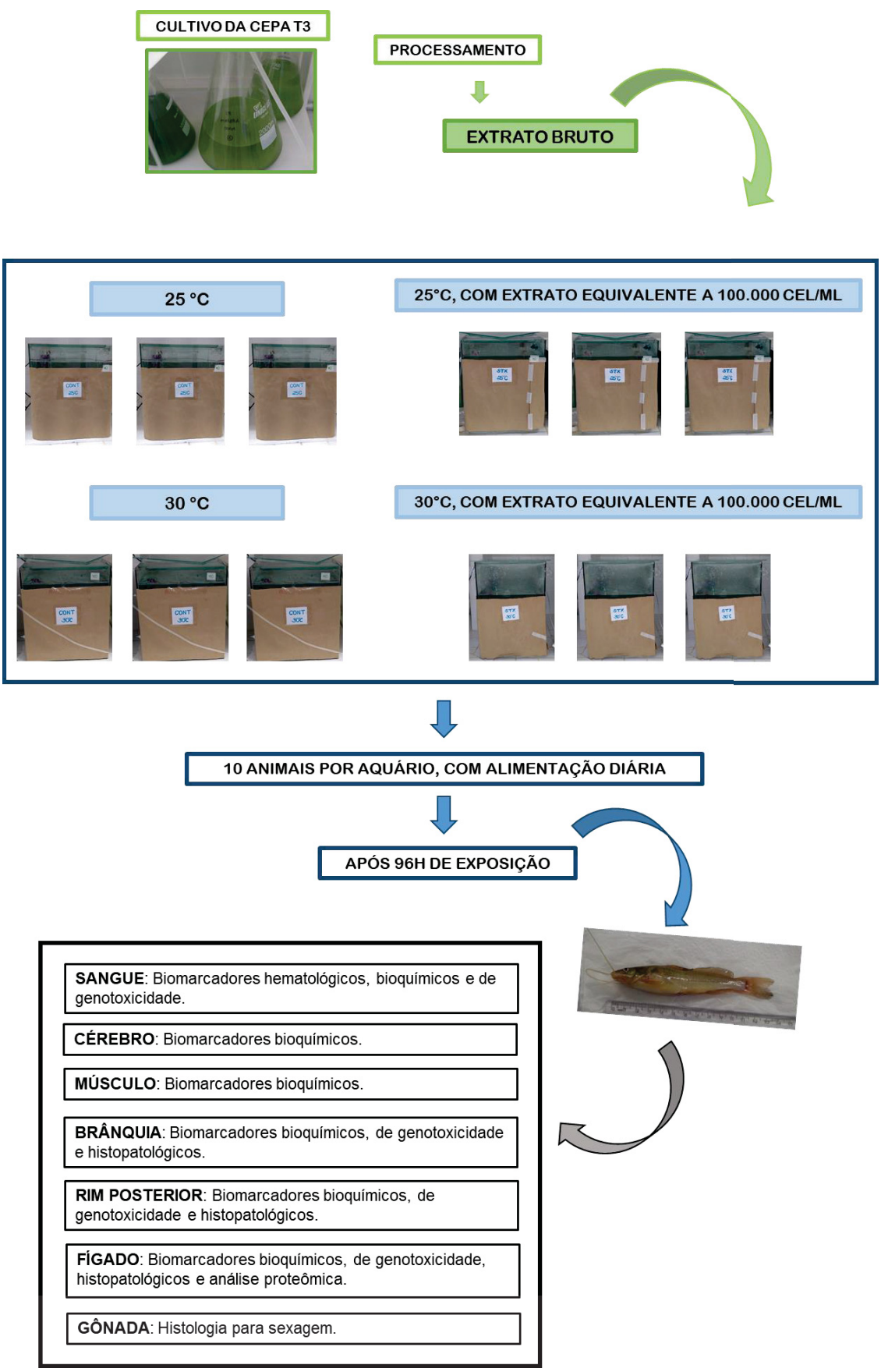
3.1 OBJETIVO GERAL

Compreender como a espécie neotropical de peixe *Rhamdia quelen* responde à diferentes condições térmicas e ao extrato bruto da cianobactéria *Raphidiopsis raciborskii*, por meio de diferentes biomarcadores de contaminação ambiental.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar como juvenis de *Rhamdia quelen* respondem, após exposição de 96h à duas diferentes condições térmicas, utilizando os biomarcadores hematológicos, bioquímicos, de genotoxicidade e histopatológicos;
- Avaliar o perfil de proteínas do fígado de juvenis de *Rhamdia quelen* após exposição de 96h à duas diferentes condições térmicas;
- Avaliar, por meio da análise de biomarcadores hematológicos, bioquímicos, de genotoxicidade e histopatológicos, como juvenis de *Rhamdia quelen* respondem à exposição de 96h ao extrato bruto da cianobactéria *Raphidiopsis raciborskii*, contendo neurotoxinas, sob duas condições térmicas;
- Avaliar o perfil de proteínas do fígado de juvenis de *Rhamdia quelen* após a exposição de 96h ao extrato bruto da cianobactéria *Raphidiopsis raciborskii*, contendo neurotoxinas, sob duas diferentes condições térmicas.

ESQUEMA GERAL DA METODOLOGIA



CAPÍTULO 1

O aumento da temperatura por curto prazo aumenta os impactos sobre um peixe neotropical

“Short time temperature rise increases impacts on a Neotropical catfish”, a ser submetido na Journal of Thermal Biology

Short time temperature rise increases impacts on a Neotropical catfish

Maiara Vicentini, Jacqueline Beatriz Kasmirski Pessatti, Maiara Carolina Perussolo, Juliana Roratto Lirola, Fellip Rodrigues Marcondes, Natalia do Nascimento, Maritana Mela, Marta Margarete Cestari, Viviane Prodocimo, Helena Cristina da Silva de Assis

Abstract

Climate change has been one of the most discussed topics in the world. The global warming is characterized by an increase in global temperature, not only of air, but also of aquatic environments. This change can affect several organisms, and can even lead to their local extinction. Thus, it is necessary to understand how different species respond to these changes. The study aimed to evaluate how the Neotropical catfish species *Rhamdia quelen* responds to temperature increases. The animals were exposed to temperatures of 25 and 30 °C for 96 hours, after acclimatization for seven days with a gradual increase in temperature. The fish were anesthetized, the blood collected and after the euthanasia, brain, liver, posterior kidney, gills, muscle and gonads were sampled. The gonads were used only for sexing, while other tissues were used for the analysis of hematological, biochemical, genotoxicity and histopathological biomarkers. Both sexes presented an increase in gonadosomatic index with the temperature rise, showing that increase in temperature may be able to alter this species reproductive cycle. Males presented increase in blood glucose, leukopenia in females and blood genotoxicity in males and females. It was not observed increase in macrolecules or damage in the others tissues, however the antioxidant system affected differently between sex. This study showed that juveniles of *R. quelen* can tolerate temperatures of 30 °C for 96 hours, higher than usual. However, several changes, such as hematological alterations and antioxidant system enzymes activation were observed, with sex differences in the biomarkers responses.

Key words: Biomarkers, global warming, *Rhamdia quelen*

Highlights

Temperature rise was genotoxic to catfish.

Antioxidant system of liver, kidney and gill can be activated by temperature rise.

Males and females responded differently to temperature rise.

1. Introduction

Climate change has been one of the most debated issues worldwide. Global warming, characterized by an average global temperature increase, is caused by an atmospheric concentration increase of gases such as carbon dioxide and methane (Le Treut et al., 2007). The anthropic activities intensification, such as industrial activity, agriculture, burning fossil fuels, deforestation and pollution, have caused the accumulation of these gases in the atmosphere making these alterations more intense (Le Treut et al., 2007; Shahzad, 2015). Based on this planet's adverse conditions aggravation, predicting changes in Earth's temperature has become the goal of many scientists around the world. In return, government officials try (or should try) limit this temperature increase. From 1850-1900 to 2011-2020 were estimated that global temperature increase at least 1.09 °C (IPCC, 2021). Naturally, the temperature can vary spatially and temporally (Kirillin & Shatwell, 2016; Ren et al., 2020), however extreme phenomena can be more frequent due to climate change (Le & Zao, 2021).

The global temperature alterations can cause several negative effects, such as affecting the environment and its biota (Pecl et al., 2017). Among the various organisms that can be affected are fish (Pörtner and Knust, 2007; Logez and Pont, 2012). These animals, as they are eurithermal and are in direct contact with water, can present different changes in the face of such disturbance, such as hypoxia tolerance, heat shock response, metabolic adjustments and behavioral changes (Pörtner and Peck, 2010, Collins et al., 2021). The high water temperature can inhibit genes related to reproductive regulation axis in these animals, leading to gonadal regression and the spawning impairment (Miranda et al., 2013). The increase in temperature can also immunocompromise host species, potentially facilitating the spread of infectious diseases (Dittmar et al., 2014). Biochemical, hematological, genotoxic and histopathological alterations can also be found in different fish species, due to the temperature rise (Anitha et al., 2000; Ahmad et al., 2011; Dietrich et al., 2018; Ashaf-Ud-Doulah et al., 2019).

Rhamdia quelen is a fish species widely used for human consumption and for studies of environmental contamination, as well as found in public water supply reservoirs in Brazil. This is a catfish found from southern South America to southern Mexico, considered eurithermal (Chippari-Gomes et al., 1999). In Brazil, few studies

with this fish species and temperature have been developed, however, dealing with lethal and non-sublethal effects. *R. quelen* resists large temperature fluctuations, with lower lethal temperature as 3 °C and higher lethal temperature as 32 °C (Garcia et al., 2018). This species fry enduring temperatures of 15 to 34 °C, with greater growth at higher temperatures (Gomes et al., 2000). However, even if they hatch more quickly at 30 °C, some anatomical defects are more observed at this temperature, such as cardiac edema (Rodrigues-Galdino et al., 2009). Thus, its thermal ideal is in the range of 22 to 28 °C (Montanha et al., 2011). In the captivity, this adult fish species were found at 19 to 23 °C (Figueredo et al., 2014).

In the face of global warming, more studies are needed that focus mainly on individual and sublethal effects, to prevent species from being totally lost (Portner and Knust, 2007). The organism response can alter its interactions with other organisms (intra or interspecific), leading to a domino effect, even in organisms resistant to such disturbance (Walther et al., 2002; Sérgio et al., 2017). This study aimed to evaluate how female and male Neotropical catfish *Rhamdia quelen* respond to the temperature rise. Different biomarkers, normally used to assess the environmental contamination impact, were analyzed, in order to show the temperature sublethal possible effects on freshwater species.

2. Material and methods

2.1. Acclimation and experimental design

Juveniles (male and female) of *Rhamdia quelen* (12.45 ± 1.06 cm and 15.82 ± 4.21 g), from a pisciculture, were acclimatized for 30 days at the Environmental Toxicology Laboratory (Paraná, Brazil), in tanks containing filtered water, constant aeration, controlled photoperiod (12h) and controlled room temperature (25 °C). Fish were divided at two groups and each one were acclimated at the bioassay temperature (25 and 30 °C), using a thermostat. For 30 °C, the acclimatization temperature started at 25 °C and was increased by one degree per day. These two temperatures were choose based at ideal temperature for the fish and simulating a water temperature rise, respecting the animal thermal amplitude (Gomes et al., 2000; Montanha et al., 2011).

After seven acclimatization days at the experiment temperature, the animals were transferred to 50 L aquaria at experimental temperatures and aeration. Each aquarium contained ten animals and the experiment was carried out in triplicate, totaling 30 animals per group. During this experiment, fish were daily fed with commercial feed (Laguna® Brazilian Fish 32), with the feed being spread throughout the aquarium to ensure that all animals have fed properly. In the water, physical-chemical parameters were monitored, such as dissolved oxygen, pH, temperature, ammonia and nitrite. After 96h of exposure, the animals were anesthetized with benzocaine and the blood was collected. A medullary section was performed as a form of euthanasia, and gills, posterior kidney, brain, muscle, liver and gonad were sampled. The animals and organs (liver and gonad) were weighed.

The blood was stored for the analysis of genotoxicity and hematological biomarkers, as well as glucose determination. A volume of the blood was centrifuged at 2000 xg for 5 min for plasma lactate quantification. The brain was collected for biochemical biomarkers; posterior kidney and gills for the analysis of biochemical, genotoxicity and histopathological biomarkers; muscle for biochemical biomarkers; and liver for biochemical, genotoxicity and histopathological biomarkers. Gonads were used for sexing the animals through histological analysis, so that the data could be analyzed by sex separately.

This project was approved for Federal University of Paraná Animal Use Ethics Committee (CEUA, n° 1140).

2.2. Hepatosomatic and gonadosomatic index

The hepatosomatic (HSI) and gonadosomatic (GSI) index were calculated according to the following equation: (liver or gonad weight / body weight) x 100.

2.3. Hematological biomarkers

The erythrocyte count was performed using the Formol-Citrate method (Oliveira Junior et al., 2009) and the leukocyte and thrombocyte count in a blood smear (Tavares-Dias et al., 1999, 2000).

2.4. Biochemical biomarkers

Blood glucose levels were quantified using a portable glucometer (Accu-Check Performace) and plasmatic lactate was measured colorimetric method using the commercial Kit (Labtest, Brazil).

Muscle was homogenized in potassium phosphate buffer (0.1 M, pH 7.5) in the proportion of 1:10 (m/v) and centrifuged for 20 minutes, at 12000 xg, 4 °C. The supernatant was used for acetylcholinesterase activity (AChE; Ellman et al., 1961 modified to microplate by Silva de Assis, 1998). Brain was homogenized at the same conditions, but used to AChE activity and measured of lipoperoxidation (LPO; Jiang et al., 1992) and carbonylated proteins (PCO; Levine et al., 1994). Liver, posterior kidney and gill was homogenized in potassium phosphate buffer (0.1 M, pH 7.0) in the proportion of 1:10; 1:10 and 1:5 (m/v), respectively. After centrifuged for 30 minutes, at 15000 xg, 4 °C, the supernatant was used to different biomarkers: glutathione S-transferase (GST; Keen et al., 1976), superoxide dismutase (SOD; Gao et al., 1998), catalase (CAT; Aebi, 1984), glutathione peroxidase (GPx; Hafeman et al., 1974), non-protein thiols (GSH; Sedlak & Lindsay, 1968) and LPO. For the carbonic anhydrase (CA), posterior kidney and gill was homogenized in 10% of phosphate buffer (10 mM, pH 7.4) and the supernatant measured carbonic anhydrase by the method described by Vitale et al. (1999). For all these tissues and samples, total protein was measured following Bradford (1976).

2.5. Genotoxicity biomarkers

For micronucleus assay, 2000 erythrocytes for each animal were examined at a blood smear stained with Giemsa 10% and micronuclei and nuclear abnormalities were analyzed (Hooftman and De Raat, 1982; Carrasco et al., 1990).

For DNA break, comet assay was performing according to Speit and Hartmann (2005) modified by Ramsdorf et al. (2009). Blood (10 µL) was placed into 500 µL of fetal bovine serum. About 10 mg of the liver and posterior kidney and one gill arch was placed into 250 µL of the serum. After the samples preparation, 100 nucleoids were assessed visually and according to its “tail” proportion was given to them a value from undamaged to maximally damaged (Collins et al. 1995). The nucleoids with small or non-existent visible head and large diffuse tails were not considered.

2.6. Histopathological biomarkers

Gonad, liver and posterior kidney fragments, as well as one gill arch, were fixed in ALFAC solution (80% alcohol, formaldehyde and glacial acetic acid). The samples were dehydrated in alcoholic series, diaphanized in xylol and included in Paraplast®. Histological sections were stained with hematoxylin-eosin (HE). Gonadal slides were analyzed for sex determination. Other tissues slides were analyzed to determine the injury index (Bernnet et al., 1999).

2.7. Data analysis

Levene and Shapiro-Wilk tests were used to test the homoscedasticity and normality, respectively. Data were submitted to the *t* test or Mann Whitney U-test, according to the assumptions, with $p \leq 0.05$. Non-Metric Multidimensional Scaling (nMDS) was performed to visualize the distribution in the groups in only two dimensions. All analyzes were performed in an R environment.

3. Results

3.1. Water parameters

The mean water physical-chemical parameters (± standard error) to 25 °C were: temperature of 26.08 ± 0.25, pH of 6.93 ± 0.03, ammonia of 1.58 ± 0.24 ppm, dissolved oxygen of 4.17 ± 0.27 ppm, and 0 ppm of nitrite. To 30 °C were: temperature of 30.07 ± 0.24, pH of 6.83 ± 0.03, total ammonia of 1.25 ± 0.20 ppm, dissolved oxygen of 3.67 ± 0.36 ppm, and 0.45 ± 0.03 ppm of nitrite.

3.2. Hepatosomatic and gonadosomatic index

The hepatosomatic index decreased with temperature increase in males (Figure 1A), while the gonadosomatic index increased in females and males in this same condition (Figure 1B).

3.3. Hematological biomarkers

The hematological parameters such as number of erythrocytes and thrombocytes did not change, however leukocytes numbers were reduced in females with increasing temperature (Figure 2A-C).

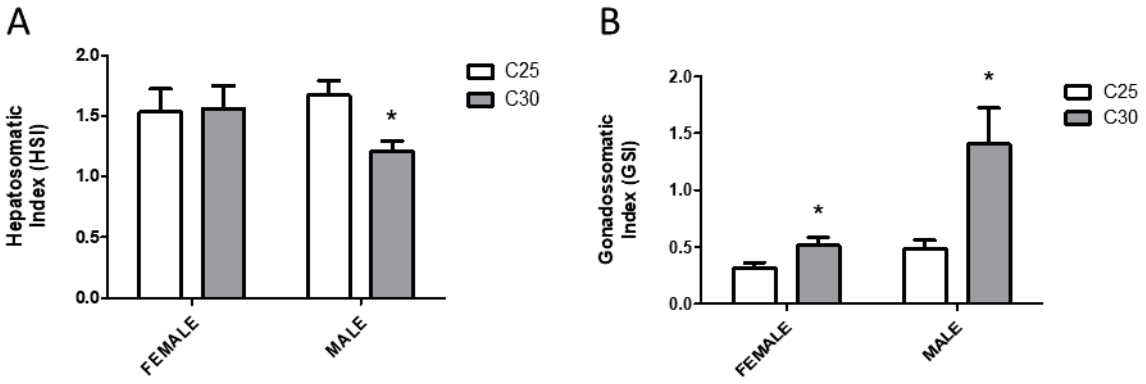


FIGURE 1: Hepatic (A) and gonadosomatic (B) indexes, in males and females (mean ± standard error). * representing significant difference by t test.

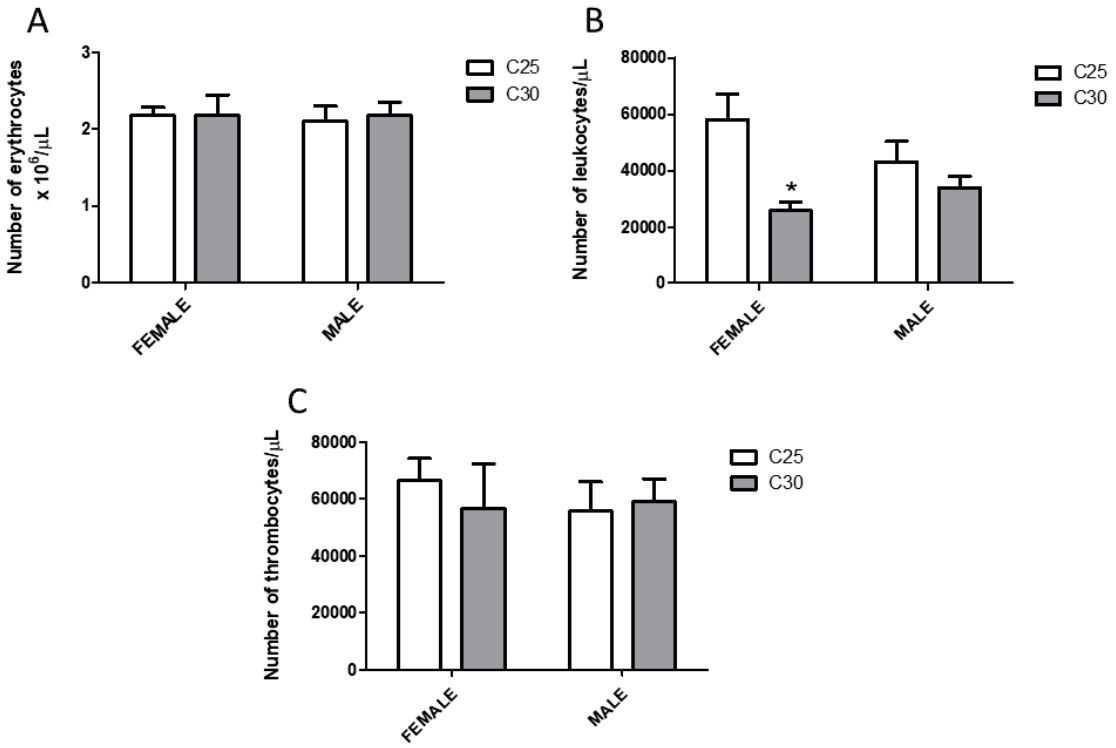


FIGURE 2: Hematological biomarkers (mean ± standard error). * representing significant difference by *t* test. A: Erythrocytes, B: Leukocytes, C: Thrombocytes.

3.4. Biochemical biomarkers

Blood glucose increased with increasing temperature in males, while the plasmatic lactate did not change. In females, these blood biochemical biomarkers were not altered.

The brain and muscular acetylcholinesterase activity not changed. The lipoperoxidation or protein carbonylation not alters in brain tissue. In the liver an increase in GST activity in males with the increase in temperature was observed. In the posterior kidney, an increase in SOD activity was observed in females. In the gills, the temperature rise was able to increase the activity of GPx and carbonic anhydrase in males and decrease lipoperoxidation in females (Table 1 and 2).

3.5. Genotoxicity biomarkers

Total erythrocyte nuclear morphological changes (micronuclei, bebbleed, vacuolated, notched, lobbed) in females and DNA break in males were increased (Figure 3A-B, Supplementary Table 1). The other tested tissues did not present genotoxicity (Supplementary Table 2).

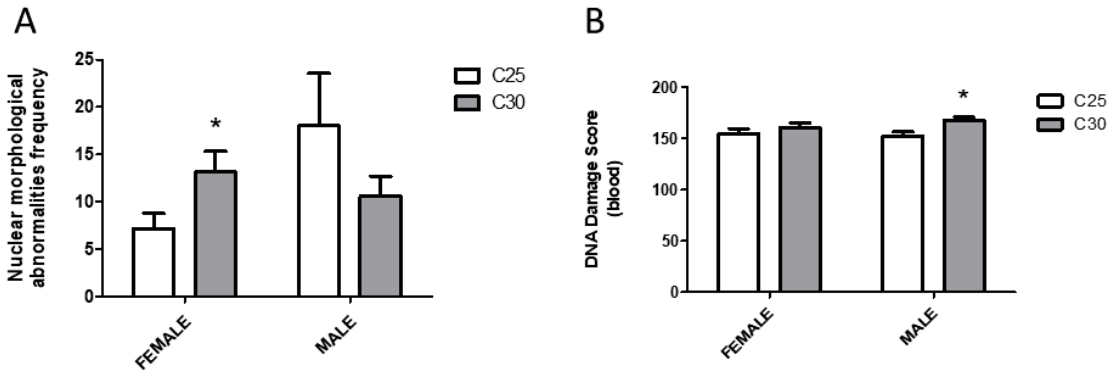


FIGURE 3: Blood genotoxic biomarkers (mean ± standard error). * representing significant difference by *t* test. A: Nuclear Morphological Abnormalities, B: Comet assay.

TABLE 1: Female biochemical biomarkers at 25 °C and 30 °C.

Tissue	Biomarker	25°C	30°C
Blood	Glucose	33.75 ± 4.22 [a]	47.36 ± 5.18 [a]
	Lactate	3.37 ± 0.28 [a]	3.98 ± 0.49 [a]
Muscle	AChE	247.40 ± 18.97 [a]	231.53 ± 11.96 [a]
Brain	AChE	116.56 ± 7.74 [a]	114.79 ± 5.92 [a]
	LPO	26.83 ± 5.45 [a]	24.86 ± 2.68 [a]
	PCO	278.10 ± 12.81 [a]	304.85 ± 16.94 [a]
Liver	GST	136.36 ± 5.19 [a]	136.40 ± 6.43 [a]
	SOD	307.02 ± 18.12 [a]	319.45 ± 16.99 [a]
	CAT	76.36 ± 5.92 [a]	84.51 ± 5.23 [a]
	GPx	168.61 ± 19.87 [a]	159.63 ± 15.40 [a]
	GSH	2.32 ± 0.29 [a]	2.33 ± 0.44 [a]
	LPO	14.47 ± 2.04 [a]	13.44 ± 1.82 [a]
Kidney	GST	62.10 ± 4.40 [a]	70.54 ± 6.73 [a]
	SOD	62.75 ± 3.71 [a]	84.01 ± 4.15 [b]
	CAT	5.33 ± 0.87 [a]	5.98 ± 0.99 [a]
	GPx	18.58 ± 1.54 [a]	17.90 ± 1.37 [a]
	LPO	9.14 ± 1.03 [a]	11.17 ± 2.10 [a]
	CA	3.00 ± 0.44 [a]	2.85 ± 0.32 [a]
Gill	GST	3.33 ± 0.25 [a]	3.38 ± 0.24 [a]
	SOD	106.83 ± 8.74 [a]	95.56 ± 8.15 [a]
	CAT	3.35 ± 0.40 [a]	5.30 ± 0.76 [a]
	GPx	19.12 ± 1.38 [a]	21.94 ± 0.96 [a]
	LPO	15.12 ± 1.45 [a]	10.37 ± 1.23 [b]
	CA	6.65 ± 0.66 [a]	5.37 ± 0.56 [a]

* Data represented as mean ± standard error, with the following units: glucose in $mg.dL^{-1}$; lactate: $mmol.L^{-1}$; acetylcholinesterase (AChE) in $nmol.min^{-1}.mg$ of $protein^{-1}$; glutathione S-transferase (GST) in $nmol.min^{-1}.mg$ of $protein^{-1}$; superoxide dismutase (SOD) in U of SOD.mg $protein^{-1}$; catalase (CAT) in $\mu mol.\ min^{-1}.mg$ of $protein^{-1}$; glutathione peroxidase (GPx) in $nmol.\ min^{-1}.mg$ of $protein^{-1}$; non-protein thiols (GSH) in µg GSH.mg of $protein^{-1}$; lipoeproxidation (LPO) in [hydroperoxides] $nmol.\ min^{-1}.mg$ of $protein^{-1}$; protein carbonylation (PCO) in [hydrazones] $pmol.mgprotein^{-1}$ and carbonic anhydrase (CA) in activity.mg of $protein^{-1}$. Different letters represents significant difference between groups.

TABLE 2: Male biochemical biomarkers at 25 °C and 30 °C.

Tissue	Biomarker	25°C	30°C
Blood	Glucose	31.00 ± 1.85 [a]	42.90 ± 3.49 [b]
	Lactate	2.73 ± 0.19 [a]	2.66 ± 0.53 [a]
Muscle	AChE	256.50 ± 13.41 [a]	222.99 ± 11.50 [a]
Brain	AChE	123.69 ± 5.49 [a]	117.26 ± 3.42 [a]
	LPO	33.28 ± 3.24 [a]	23.79 ± 3.38 [a]
	PCO	197.48 ± 23.78 [a]	194.11 ± 11.05 [a]
Liver	GST	133.75 ± 4.97 [a]	152.15 ± 5.61 [b]
	SOD	343.97 ± 13.04 [a]	326.82 ± 13.28 [a]
	CAT	89.08 ± 3.52 [a]	90.55 ± 5.85 [a]
	GPx	184.73 ± 17.77 [a]	199.41 ± 14.56 [a]
	GSH	2.51 ± 0.23 [a]	2.59 ± 0.27 [a]
	LPO	14.26 ± 1.42 [a]	12.27 ± 1.40 [a]
Kidney	GST	69.22 ± 4.81 [a]	69.13 ± 4.13 [a]
	SOD	76.60 ± 6.44 [a]	77.57 ± 3.89 [a]
	CAT	5.50 ± 0.56 [a]	6.02 ± 0.56 [a]
	GPx	22.46 ± 2.13 [a]	16.70 ± 0.74 [a]
	LPO	9.82 ± 0.82 [a]	10.26 ± 1.24 [a]
	CA	1.61 ± 0.16 [a]	2.12 ± 0.38 [a]
Gill	GST	3.43 ± 0.11 [a]	3.55 ± 0.12 [a]
	SOD	88.56 ± 3.53 [a]	89.73 ± 6.40 [a]
	CAT	4.30 ± 0.40 [a]	3.68 ± 0.42 [a]
	GPx	20.61 ± 0.92 [a]	23.39 ± 0.92 [b]
	LPO	9.89 ± 1.07 [a]	10.39 ± 0.80 [a]
	CA	2.44 ± 0.40 [a]	6.07 ± 0.52 [b]

* Data represented as mean ± standard error, with the following units: glucose in $mg.dL^{-1}$; lactate: $mmol.L^{-1}$; acetylcholinesterase (AChE) in $nmol.min^{-1}.mg\ of\ protein^{-1}$; glutathione S-transferase (GST) in $nmol.min^{-1}.mg\ of\ protein^{-1}$; superoxide dismutase (SOD) in U of SOD.mg $protein^{-1}$; catalase (CAT) in $\mu mol.\ min^{-1}.mg\ of\ protein^{-1}$; glutathione peroxidase (GPx) in $nmol.\ min^{-1}.mg\ of\ protein^{-1}$; non-protein thiols (GSH) in µg GSH.mg of $protein^{-1}$; lipoeproxidation (LPO) in [hydroperoxides] $nmol.\ min^{-1}.mg\ of\ protein^{-1}$; protein carbonylation (PCO) in [hydrazones] $pmol.mgprotein^{-1}$ and carbonic anhydrase (CA) in activity.mg of $protein^{-1}$. Different letters represents significant difference between groups.

3.6. Histopathological biomarkers

In the liver, few fish from both thermal conditions presented necrosis, leukocyte infiltration and sinusoids dilation. In the gills, at 25°C some organisms showed hyperplasia of the epithelial tissue. At 30°C, in addition to this hyperplasia, one animal presented an aneurysm In the kidneys cases of leukocyte infiltration were

observed for both temperatures (Supplementary Table 3). Therefore, the three tissues analyzed did not show significant differences for the injury index.

3.7. Multivariate analysis

The nMDS analysis showed response differentiation between sex and response differentiation between temperatures in males (Figure 4).

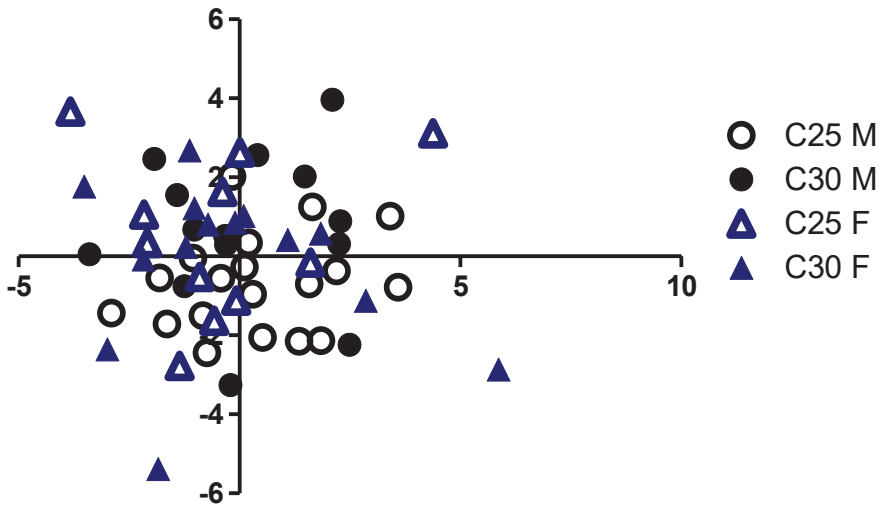


FIGURE 4: nMDS representation. C25 M: males at 25°C; C30 M: males at 30°C; C25 F: females at 25°C; C30 F: females at 30°C.

4. Discussion

The temperature is one of the main aquatic ecosystem functioning components affected by climate change. Its alteration can strongly affect the ecosystem structure, resulting in species loss (Moody et al., 2019). In this study, temperature was able to alter different parameters at the catfish *Rhamdia quelen*, highlighting differences between females and males that normally are not studied.

Somatic index calculations are common in studies, mainly about population studies (Louiz et al., 2009). In this study, the gonadosomatic index (GSI) increased in males and females with the temperature increase. GSI determines the reproductive condition and is normally highest in the reproductive period where the gonadal tissue increases (Del Fresno et al., 2020). The reproductive period can occurs in the summer for many fishes, where the photoperiod and at higher temperatures provide

more favorable conditions (Miranda et al., 2013). *Rhamdia quelen* is a species that has this reproductive cycle type, with a peak in the spring and another in the summer (Gomes et al., 2000). With this, the increase in temperature may be able to alter the species' reproductive cycles. However, as it has already been shown that larvae can hatch at higher temperatures (such as 30 °C), but presenting abnormalities. The increase in temperature can affect the thermocycle and be an external indicator for the animal to enter the reproductive period (Rodrigues-Galdino et al., 2009; Servili et al., 2020).

However, the hepatosomatic index (HSI) presented differences between female and male catfish. HSI indicates the liver proportion in relation to the animal's body, and the temperature increase reduced the HSI only in males. This reduction with increasing temperature has already been described for other species, such as sea bass *Dicentrarchus labrax*, that lives in waters ranging from 5 to 24 °C (Islam et al., 2020). The inverse relationship between HSI and GSI observed in *Rhamdia quelen* male can indicate the energy was transferred from liver to gonads, that occurs in reproductive period, as noted for the freshwater goldfish *Carassius auratus* (Tizkar et al., 2016), showing that the temperature rise may be able to advance the reproductive period of these male catfih.

Hematological biomarkers are widely used to assess the animal health, since they can be the result of many diseases or affected by the animal habitat (Ahmad et al., 2011). The change in blood cells, such as leukocytes numbers, can be considered a primary blood disorder (Burgos-Aceves et al., 2019). In females, the leukocytes number decreased by 55% at 30 °C compared to 25 °C. This leukopenia demonstrated that the increase in temperature can compromise the female catfish immune system, since leukocytes influence the immediate responses to inflammation or infection by being able to fight invading cells (Fisher et al., 2006).

In addition, the genotoxicity was observed, differently between female and male. Females presented increase in nuclear morphological abnormalities according to temperature increase, the same response found for freshwater subtropical carp *Labeo rohita* and sea bass *Dicentrarchus labrax* (Ashaf-Ud-Doulah et al., 2019; Islam et al., 2020). However, the males from our study presented erythrocytes DNA break with temperature increase, different from females. This means that male erythrocytes are more susceptible to deleterious genetic processes (Tatin et al., 2021). Temperature is also known to be a factor that can lead to an increase in reactive

oxygen species that can cause macromolecules damage, such as DNA (Cheng et al., 2018). It is already known that seasonal temperature variations may be able to affect the DNA migration in *Cyprinus carpio* cells, a subtropical freshwater carp (Frenzilli et al., 2009). Thus, the increase in temperature can affect the genetic material from the DNA to the chromosomal level, as evidenced by DNA break in males and the nuclear abnormalities in females, respectively.

The temperature increase was responsible for altering mainly biochemical parameters at different tissues. Blood glucose is an important temperature response to be considered a secondary response to heat stress (Makaras et al., 2020). *R. quelen* male blood glucose was increased with the temperature rise. An increase in plasmatic glucose was also observed in *R. quelen* at 31 °C compared to 23 °C, both in acute (12h) and chronic (21 days) exposure, without differentiating the sex (Lermen et al. 2004). The present study showed that this ocurred mainly in males. Blood glucose increase was already observed on other freshwater fish species, but with some differences. On the freshwater subtropical carp *Labeo rohita,* the blood glucose levels increased within 7 exposure days to 36 °C, and declined again within 60 exposure days (Ashaf-Ud-Doulah et al., 2019). On other subtropical carp, *Cyprinus carpio communis*, the increase in plasma glucose occurred on exposure to 28 °C for 30 days, with a reduction in these glucose levels at 32 °C (Ahmad et al., 2011).

Glucose is the main substrate for energy production (Kang et al., 2021). Its increase in males probably indicates that the energy demand for these organisms has increased. To supply this demand, glucose can be mobilized to other tissues such as brain and liver, being requested for metabolic functions (Soengas and Aldegunde, 2002; Sun et al., 2019). The hepatic glutathione S-transferase activity, a phase II biotransformation enzyme, increased in male according to temperature rise. In addition to be a key enzyme for the metabolism of many substances, GST can also act as an antioxidant enzyme (Paul et al., 2021). The temperature out of what is considered the organism optimum temperature, can activate the antioxidant system as a way of preventing and combating reactive oxygen species produced by this abiotic factor (Pörtner and Peck, 2010; Cheng et al., 2018). In this study, different antioxidant system enzymes were induced as response to temperature increase: GST in male livers, glutathione peroxidase (GPx) in male gills and superoxide dismutase (SOD) in female kidneys. This induction of different antioxidant enzymes

may be one of the reasons for not having seen an increase in oxidative damage in these tissues, such as lipoperoxidation (LPO) and DNA damage (Lushchak, 2011).

Temperature increase lead to induction on carbonic anhydrase activity of the male gills. Opposite result to that found for tilapia *Oreochromis mossambicus* exposed to 30 °C to 7 and 14 days, compares to 25 °C (Kaya et al., 2016). The carbonic anhydrase is a important branchial enzyme, acting on ion uptake, acid base regulation and respiration in freshwater fish (Evans and Claiborne, 2009; Lionetto et al.,2012). Thus, an increase in this enzyme could be a compensatory response to maintain its role in plasma ion uptake and/or acid base balance in order to maintain the homeostasis (e.g. Evans and Claiborne, 2009; Gilmour, 2012).

As can be observed, in this study, there was a difference in responses to the temperature rise between female and male fish. Castañeda-Cortés et al. (2020) studied the high temperature effects on genome transcription on the initial development of fish *Oryzias latipes*. The authors found no sexual dimorphic changes, in other words, heat stress was independent of genotypic sex. However, studies have already found changes in the fish reproductive axis caused by the increase in temperature (Miranda et al., 2013). Thus, the animal response to the temperature increase may be influenced by the animal sex. The present study indicates that other biomarkers may respond differently between sexes, with a higher male sensitivity to heated thermal stress, as showed at multivariate analysis.

5. Conclusion

In order to survive to temperature changes, the organism must be able to tolerate changes or be able to adapt to such new conditions, altering its cellular systems or behavior. Juveniles of *Rhamdia quelen*, a Neotropical catfish, can tolerate temperatures of 30 °C, higher than usual, for a period of 96 h, however with several hematological, biochemical and genotoxic responses. Temperature rise caused leukopenia in females, compromising the immune system, for example. Differences in sex responses were found. This result highlights the importance to study the sex responses separately to better understand how the increase in temperature can alter reproductive functions, since the males showed greater sensitivity to the increase in temperature than females, at least for short higher temperature exposures. This is

important to understand the physiology of this species in warming waters since this fish specie are found in rivers reservoirs and in aquaculture.

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Supplementary Data

Table S1: Erythrocyte nuclear morphological changes in *Rhamdia quelen* erythrocytes exposed to 25°C and 30°C for 96 h.

Sex	Alterations type	*25°C*	*30°C*
Female	Micronuclei	1	1
	Blebbed	17	62
	Lobbed	1	2
	Notched	11	30
	Vacuolated	49	88
	Binucleate	0	1
	TOTAL	79	184
Male	Micronuclei	1	0
	Blebbed	59	42
	Lobbed	3	2
	Notched	39	27
	Vacuolated	204	77
	Binucleate	2	0
	TOTAL	306	148

Table S2: DNA damage measured by comet assay in *Rhamdia quelen* exposed to 25°C and 30°C for 96 h.

Sex	*Tissue*	*25°C*	*30°C*
Female	Liver	165.82 ± 28.13	165.77 ± 27.41
	Gill	165.80 ± 21.13	154.54 ± 25.68
	Kidney	166.36 ± 29.93	164.08 ± 24.45
Male	Liver	152.21 ± 17.19	158.62 ± 29.07
	Gill	155.06 ± 24.04	154.79 ± 29.65
	Kidney	144.06 ± 41.85	158.00 ± 19.22

* Data represented as mean ± standard error of score damage.

Table S3: Histopathological alterations in *Rhamdia quelen* exposed to 25°C and 30°C for 96 h. The alterations are represented by the number of animals with a given change by the total number of animals in the group.

Tissue	*Sex*	*Type*	*25°C*	*30°C*
Liver	Female	Injury Index (mean)	2.55	2.29
		Necrosis (%)	2/11	3/14
		Leukocyte infiltration (%)	4/11	1/14
		Sinusoids dilation (%)	0/11	1/14
	Male	Injury Index (mean)	2.00	1.71
		Necrosis (%)	3/19	2/14
		Leukocyte infiltration (%)	2/19	2/14
		Sinusoids dilation (%)	3/19	1/14
Gill	Female	Injury Index (mean)	0.36	0
		Hyperplasia of the epithelial tissue (%)	1/11	0/14
		Aneurism (%)	0/11	0/14
	Male	Injury Index (mean)	0	0.43
		Hyperplasia of the epithelial tissue (%)	0/19	1/14
		Aneurism (%)	0/19	1/14
Kidney	**Female**	Injury Index (mean)	0.73	0.57
	Male	Injury Index (mean)	0.63	1.14

CAPÍTULO 2

Como o aumento da temperatura afeta um peixe de água doce? Uma abordagem proteômica

“How does temperature rise affect a freshwater catfish? A proteomic approach”, a ser submetido para Science of Total Environment

How does temperature rise affect a freshwater catfish? A proteomic approach

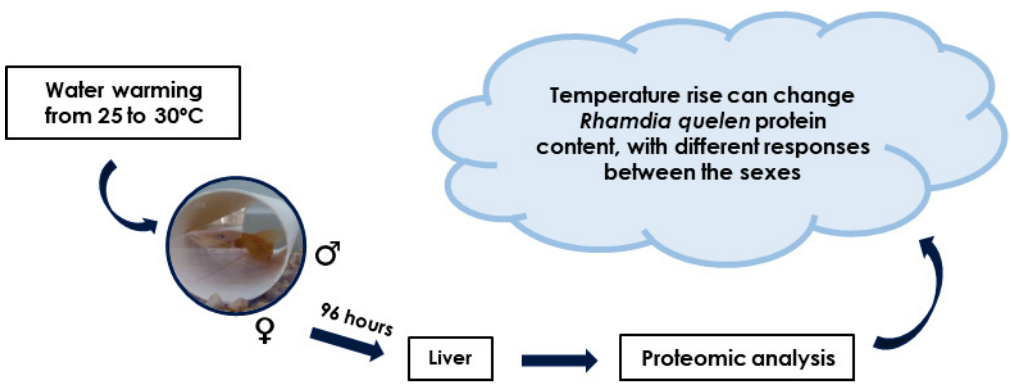
Maiara Vicentini, Denina Simmons, Helena Cristina da Silva de Assis

Abstract

Climate change is a hotly debated topic due to all its consequences. Changes in the temperature can affect aquatic organisms and it is important to understand and to detect earlier signals of biological effects. This study aimed to analyze how a Neotropical fish species responds to temperature increases, using proteomic analysis as a tool. For this, fish of the species *Rhamdia quelen*, male and female, were exposed to two temperatures: 25 °C and 30 °C. After 96h, the animals were anesthetized, euthanized and the liver was collected for proteomic analysis. Using freely available online software and databases (e.g. MetaboAnalyst, Gene Ontology and UniProt), altered proteins in both sexes were defined: 42 in females and 62 in males. Differences between the two temperatures were observed mainly in the energy production and amino acid metabolic pathways. In females, the effects were apparently compensated with energy production, whereas males presented an energy deficit. In addition, immune response was altered, indicating that effects at lower levels of biological organization could serve as a predictor of higher-level effects when temperature rise affects wildlife populations.

Key words: climate change, proteomics, temperature rise, Neotropical fish

Graphical abstract:



Highlights:

Temperature rise changes catfish hepatic protein content.
Proteins related at cellular process and structure were affected with temperature rise.
Male catfish presented an energy deficit.

1. Introduction

Climate change is affecting the world, drawing the attention of scientists and governments. Release of gases, such as carbon dioxide and methane, to the atmosphere leads to the greenhouse effect. This process, which results in global temperature increase, is a natural process of the planet. However, the process is being augmented due to human activities, including the burning of fossil fuels and agriculture of animals (Shahzad, 2015). The Paris Agreement, in 2015, proposed a limit of 1.5 °C increase in global temperature, the same average increase that occurred between 1850 and 2010 in Brazil (Artaxo, 2014; Allen et al., 2018). As the air temperature affects the water temperature, it is important to study what these changes will bring not only in oceanic waters, but also in continental waters.

Teleost fish are useful models for researching the effects of temperature changes in aquatic environments. They are ectotherm animals and spend their lives in direct water contact, and they utilized dissolved oxygen of the water for respiration. These animals vulnerability to climate change depends on stage-specific thermal tolerance, with spawning adults and embryos being the most vulnerable stages (Dalhke et al., 2020). In addition, some species are widely consumed by humans for sustenance, placing an additional pressure on fish populations. *Rhamdia quelen* is an omnivorous catfish species with a habitat range from southern South America to Mexico, and that is consumed by humans because it has a pleasant meat flavor and lacks intramuscular spines, as well as being easy to cultivate (Gomes et al., 2000; Montanha et al., 2011). The lower and higher lethal temperature for this species is 3 and 32 °C, respectively, with 22 to 22 °C as ideal temperature range (Montanha et al., 2011; Garcia et al., 2018).

Proteins are involved in most cellular and physiological aspects of living organisms (Dietrich et al., 2019). Temperature can affect protein bonds and cause protein denaturation, as well as affect the rate of enzymatic reactions (Currie and Schulte,

2014). Thus, protein quantification and identification can be used to understand the effects of temperature on aquatic animals. Proteomics is a technique that attempts to characterize the total protein complement in a tissue or biofluid under specific conditions (Snape et al., 2004). With this technique it is possible to understand, which biological process can be alters, such as toxicology, reproduction and thermal change responses (Forné et al., 2010). In Atlantic salmon liver, for example, proteome analysis showed that transcription/translation mechanisms, protein degradation and cytoskeletal components were down-regulated and amino acid degradation and oxidative stress were up-regulated in chronic exposure to higher temperatures (Nuez-Ortín et al., 2018). In acute exposure, the liver of *Channa striatus*, a freshwater fish, showed an increase in protein to the antioxidant system and chaperones under heat stress conditions (Mahanty et al., 2016). For *Rhamdia quelen*, this technique was used to study the effects of waterborne Diclofenac exposure (Ribas et al., 2017). Proteome responses can be used to understand and predict the effects on higher physiological systems (Liang et al., 2018). As climate change accelerates, scientists need to know how subtle increases in global temperature will affect the physiological function of different biological species. Furthermore, it is important to understand the difference in response mechanisms to this heat stress between the two sexes, since there are few studies that attempt to make this differentiation (Edmands et al., 2021). For this, the aim of this study is to evaluate temperature effects on the hepatic proteome of male and female South American catfish, *Rhamdia quelen*. The main study hypothesis is that the increase in temperature will alter pathways involved with metabolism and energy production, with this response being different for females and males.

2. Material and methods

2.1. Fish acclimation and experimental design

This project was approved by the Animal Use Ethics Committee of Federal University of Paraná, under number 1140. Male and female juveniles of *Rhamdia quelen* (Quoy & Gaimard, 1824) (12.45 ± 1.06 cm and 15.82 ± 4.21 g), obtained from Western Paraná State University, were acclimatized for 30 days at Federal University of Paraná. During acclimation, the animals were kept in tanks with filtered water,

static renewal, constant aeration, controlled photoperiod (12h) and controlled room temperature (25 ± 1°C), with food once a day (Laguna® Brazilian Fish 32). After this period, the animals were divided into two groups to be acclimatized to the experiment temperature for a week, with the addition of one degree per day. The temperatures chosen were 25 and 30 °C, based on previous studies with this species (Gomes et al., 2000; Montanha et al., 2011).

Three 50 L aquarium was used for each treatment, containing 10 animals per one, not yet sexed because they are juveniles, totaling 30 animals per treatment and ensuring that there would be at least three animals of each sex per group. During the experiment, the fish were fed once a day (Laguna® Brazilian Fish 32). The mean water physical-chemical parameters were: pH of 6.9 ±0.1, ammonia of 1.4 ± 0.2 ppm, dissolved oxygen of 3.8 ± 0.2 ppm, and 0.2 ± 0.1 ppm of nitrite. After 96h, the fish were anesthetized with benzocaine, euthanized by medullary section and their gonads and liver were removed. The gonads were fixed, embedded, cut and stained in hematoxylin/eosin to sex the animals. The livers were kept at -80°C. After sexing through histology, three males and three female livers from each group were chosen for proteomic analysis.

2.2. Proteomic analysis

About 10 mg of liver was used for protein extraction with lysis buffer (SDS 4%; DTT 0.1M; Tris HCl 0.1M pH 7.5) at 1:5 (m/v) proportion. This homogenization process consisted of repeating the following cycle three times: two minutes using a Minilys® in mean speed and three minutes at 95°C. This protein extract preparation was carried out based on the standard operating procedure of Fundação Oswaldo Cruz Paraná (FIOCRUZ-PR), under number ICC-POP.121. After one hour in an ultrasonic bath followed by centrifugation at 16000 x g for 5 minutes, the supernatant was stored and its protein concentration was measured in Nanodrop®. After add sample buffer (SDS 2%; Tris HCl 80mM pH 7.5; glycerol 12%; βME 5%; bromofenol blue 0.005%) in 40 $\mu g.\mu L^{-1}$, the solution was heated to 95 °C for 5 minutes, with agitation. The samples were applied in SDS PAGE with subsequent staining with Coomassie Blue 0.1%, according to the standard operating procedure of FIOCRUZ-PR, under number ICC-POP.120. Each lane, representing the sample, was cut from the gel, packed in microtubes and bleached in 25 $mmol.L^{-1}$ ammonium bicarbonate

solution in 50% ethanol. The resulting gel was dehydrated in alcohol and the precipitate was dried in speed vacuum with a pressure of 0.1. These dry samples were reduced with DTT (10 $mmol.L^{-1}$) for 60 minutes at 56°C and alkylated with iodacetamide solution (50 $mmol.L^{-1}$) for 45 minutes at 25°C. After washing with ammonium bicarbonate (ABC) solution (50 $mmol.L^{-1}$), dehydration and drying, the trypsin solution (12.5 $ng.\mu L^{-1}$) diluted in ABC solution (50 $mmol.L^{-1}$) was added and the samples kept at 4 ° C for 20 minutes. The excess trypsin was removed and then the samples were kept in digestion buffer (ABC, 50 $mmol.L^{-1}$) for 16 to 18 hours. The peptides were extracted from these samples using trifluoroacetic acid (TFA) 3% and acetonitrile (ACN) 30%. The supernatant was dried in speed vacuum and purified by StageTips-C18, according to the standard operating procedure of FIOCRUZ-PR, under number ICC-POP.116 and ICC-POP.119.

The samples were analyzed at the Easy-nLC 1000 liquid chromatograph coupled to LTQ Orbitrap XL ETD hybrid mass spectrometry facility P02-004/Carlos Chagas Institute - Fiocruz Paraná, using MaxQuant version 1.6.10.43 to analyze the mass spectra and the Siluriform database from NCBI to identify proteins, first the female samples and then the male samples. Other information are presented at supplementary material (Table S1).

2.3. Data analysis

The LFQ Intensity data were analyzed using MetaboAnalyst 3.0 (Pang et al., 2020), with t test. The proteins with raw p-values < 0.05 were considered with significant change and were chosen for the next step. The gene symbols were searched using protein Blast, from NCBI, against the *Danio rerio* database (taxid: 7955), accessed at 2020 May, always choosing the genes with the greatest homology from those available. After that, the equivalent gene symbols for proteins with negative and positive log2(FC) were uploaded to the Gene Ontology (GO) gene enrichment analysis separately as two groups. The GO biological process and molecular function terms were identified, and then the same data were uploaded to the STRING platform, to visualize how these proteins were related, as well as uploaded to Kyoto Encyclopedia of Genes and Genomes (KEGG), to identify their pathways. UniProtKB (*Danio rerio*) and Zebrafish Information Network (ZFIN database) were used to find additional information about function and subcellular

location, and missing information was identified by literature search. The UniProt entry name is on the Supplementary Data.

3. Results

The hepatic proteome results for females presented a total of 1325 proteins at 25°C and 1467 at 30°C, which 18 proteins that significantly decreased in abundance and 24 proteins that increased according to temperature increase (Table S2-5). In the males, a total of 924 proteins at 25°C and 957 at 30°C was detected, which 35 proteins that significantly decreased in abundance and 27 proteins were that increased (Table S6-9).

Among the altered proteins in females and males, the GO molecular functions included binding, catalytic activity, structural molecule activity, transport activity and regulatory translation activity (Table 1). Regarding the GO biological processes, the altered proteins in females were involved in biological regulation, cell organization or biogenesis, cellular process, development process, growth, localization, locomotion, metabolic process, multicellular organizational process, response to stimulus, and signaling. In males, the altered proteins were involved in biological regulation, cell organization or biogenesis, cellular process, development process, growth, localization, metabolic process, multicellular organizational process, biological adhesion, cell population proliferation and immune system process (Table 2).

4. Discussion

Temperature affects the rates of important processes for animals, such as metabolism, biochemical reactions, biophysical processes and molecules functional states (Hill et al., 2012). In salmon, for example, temperature increase from 15 to 21°C affects transcription, translation, protein and amino acid degradation, as well as cytoskeleton components (Nuez-Ortín et al., 2018). In the present study, *Rhamdia quelen* presented changes in hepatic proteins related to (1) Cell structure, organization, protein synthesis, and molecular transport; (2) Energy production; (3) Metabolic processes; and (4) Cellular differentiation and immune response.

4.1. Cell structure, organization, protein synthesis and molecular transport

To maintain function, the cell needs to be correctly structured and organized. The cytoskeleton and its filaments are important for regulating processes such as mobility, tissue contraction, cell division and cell signaling (Madeira et al., 2016). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh, which is involved in the microtubules binding and cell apoptosis), and actin-related protein 2/3 complex (Arpc3, which is linked to actin and plays a role in apoptosis), were decreased and increased in abundance at 30°C, respectively, in female fish. In males, tropomyosin 3 (Tpm3) and epiplakin 1 (Eppk1) were increased in abundance, while tubulin alpha chain (Tuba8l2) and nascent polypeptide-associated complex subunit alpha (Naca) were decreased at 30°C, all of which are associated with the cytoskeleton. Those observed changes in protein abundance might correspond to a cytoskeleton reorganization expected and encountered for adverse conditions on fish, including thermal changes (Pédron et al., 2017).

TABLE 1: Proteins molecular function altered in females and males of the catfish *Rhamdia quelen* exposed to a temperature increase (from 25 to 30 ºC) for 96h.

GO Term	GO Description	D/I	Females		Male	
			Count	Proteins	Count	Proteins
GO:0005488	Binding	D	5	Shmt2, Anxa6, Sept2, Rplp0, Anxa13	10	Rps16, Rpl22, Pabpc1b, Tuba8l2, Rps10, Rpl4, Eif5a, Fth1a, Acox1, Rack1
		I	11	Arpc3, Hsp90ab1, Eef2b, Eif4a2, Esyt1b, Hspa8, Kars, Calr3b, Rcn3, Hspa5, Hsp90b1	8	Hbaa1, Pygl, Lgals9l1, H2ax, Tpm3, Gnmt, Eppk1
GO:0003824	Catalytic activity	D	5	Shmt2, Fbp1b, Sept2, Hal, Taldo1	7	Grhprb, Uqcrc2b, Mat1a, Acaa1, Fth1a, Acox1, Pdhb
		I	8	Eef2b, Uqcrc2b, Hspa8, Kars, Eprs, Gamt, Larsb, Hspa5	13	Aldh9a1a, Hbaa1, Pygl, Agmat, Mdh1aa, Bcat1, Gbe1b, Got1, Ctsc, Hpx, Gnmt, Sdr16c5b
GO:0005198	Structural molecule activity	D	1	Rplp0	6	Rps16, Rpl22, Tuba8l2, Rps10, Rpl4, Rpl36
		I	0	-	0	-
GO:0005215	Transport activity	D	1	Anxa6	0	-
		I	0	-	1	Hbaa1
GO:0045182	Regulatory translation activity	D	0	-	1	Eif5a
		I	2	Eef2b, Eif4a2	0	-

* D: decreased abundance; I: increased abundance.

TABLE 2: Biological process proteins altered in females and males of the catfish *Rhamdia quelen* exposed to a temperature increase (from 25 to 30 ºC) for 96h.

GO Term	GO Description	D/I	Females		Male	
			Count	Proteins	Count	Genes
GO:0065007	Biological regulation	D	3	Shmt2, Anxa6, Anxa13	7	Cirbpa, Pabpc1b, Eif5a, Acaa1, Fth1a, Acox1, Rack1
		I	4	Arpc3, Hsp90ab1, Calr3b, Hspa5	4	Lgals9l1, Rab8a, H2ax, Gnmt
GO:0071840	Cell organization or biogenesis	D	3	Anxa6, Sept2, Rplp0	4	Rps16, Tuba8l2, Uqcrc2b, Rps10
		I	4	Arpc3, Sec13, Tmed2, Uqcrc2b	7	Rab8a, H2ax, Tpm3, Col6a3, Hpx, Gnmt, Eppk1
GO:0009987	Cellular process	D	7	Shmt2, Anxa6, Fbp1b, Sept2, Rplp0, Anxa13, Ddx39b	15	Cirbpa, Eif3m, Rps16, Rpl22, Pabpc1b, Tuba8l2, Uqcrc2b, Rps10, Eif5a, Acaa1, Fth1a, Acox1, Rack1, Pdhb, Rpl36
		I	14	Arpc3, Sec13, Hsp90ab1, Eef2b, Tmed2, Eif4a2, Uqcrc2b, Hspa8, Kars, Calr3b, Eprs, Larsb, Hspa5, Hsp90b1	16	Hbaa1, Pygl, Agmat, Mdh1aa, Lgals9l1, Bcat1, Rab8a, H2ax, Got1, Tpm3, Ctsc, Col6a3, Hpx, Gnmt, Eppk1
GO:0032502	Development process	D	1	Anxa6	0	-
		I	0	-	1	Col6a3
GO:0040007	Growth	D	1	Anxa6	0	-
		I	0	-	1	Col6a3
GO:0051179	Localization	D	3	Anxa6, Sept2, Anxa13	2	Uqcrc2b, Fth1a
		I	4	Sec13, Tmed2, Uqcrc2b, Hspa8	2	Lgals9l1, Rab8a
GO:0040011	Locomotion	D	1	Anxa6	0	-
		I	0	-	0	-
GO:0008152	Metabolic process	D	4	Shmt2, Fbp1b, Rplp0, Ddx39b	12	Cirbpa, Eif3m, Rps16, Rpl22, Pabpc1b, Uqcrc2b, Eif5a, Acaa1, Acox1, Rack1, Pdhb, Rpl36
		I	7	Eef2b, Eif4a2, Uqcrc2b, Kars, Eprs, Larsb, Hspa5	13	Hbaa1, Pygl, Agmat, Mdh1aa, Lgals9l1, Bcat1, Gbe1b, H2ax, Got1, Ctsc, Hpx, Gnmt
GO:0032501	Multicellular organizational process	D	1	Anxa6	0	-
		I	0	-	3	Lgals9l1, Tpm3, Col6a3
GO:0050896	Response to stimulus	D	2	Shmt2, Anxa6	1	Acaa1
		I	4	Hsp90ab1, Hspa8, Calr3b, Hspa5	2	Rab8a, Eppk1
GO:0023052	Signaling	D	1	Anxa6	0	-
		I	2	Calr3b, Hspa5	0	-
GO:0022610	Biological adhesion	D	0	-	0	-
		I	0	-	1	Lgals9l1
GO:0008283	Cell population proliferation	D	0	-	0	-
		I	0	-	1	Lgals9l1
GO:0002376	Immune system process	D	0	-	0	-
		I	0	-	1	Lgals9l1

* D: decreased abundance; I: increased abundance.

Protein production is also extremely important to maintain cell structure and function, with the ribosome being the basis structure for this function. Proteins related to this non-membranous organelle are important for cell growth (Lempiäinen and Shore, 2009). The eukaryotic translation elongation factor 2b (Eef2b), which was increased in females at 30°C, acts in ribosome binding, being therefore extremely important for protein synthesis (Liang et al., 2014), showing greater recruitment of protein synthesis in females. However, Rplp0, a component protein of the 60S ribosome, was also reduced at 30°C in these females, showing an imbalance in this process since there is an increase in ribosomal synthesis protein, but not in a ribosomal component. In males, many ribosomal proteins were reduced in abundance at 30°C. The ribosomal protein S16 (Rps16), ribosomal protein S10 (Rps10), ribosomal protein P2 (Rplp2), 60S ribosomal protein L36 (Rpl36), ribosomal protein L22 (Rpl22), ribosomal protein L4 (Rpl4), ribosomal protein P1 (Rplp1) and guanine nucleotide-binding protein (Rack1) are involved in ribosomal processes, since translation to being a constituent subunit. This demonstrate that temperature increase could cause a deficiency in ribosomal biogenesis in males and, consequently, in protein synthesis.

Chaperones are highly conserved proteins that promote the correct folding and assembly of these newly synthesized proteins, being one of the most important cellular stress response components (Sumanas et al., 2003; Somero, 2020). Therefore, proteins can be synthesized and still not be able to function properly. Heat shock proteins (HSP) are chaperones that can refold proteins and play a role in reducing and repairing protein damage (Somero, 2010). In females, some chaperone proteins (Hsp90ab1, Hspa8, Hspa5, Hsp90b1 and Calr3b), that can interact with each other, were increased in abundance at 30°C. Thus, the positive regulation of these proteins demonstrates an organism's response to avoid protein damage. However, in males, the only altered chaperone, heat shock protein 9 (Hspa9), was decreased at 30°C. Hspa9, which belongs to the heat shock proteins 70 family and acts in the mitochondria, can regulate apoptosis (Currie and Schulte, 2014; Rupik et al., 2011), indicating that changes in programmed apoptosis can occur in males at increased temperatures.

In addition to being correctly synthesized and refolded by chaperones, proteins must also be properly transported. In females, proteins related to intracellular protein transport were increased, including Ras-related protein Rab-2A (Rab2a),

transmembrane emp24 domain trafficking protein 2 (Tmed2) and solute carrier family 25 alpha (Slc25a5). In males, proteins related to intracellular protein transport were altered at 30°C, as well: Ras-related protein Rab-8A (Rab8a) was increased and Naca was decreased. Increased temperature also increased proteins related to RNA transport in females, Sec13 homolog (*S. cerevisiae*, Sec13) and eukaryotic translation initiation factor 4A (Eif4a2), while one protein involved in RNA transport, the polyadenylate-binding protein (Pabpc1b) was decreased in males. Along with changes in cytoskeletal proteins, changes in all these transport proteins in males and females may also indicate cellular reorganization in a high temperature reflex, since intracellular transport is fundamental for cell function and survival, especially for proteins (Hirokawa et al., 2009). However, a decrease in transport proteins, such as Naca, may indicate that certain proteins are not being transported correctly, which can affect other functions in the cell.

Extended synaptotagmin-like protein 1b (Esyt1b) was increased in females and apoliprotein Bb (Apobb.1), sterol carrier protein 2a (Scp2a) and high density lipoprotein binding protein (Hdlbpa) were decreased at 30°C in males, and all of these proteins are related to lipid and cholesterol transport. Lipids are essential plasma membrane components, and temperature is known to regulate membrane fluidity (Li et al. 2018). Hdlbpa and Scp2a are cholesterol transport proteins, and their decreased abundance can reduce transport of cholesterol to the plasma membrane, and thus, reduce membrane fluidity (Mathew et al., 2018). This more fluid membrane can make the cell more susceptible to other agents and affect the DNA (Eckl and Bresgen, 2017).

Some co-factors, such as calcium and manganese, are also important in maintaining cellular organization and processes. Calcium is an ion related to muscle contraction, homeostasis and growth, being absorbed mainly by fish gills and intestines (Flik et al., 1996). In the liver, the induction of calcium-binding proteins can occurs in the presence of estrogen (Chan, 1972). Annexin is a superfamily of calcium-related proteins (Morgan et al., 2004). In females, the annexins 6 and 13 (Anxa6 and Anxa13) were decreased at higher temperature, which suggests altered membrane fluidity, cytoskeleton assembly and vesicle transport. In particular, Anxa6 is involved in mitochondrial calcium homeostasis, in muscle contraction regulation and membrane related-process (Buzhynskyy et al., 2009). Other proteins that were also related to calcium binding in females at increased temperature were Esyt1b,

calreticulin 3b (Calr3b) and reticulocalbin 3 (Rcn3). These three proteins were increased in females, and could be a compensation mechanism for the body to maintain cellular homeostasis, since the annexins were down.

4.2. Energy production

Increase in temperature raises metabolic rate and, consequently, the need for energy leading to a metabolic remodeling state to compensate for the increased energy demand (Nuez-Ortíz et al., 2018). This energy can be supplied in the form of adenosine triphosphate (ATP), produced by cellular respiration that occurs in different stages: during (1) glycolysis, (2) the Krebs (or citric acid) cycle, and (3) oxidative phosphorylation (Hill et al., 2012). In the present study, females at 30°C had decreased UTP--glucose-1-phosphate uridylyltransferase (Ugp2b) protein, which participates in galactose metabolism and which results in glucose (dre00052, KEGG). Glucose is used at the beginning of cellular respiration, suggesting that in females, glycolysis may be affected by lack of substrate. Another pathway that can affect glucose availability for cellular respiration is insulin signaling. Among the proteins involved in this signaling pathway are alpha-1, 4 glucan phosphorylase (Pygl) and fructose-1, 6-bisphosphatase 1b (Fbp1b). In males, Pygl was increased at 30°C and this protein can catabolize glycogen, an important glucose source. However, in females, Fbp1b was decreased. This protein is associated with several pathways that result in the glucose formation, such as pentose phosphate pathway (dre00030, KEGG). Its reduction reinforces the deficit of substrate for cellular respiration in females and indicates reproductive damage since this protein can also increase in response to estradiol, an important female hormone (Porseryd et al., 2018).

Glycolysis is a glucose catabolic process, which results in two ATP molecules and pyruvic acid (Polakof et al., 2012). In females, two proteins involved in this pathway, Fbp1b and Gapdh, were decreased at 30°C, showing a deficit in energy and by-products of cellular respiration first stage. In males, acetyltransferase component of pyruvate (Dlat) and pyruvate dehydrogenase E1 component (Pdhb) were decreased and 4-trimethylaminobutyraldehyde dehydrogenase (Aldh9a1a.1) was increased at 30°C. Pdhb is involved in two glycolysis reactions, which means the change in this protein in males could lead to a pyruvic acid deficit important to Krebs

cycle (dre00010, KEGG). Aldh9a1a.1 act in the end of the pyruvate metabolism (dre00620, KEGG), but its increase in males may not be enough to balance the reduction in Dlat and Pdhb.

Pyruvate is transformed into acetyl CoA in the mitochondrial matrix. Malate dehydrogenase (Mdh1aa), hydroxyacylglutathione hydrolase (Hagh), Aldh9a1a.1, Pdhb, Dlat and glyoxylate reductase/hydroxypyruvate reductase b (Grhprb) are examples of proteins that act in the pyruvate metabolism (dre00620, KEGG). In males at 30°C, three of these proteins (Mdh1aa, Hagh and Aldh9a1a.1) were increased and three were decreased (Pdhb, Dlat and Grhprb). Acetyl CoA production, fuel for the Krebs cycle, can also be affected in other pathways. Pantothenate and CoA biosynthesis pathway can result in CoA production, and the branched-chain-amino-acid aminotransferase (Bcat1), which increased in males at 30°C, is part of this pathway (dre00770, KEGG). Glyoxylate and dicarboxylate metabolism can utilize metabolites that are involved in pyruvate metabolism and includes proteins like mitochondrial serine hydroxymethyltransferase (Shmt2), Mdh1aa and Grhprb (dre00630, KEGG). In this study, Shmt2 decreased in females, Mdh1aa increased in males and Grhprb decreased in males at 30°C. Thus, compensation maybe occurring in pyruvate metabolism in males, to account for the additional demand for oxaloacetate and citrate created by glyoxylate and dicarboxylate metabolism, which was not observed in females (Hill et al., 2012).

In this study, proteins related with guanosine triphosphate (GTP) were altered and GTP-binding proteins constitute a regulatory molecule superfamily (Lee et al., 1998). GTPases are regulators of adhesion, cytoskeletal organization and apoptosis, as well as catalyze the conversion of GTP to inorganic phosphate, producing ATP (Carvalho et al., 2015). In females, the GTPase protein septin 2 (Sept2) was decreased at 30°C. However, Eef2b and Rab2a, with GTPase activity, were increased. In males, Tuba8l2, a cytoskeletal protein that binds GTP and has GTPase activity, was decreased at 30°C, while Rab8a, with GTPase activity and GTP binding, was increased in abundance – just as in females. The cellular respiration second stage needs guanosine diphosphate (GDP) which will be transformed into GTP. It donates its terminal phosphate group to adenosine diphosphate (ADP) which results in the formation of ATP and GDP (Hill et al., 2012). Thus, protein changes related to GTP may have some influence on energy metabolism.

The last stage of energy production is the electron transport chain following oxidative phosphorylation that occurs in mitochondria. The ubiquinol-cytochrome c reductase core protein (Uqcrc2b), a mitochondria protein that acts as endopeptidase and be part of electron transport complex (dre00190, KEGG), was increased in abundance in females at 30°C, perhaps as a way to compensate for the reduction in other proteins involved in the previous steps, including Ugp2b, Fbp1b, Gapdh, Shmt2 and Sept2. In males, Uqcrc2b and ATP synthase membrane subunit (Atp5md), which is present in the mitochondria membrane, were both decreased at 30°C. Thus, in males, the combined effect of changes in the cellular respiration process, energy production may be compromised as temperatures increased in this study, which could be explored in future studies by quantifying glucose and lactate concentrations, for example.

In addition to cellular respiration, energy can be produced through fatty acid beta-oxidation. In this catabolic process, successive molecules of acetyl-CoA and NADH are produced. Acetyl-CoA can be used in the Krebs cycle to produce more NADH, which are used in oxidative phosphorylation to produce ATP (Tocher, 2003). The medium-chain acyl-CoA ligase (Acsf2) catalyzes the initial reaction in fatty acid metabolism, by forming a thioester with CoA, and was increased in females at 30°C, suggesting an increased energy requirement for female liver tissues, which could be due to changes earlier in the respiratory chain (Cline et al., 2020). In male fish from the present study, the opposite happened. Four proteins involved in beta-oxidation processes in mitochondria and peroxisomes were decreased at 30°C (acyl-coenzyme A oxidase (Acox1), acetyl-CoA acyltransferase (Acaa1), Scp2a and 17-beta-hydroxysteroid dehydrogenase (Hsd17b4); dre01212, KEGG). In males, this suggests that two other pathways involved with fatty acid beta-oxidation, (alpha-linolenic acid metabolism (Acox1 and Acaa1; dre00592, KEGG) and the signaling pathway for receptors activated by peroxisome proliferators (Acaa1, Scp2a and Acox1; dre03320, KEGG) were affected by temperature, further compromising energy production in males.

Carbohydrate metabolism is also essential because it is related to the energy supply for osmoregulation, with the liver being the central carbohydrate reserve (Tseng and Hwang, 2008). The increase in temperature in the present study caused changes in proteins that are involved in the metabolism of starch and sucrose (increased Fbp1b and reduced Ugp2b in females, and increased Pygl and glucan

(1,4-alpha-)-branching enzyme (Gbe1b) in males). In males, increased Pygl and Gbe1b could indicate an increase in energy demand to compensate for perturbations in aerobic cellular respiration (Wen et al., 2018). In females, an increase in Fbp1b suggests that fructose and mannose metabolism may also affected (dre00021, KEGG).

4.3. Metabolic processes

Many proteins involved in amino acid pathways were affected by increased temperature in the present study. For example, the glycine, serine and threonine metabolism pathways (dre00260, KEGG): in females, decreased abundance of Shmt2, increased abundance of guanidinoacetate N-methyltransferase (Gamt) and increased pipecolic acid oxidase (Pipox) occurred, while in males, there was decreased abundance of glyoxylate reductase/hydroxypyruvate reductase b (Grhprb), and increased levels of Pipox, glycine N-methyltransferase (Gnmt) and amine oxidase (Mao) at 30°C. The balance of protein perturbations in these pathways may demonstrate a compensation mechanism in both sexes. The products of the glycine, serine and threonine metabolism pathways are important to the production of creatine, tryptophan and pyruvate - a factor for cellular respiration. Tryptophan metabolism, along with its phenylalanine/tyrosine biosynthesis, were also affected (dre00400, KEGG). In females, phenylalanine hydroxylase (Pah) was decreased at 30°C. In males, aspartate aminotransferase (Got1), Aldh9a1a.1, and Mao were increased. Tryptophan is an essential amino acid and also a precursor of serotonin (Hseu et al., 2003). Temperature rise increased serotonin levels (stomach and gut) in Antarctic fish (Vargas-Chacoff et al., 2019), supporting the results for males in this present work with Neotropical fish. Serotonin can be involved with social behavior (Winberg and Thörnqvist, 2016), so it is possible that increased temperature could also influence the behavior of male catfish, a hypothesis that should be tested in future studies.

The catabolism of histidine was also affected by temperature. In females, the histidine ammonia-lyase (Hal) and formimidoyltransferase cyclodeaminase (Ftcd) proteins were decreased at 30°C, which are involved in the conversion phases of L-histamine to urochanate, which is further converted to L-glutamate (dre00340, KEGG). Thus, an increase in L-histidine may occur. The histamine, that is an

inflammatory process response, is synthesized from L-histidine, and its accumulation can also influence this type of process in female fish (Galindo-Villegas et al., 2016). In males, decreased histamine N-methyltransferase (Hnmt) and an increase in the Mao, suggest that these fish were trying to maintain histamine homeostasis even at 30°C (dre00340, KEGG).

Arginine is another amino acid with proteins involved in its metabolism that were altered by temperature. In females, the Gamt protein was increased, which is responsible for one of the reactions that converts arginine to creatine, leading to an increase in creatine in liver (dre00330, KEGG). Creatine is a non-essential amino acid transported to the white muscle for ATP production when the energy demand is greater, for example in cases of migration over long distances (McFarlane et al., 2001). In males, the alteration occurred in different pathway points, including arginine biosynthesis, with increased abundance of agmatine ureohydrolase (Agmat), Got1, Mao and Aldh9a1a.1 at 30°C (dre00330, KEGG). Arginine is involved in different metabolic pathways, from protein and creatine synthesis, to being used as a precursor to nitric oxide synthesis in the immune process (Habte-Michael, 2020). So increased arginine biosynthesis proteins may be related to the immune system in the males from the present study.

As previously mentioned, temperature can affect RNA levels. In this case, temperature increase caused an increase in tRNA biosynthesis in females, with an increase in abundance of glutamyl-prolyl-tRNA synthetase (Eprs1), leucyl-tRNA synthetase (Larsb) and lysine--tRNA ligase (Kars1), related to glutamine, leucine and lysine biosynthesis, which demonstrates that these amino acids were being increasingly recruited. On the other hand, the proteins in the lysine degradation pathway were increased both in females (Pipox) and in males (Pipox and Aldh9a1a.1), suggesting that amino acids were used as a substrate for carnitine synthesis, which is used in the transport of fatty acids from the cytosol to the mitochondria (Li et al., 2009).

In males, other pathways related to amino acids were altered at 30°C. The biosynthesis and degradation pathways of valine, leucine and isoleucine had a reduced Acaa1 and increased branched-chain-amino-acid aminotransferase (Bcat1) and Aldh9a1a.1, with Bcat1 being responsible for the last reaction of all these amino acids biosynthesis and the first reaction of degradation, that is, controlling the process (dre00290, KEGG). Proteins in the cysteine and methionine metabolic

pathways were also altered, with the increase of Got1, Bcat1 and malate dehydrogenase (Mdh1aa); and decrease of S-adenosylmethionine synthase (Mat1a), which is mainly responsible for methionine recuperation (dre00270, KEGG). In alanine, aspartate and glutamate metabolism, the temperature increase the Got1, a protein related to reactions before to the Krebs cycle (dre00250, KEGG). Related to beta alanine metabolism, Aldh9a1a.1 was up regulated and Acox1 was down regulated, and this amino acid is a constituent of other molecules such as carnosine and anserine which are antioxidants (Li et al., 2009). Amino acids have many functions and overall , decreased amino acids can cause energy deficits in cells, while increased abundance can provide a greater energy expenditure for the production of proteins (Ballantyne, 2001).

Some vitamin-related proteins were also altered by 30°C, and it is known that they are essential for the proper functioning of the body. In females, Pah and biliverdin reductase B (Blvrb) were reduced, which could suggest folate biosynthesis and riboflavin metabolism could be altered, respectively. In long term, this possible folate reduction may result in chronic haemolytic anemia (Clauss et al., 2008). Blvrb acts specifically in riboflavin degradation and its decrease can lead to a reduction in factors necessary for other reactions, such as the reduction of FAD used to regenerate glutathione (Hansen et al., 2015). In males, other vitamin-related pathway proteins were affected by temperature increase. Increased Aldh9a1a.1 and short chain dehydrogenase/reductase (Sdr16c5b) could indicated increased metabolism of ascorbate and aldarate, respectively. Arcorbate and retinol pathways, among many functions, can also increase the antioxidant capacity, thus could be increased in males in higher temperatures to fight reactive oxygen species (Dabrowski and Ciereszko, 2001; Jiang et al., 2019).

Metabolic detoxification processes can also be altered by 30°C, especially in the liver, which is the main metabolism organ. In females, proteins of pentose and glucuronate interconversion pathway (Ugp2b) and pentose phosphate pathway (Fbp1b and transaldolase (Taldo1)) were decrease in abundance. Conjugation of glucuronic acid produced by this pathway is important for xenobiotic detoxification and the pentose pathway phosphate is an alternative route for glucose catabolism that produces NADPH, which could be used by detoxification enzymes (Wen et al., 2018). Thus, we suggest that female detoxification can be compromised by the increase in temperature and should be better studied. Likewise, bile acids are

important for detoxification (Hauser-Davis et al., 2012). In males, the decreased abundance of Hsd17b4 and Scp2a suggests that primary biosynthesis of bile acids was affected (dre00120, KEGG), which could also compromise detoxification capability.

Metabolic process such as detoxification can cause an imbalance between the antioxidant system and oxidative species leading to oxidative stress, and this imbalance occurs mainly in mitochondria (Nuez-Órtin et al., 2018). Proteins related to oxidation and reduction, which are a source of reactive oxygen species (ROS), were decreased in males, including phytanoyl-CoA dioxygenase domain-containing protein (Phyhd1), Zgc:56585 (Zgc:56585) and Hspa9 at 30°C. Also in males, ferritin (Fth1a) was decreased and hemoglobin (Hbaa1) was increased at 30°C, and both are involved in removing ROS that could be formed by metabolism (as mentioned previously). Fth1a is important to maintain iron homeostasis, a molecule that can suffer oxidation and produce ROS (Chen et al., 2020). Hbaa1 is a protein that acts on oxygen transport and is involved in the catabolic process of the reactive hydrogen peroxide species (Campo et al, 2008). This balance with greater reduction of proteins involved in the ROS production can be positive for the organism, reducing factors that lead these hepatocytes to oxidative stress. In addition, as shown previously, there was a change in proteins related to energy production, following an energy deficit in males. This increase in hemoglobin may also indicate a greater demand for oxidative respiration, as an attempt to fill the energy deficit.

In case of alterations in these cellular processes, it is important that the organism is able to destroy the problematic cell or its problematic parts, such as an autophagic process. This is a subcellular degradation process in which eukaryotic cells can recycle their intracellular substances, thus maintaining the health of the cell and the organism (Xia et al., 2019). In males, cathepsin D protein (Ctsd) was increased at 30°C, which could indicate increased autophagy (dre04140, KEGG). Proteins involved in necroptosis (dre04217, KEGG), programmed cell necrosis by genetic factors, were also altered in males with a reduction in Fth1a, and an increase in Pygl and histone H2A (Hist1h2a4) at 30°C. In females, there was also an increase in Slc25a, which is associated with cellular senescence. Autophagy, a lysosome-dependent degradation pathway, necrosis and senescense could be a response to a stress, to maintain homeostasis (Xia et al., 2019).

4.4. Cellular differentiation and immune response

Cellular differentiation is extremely important to organism function. Particularly in the case of erythrocyte differentiation, as this cell type is involved in the oxygen transport throughout the body and its reduction leads to anemia (Kulkeaw and Sugiyama, 2012). Females had decreased abundance of Hal at 30°C, a protein involved in hematopoietic stem cell differentiation (Han et al., 2016). Males had decreased ribosomal protein (Rplp1) at 30°C, which is involved in erythrocyte differentiation (Yadav et al., 2014).

Correct cell differentiation is also important since leukocytes, another blood cell type, help with responses against pathogens. The immune system can be altered by many factors, including temperature (Bowden, 2008). In females, temperature increase resulted in increased abundance of Calr3b, involved in phagosome decreased (dre04145, KEGG). These results suggest that temperature can modulate the immune system, as observed in European seabass, *Dicentrarchus labrax*, exposed to 32°C for 30 days (Islam et al., 2020).

Innate immunity is the base immune defense, the first mechanism to be activated (Tort et al., 2003). In males, temperature increase decreased Naca and increased Hist1h2a4, two proteins that are involved in innate defense (Li et al., 2015, Kong et al., 2017), and the inflammatory process (Relb and Goldammer, 2018). Adaptive immunity is the organism's response to pathogens with which they have previously had contact, and CD4 cells and antibodies are involved in this type of immune response (Nakanichi et al., 2015). In males, a galectin (Lgals911), which negatively regulates the proliferation of CD4 cells, was increased in abundance, suggesting decreased recruitment of this cell type and impact the adaptive immune response. For both sexes, increased abundance of proteins related to the immune system could also indicate an autoimmune response of the body to tissue damaged by temperature (Dittmar et al., 2014).

5. Conclusion

Increased temperature affected proteins mainly in the production of energy and in metabolic pathways related to amino acids, lipids, and carbohydrates, both in males and females, at 96 hours of exposure. However, the differential abundance of

proteins within pathways was apparently balanced in female fish, which suggests compensation in the case of energy production, where proteins participating in the same process were both increased and decreased. Most often, the abundance of proteins in male fish was decreased, which suggests that males could experience a greater energy deficit with increased water temperature compared to females. In addition, proteins in males also indicated a potential deficit in protein synthesis. All of these protein changes could also affect oxidative stress and immune regulation pathways, affecting long-term survival in a warmer world. This study demonstrated that proteomics is a useful tool to detect the sublethal effects of rising temperatures. The proteomics use can help predict which effect temperature could cause and help to understand how temperature (and climate change) changes the planet aquatic biota.

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Supplementary Data

Table S1: MaxQuant parameters.

Parameter	Value
Version	1.6.10.43
Date of writing	2020 August, 4
Include contaminants	True
Use normalized ratios for occupancy	True
Minimum peptide length	7
Minimum score for unmodified peptides	0
Minimum score for modified peptides	40
Minimum unique peptides	0
Minimum peptides	1
Modifications included in protein quantification	Oxidation (M);Acetyl (Protein N-term)
Stabilize large LFQ ratios	True
Separate LFQ in parameter groups	False
Require MS/MS for LFQ comparisons	True
Calculate peak properties	False
Maximum peptide mass [Da]	4600
Minimum peptide length for unspecific search	8
Maximum peptide length for unspecific search	25
Evaluate variant peptides separately	True
MS/MS tol. (FTMS)	20 ppm
Top MS/MS peaks per Da interval. (FTMS)	12
Da interval. (FTMS)	100
MS/MS deisotoping (FTMS)	True
MS/MS deisotoping tolerance (FTMS)	7
MS/MS deisotoping tolerance unit (FTMS)	Ppm
MS/MS higher charges (FTMS)	True
MS/MS water loss (FTMS)	True
MS/MS ammonia loss (FTMS)	True
MS/MS dependent losses (FTMS)	True
MS/MS recalibration (FTMS)	False

Table S2: *Rhamdia quelen* female decreased proteins identification exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	Gene Symbol	Gene Name	UniProt Entry Name	UniProt Name
1709672135	*anxa13*	annexin A13	F1REH8 (F1REH8_DANRE)	Annexin
1503285027	*anxa6*	annexin A6	F1R6N3 (F1R6N3_DANRE)	Annexin
1764638154	*blvrb*	biliverdin reductase B	B8JMC1 (B8JMC1_DANRE)	Biliverdin reductase B
1764580684	*ddx39ab*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39Ab	Q803W0 (Q803W0_DANRE)	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39Ab
576888482	*fbp1b*	fructose-1,6-bisphosphatase 1b	Q7T337 (Q7T337_DANRE)	Fructose-1,6-bisphosphatase 1b
1764589250	*ftcd*	formimidoyltransferase cyclodeaminase	F1QZ40 (F1QZ40_DANRE)	Formimidoyltransferase cyclodeaminase
308321716	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	Q5XJ10 (G3P_DANRE)	Glyceraldehyde-3-phosphate dehydrogenase
1042288042	*hal*	histidine ammonia-lyase	A4FUP1 (A4FUP1_DANRE)	Histidine ammonia-lyase
1520586854	*krt5*	keratin 5	F1QV31 (F1QV31_DANRE)	Keratin 5
576886301	*pah*	phenylalanine hydroxylase	Q6PHI7 (Q6PHI7_DANRE)	Phenylalanine hydroxylase
1709658404	*pah*	phenylalanine hydroxylase	Q6PHI7 (Q6PHI7_DANRE)	Phenylalanine hydroxylase
1764585473	*rplp0*	ribosomal protein, large, P0	Q6P5K3 (Q6P5K3_DANRE)	60S acidic ribosomal protein P0
1042301029	*sept2*	septin 2	F1QQN9 (F1QQN9_DANRE)	Septin 2
1520629056	*shmt2*	serine hydroxymethyltransferase 2	A9LDD9 (A9LDD9_DANRE)	Mitochondrial serine hydroxymethyltransferase
1042358820	*sult1st2*	sulfotransferase family 1, cytosolic sulfotransferase 2	Q7ZUS4 (ST2S2_DANRE)	Cytosolic sulfotransferase 2
1520569604	*taldo1*	transaldolase 1	Q6P6Y0 (Q6P6Y0_DANRE)	Transaldolase
1709658796	*ugp2b*	UDP-glucose pyrophosphorylase 2b	Q6NWJ8 (Q6NWJ8_DANRE)	UTP--glucose-1-phosphate uridylyltransferase
1520598290	*usp14*	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)	F1R4B7 (F1R4B7_DANRE)	Ubiquitin carboxyl-terminal hydrolase

Table S3: *Rhamdia quelen* female decreased proteins information exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	log2(FC)	raw.pval	Peptides	Unique peptides	Sequence coverage [%]	Unique sequence coverage [%]	Mol. Weight [kDa]	Sequence length	Score
1709672135	-0.52399	0.013105	6	2	19.3	4.7	30.517	275	10.599
1503285027	-0.70194	0.040906	13	3	24	7.4	74.657	662	42.854
1764638154	-0.39995	0.00039435	3	2	15.5	11.1	22.76	207	15.074
1764580684	-0.40792	0.048245	14	12	44.5	38.4	48.86	427	33.578
576888482	-0.48872	0.00012823	21	12	55.2	30.6	36.632	337	323.31
1764589250	-0.39401	0.024406	8	3	14.4	5.7	59.181	540	38.017
308321716	-0.3756	0.015277	15	5	43.8	11.7	35.889	333	170.83
1042288042	-0.50495	0.032164	23	4	52.7	9.3	73.346	664	188.53
1520586854	-0.93454	0.0035127	13	0	21.7	0	58.246	548	14.978
576886301	-0.77059	0.014104	11	3	27.6	9.4	50.947	445	41.973
1709658404	-0.83678	0.005238	10	2	29.5	5.4	46.809	410	50.33
1764585473	-0.32493	0.029656	11	11	46.8	46.8	33.959	316	105.75
1042301029	-0.35148	0.049982	5	2	22.4	11.1	41.344	361	35.494
1520629056	-1.1004	0.031749	16	11	46.2	33.5	54.831	496	209.04
1042358820	-0.56024	0.022361	4	2	19.9	12	34.673	292	25.813
1520569604	-1.0459	0.038135	6	1	18.1	3.9	37.618	337	1.8209
1709658796	-0.45194	0.018538	11	1	31.1	4.5	55.235	492	22.38
1520598290	-6.2797	5.9767e-05	3	3	8.3	8.3	56.384	494	2.7722

Table S4: *Rhamdia quelen* female increased proteins identification exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	Gene Symbol	Gene Name	UniProt Entry Name	UniProt Name
1764618757	*acsf2*	acyl-CoA synthetase family member 2	Q0P4F7 (ACSF2_DANRE)	Medium-chain acyl-CoA ligase ACSF2, mitochondrial
1042303154	*arpc3*	actin related protein 2/3 complex, subunit 3	Q6ZM62 (Q6ZM62_DANRE)	Actin-related protein 2/3 complex subunit 3
1709650524	*calr3b*	calreticulin 3b	Q6DI13 (Q6DI13_DANRE)	Calreticulin
1764598874	*calr3b*	calreticulin 3b	Q6DI13 (Q6DI13_DANRE)	Calreticulin
576892150	*dhrs12*	dehydrogenase/reductase (SDR family) member 12	B0UYL5 (B0UYL5_DANRE)	Dehydrogenase/reductase (SDR family) member 12
1709635873	*eef2b*	eukaryotic translation elongation factor 2b	A0A286Y8X9 (A0A286Y8X9_DANRE)	Eukaryotic translation elongation factor 2b
1709658295	*eif4a2*	eukaryotic translation initiation factor 4A, isoform 2	F1R166 (F1R166_DANRE)	Eukaryotic translation initiation factor 4A, isoform 2
1709641409	*eprs1*	glutamyl-prolyl-tRNA synthetase 1	F1QP23 (F1QP23_DANRE)	Glutamyl-prolyl-tRNA synthetase
1709660161	*esyt1b*	extended synaptotagmin-like protein 1b	F6NZP4 (F6NZP4_DANRE)	Extended synaptotagmin-like protein 1b
318054260	*gamt*	guanidinoacetate N-methyltransferase	Q71N41 (GAMT_DANRE)	Guanidinoacetate N-methyltransferase
1465648784	*hsp90ab1*	heat shock protein 90, alpha (cytosolic), class B member 1	O57521 (HS90B_DANRE)	Heat shock protein HSP 90-beta
1503223927	*hsp90b1*	heat shock protein 90, beta (grp94), member 1	A0A286YAP4 (A0A286YAP4_DANRE)	Heat shock protein 90, beta (grp94), member 1
1023043046	*hsp90b1*	heat shock protein 90, beta (grp94), member 1	A0A286YAP4 (A0A286YAP4_DANRE)	Heat shock protein 90, beta (grp94), member 1
1520603690	*hspa5*	heat shock protein 5	Q6P3L3 (Q6P3L3_DANRE)	Heat shock protein 5
1503271866	*hspa8*	heat shock protein 8	Q90473 (HSP7C_DANRE)	Heat shock cognate 71 kDa protein
1764621901	*kars1*	lysyl-tRNA synthetase 1	Q6DHE6 (Q6DHE6_DANRE)	Lysine--tRNA ligase
1503263868	*lars1b*	leucyl-tRNA synthetase 1b	F1QFN3 (F1QFN3_DANRE)	Leucyl-tRNA synthetase b
1042301809	*pipox*	pipecolic acid oxidase	F6P928 (F6P928_DANRE)	Pipecolic acid oxidase
1520502608	*rab2a*	RAB2A, member RAS oncogene family	Q803J3 (Q803J3_DANRE)	RAB2A, member RAS oncogene family
318853127	*rcn3*	reticulocalbin 3, EF-hand calcium binding domain	F1R0M8 (F1R0M8_DANRE)	Reticulocalbin 3, EF-hand calcium-binding domain
318068042	*sec13*	SEC13 homolog, nuclear pore and COPII coat complex component	Q7T2E1 (Q7T2E1_DANRE)	SEC13 homolog (S. cerevisiae)
1764634355	*slc25a5*	solute carrier family 25 member 5	Q8JHI0 (Q8JHI0_DANRE)	Solute carrier family 25 alpha, member 5
1709639864	*tmed2*	transmembrane p24 trafficking protein 2	Q7ZW75 (Q7ZW75_DANRE)	Transmembrane emp24 domain trafficking protein 2
1520591273	*uqcrc2b*	Ubiquinol-cytochrome c reductase core protein 2b	Q6IQ59 (Q6IQ59_DANRE)	Ubiquinol-cytochrome c reductase core protein 2b

Table S5: *Rhamdia quelen* female increased proteins identification exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	log2(FC)	raw.pval	Peptides	Unique peptides	Sequence coverage [%]	Unique sequence coverage [%]	Mol. Weight [kDa]	Sequence length	Score
1764618757	1.6473	0.042154	4	3	7.9	5.6	67.638	609	20.977
1042303154	0.28737	0.049092	2	1	12.4	6.2	20.57	178	8.4135
1709650524	2.6129	0.033427	8	1	16.8	2	51.5	441	28.763
1764598874	0.84766	0.0081323	15	6	36.4	19	48.726	420	99.396
576892150	3.0614	0.00090447	3	3	9	9	36.057	324	3.2333
1709635873	1.0152	0.034214	31	2	43.1	2.3	95.56	858	20.043
1709658295	0.61006	0.014889	13	5	45.4	19.7	44.637	390	15.398
1709641409	0.53765	0.029534	3	2	2.8	1.5	194.98	1736	5.2926
1709660161	0.57381	0.010351	3	2	3.3	2	180.57	1610	2.6564
318054260	2.6343	0.014155	2	2	16.7	16.7	26.718	234	42.578
1465648784	0.49577	0.022347	29	4	45	5.5	83.848	729	323.31
1503223927	1.5758	0.031307	27	0	29.3	0	91.189	792	18.873
1023043046	1.2906	0.03172	28	1	35.7	2.5	87.072	756	297.97
1520603690	0.9722	0.03427	24	0	38.5	0	72.473	657	323.31
1503271866	0.37367	0.041677	22	0	43.3	0	71.14	649	5.4671
1764621901	6.907	0.00026208	6	2	12.1	5.7	65.995	577	4.4756
1503263868	0.54022	0.049474	5	5	7.2	7.2	121.69	1070	8.2299
1042301809	1.2279	0.045653	7	5	22.9	17.6	37.992	340	31.127
1520502608	0.53498	0.036711	8	8	48.6	48.6	23.496	212	90.015
318853127	0.49409	0.0020973	2	1	5.8	2.2	36.609	313	3.0168
318068042	0.65145	0.020612	2	2	16.6	16.6	35.205	320	5.1888
1764634355	0.41191	0.014409	11	2	42.3	6.7	32.882	298	2.8092
1709639864	1.1666	0.031083	3	1	24.9	13.9	22.788	201	8.5529
1520591273	6.9265	0.00021236	2	1	10.6	6.7	47.743	451	10.077

Table S6: *Rhamdia quelen* male decreased proteins identification exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	Gene Symbol	Gene Name	UniProt Entry Name	UniProt Name
318066051	*acaa1*	acetyl-CoA acyltransferase 1	Q6GQN6 (Q6GQN6_DANRE)	Acaa1 protein
1552122390	*acox1*	acyl-CoA oxidase 1, palmitoyl	F1R071 (F1R071_DANRE)	Acyl-coenzyme A oxidase
576892917	*acox1*	acyl-CoA oxidase 1, palmitoyl	F1R071 (F1R071_DANRE)	Acyl-coenzyme A oxidase
1042332766	*apobb.1*	apolipoprotein Bb, tandem duplicate 1	Q5TZ29 (Q5TZ29_DANRE)	Apolipoprotein Bb, tandem duplicate 1
318037591	*atp5md*	ATP synthase membrane subunit DAPIT	H0WES8 (H0WES8_DANRE)	ATP synthase membrane subunit DAPIT
1042314542	*cirbpa*	cold inducible RNA binding protein a	Q566W6 (Q566W6_DANRE)	Cold-inducible RNA-binding protein a
1042304313	*dlat*	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	Q804C3 (Q804C3_DANRE)	Acetyltransferase component of pyruvate dehydrogenase complex
318065783	*eif3m*	eukaryotic translation initiation factor 3, subunit M	Q7T3B0 (EIF3M_DANRE)	Eukaryotic translation initiation factor 3 subunit M
1042364803	*eif5a*	eukaryotic translation initiation factor 5A	Q6NX89 (Q6NX89_DANRE)	Eukaryotic translation initiation factor 5A
68161035	*fth1a*	ferritin, heavy polypeptide 1a	Q9DDT0 (Q9DDT0_DANRE)	Ferritin
1764581560	*grhprb*	glyoxylate reductase/hydroxypyruvate reductase b	F1QYH7 (F1QYH7_DANRE)	Glyoxylate reductase/hydroxypyruvate reductase b
1503185578	*hdlbpa*	high density lipoprotein binding protein a	F1R9Y8 (F1R9Y8_DANRE)	High density lipoprotein-binding protein a
318102144	*hnmt*	histamine N-methyltransferase	Q6DC37 (HNMT_DANRE)	Histamine N-methyltransferase
1764609674	*hnrnpa1b*	heterogeneous nuclear ribonucleoprotein A1b	Q803K3 (Q803K3_DANRE)	Heterogeneous nuclear ribonucleoprotein A1
1764630626	*hsd17b4*	hydroxysteroid (17-beta) dehydrogenase 4	Q8AYH1 (Q8AYH1_DANRE)	17-beta-hydroxysteroid dehydrogenase type 4
1042371850	*hspa9*	heat shock protein 9	Q7ZYY3 (Q7ZYY3_DANRE)	Heat shock protein 9
1503198368	*hspa9*	heat shock protein 9	Q7ZYY3 (Q7ZYY3_DANRE)	Heat shock protein 9
1042352260	*mat1a*	methionine adenosyltransferase I, alpha	Q7ZW04 (Q7ZW04_DANRE)	S-adenosylmethionine synthase
1042292950	*naca*	nascent polypeptide associated complex subunit alpha	Q8JIU7 (NACA_DANRE)	Nascent polypeptide-associated complex subunit alpha
1042325542	*pabpc1b*	poly A binding protein, cytoplasmic 1 b	Q6P3L1 (Q6P3L1_DANRE)	Polyadenylate-binding protein
1709640083	*pdhb*	pyruvate dehydrogenase E1 subunit beta	Q7T368 (Q7T368_DANRE)	Pyruvate dehydrogenase E1 component subunit beta
1520500282	*phyhd1*	phytanoyl-CoA dioxygenase domain containing 1	Q5U3U0 (PHYD1_DANRE)	Phytanoyl-CoA dioxygenase domain-containing protein 1
1520522351	*rack1*	receptor for activated C kinase 1	O42248 (GBLP_DANRE)	Guanine nucleotide-binding protein subunit beta-2-like 1
1709639552	*rpl22*	ribosomal protein L22	F1QG80 (F1QG80_DANRE)	Ribosomal protein L22
82217274	*rpl36*	ribosomal protein L36	Q6Q415 (RL36_DANRE)	60S ribosomal protein L36
1764638019	*rpl4*	ribosomal protein L4	Q7ZW95 (Q7ZW95_DANRE)	Ribosomal protein L4
1520586328	*rplp1*	ribosomal protein, large, P1	Q6P5K5 (Q6P5K5_DANRE)	Ribosomal protein, large, P1
1503288287	*rplp2*	ribosomal protein, large P2	Q6PBJ9 (Q6PBJ9_DANRE)	Ribosomal protein, large P2
318056052	*rps10*	ribosomal protein S10	Q7T1J9 (Q7T1J9_DANRE)	Ribosomal protein S10
1042347515	*rps10*	ribosomal protein S10	Q7T1J9 (Q7T1J9_DANRE)	Ribosomal protein S10
54039448	*rps16*	ribosomal protein S16	Q1LWH1 (Q1LWH1_DANRE)	Ribosomal protein S16
1520534824	*scp2a*	sterol carrier protein 2a	Q6P4V5 (Q6P4V5_DANRE)	Sterol carrier protein 2
1764586305	*tuba8l2*	tubulin, alpha 8 like 2	Q6PC95 (Q6PC95_DANRE)	Tubulin alpha chain
1503205142	*uqcrc2b*	ubiquinol-cytochrome c reductase core protein 2b	Q6IQ59 (Q6IQ59_DANRE)	Ubiquinol-cytochrome c reductase core protein 2b
1520569133	*zgc:56585*	zgc:56585	F1QBQ6 (F1QBQ6_DANRE)	Zgc:56585

Table S7: *Rhamdia quelen* male decreased proteins information exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	log2(FC)	raw.pval	Peptides	Unique peptides	Sequence coverage [%]	Unique sequence coverage [%]	Mol. Weight [kDa]	Sequence length	Score
318066051	-1.2792	0.046936	3	2	13.6	11	43.768	418	20.328
1552122390	-1.0511	0.0066184	7	3	15.5	7.9	74.252	660	91.505
576892917	-0.82731	0.04113	9	4	20.5	10.3	74.079	660	35.144
1042332766	-7.5081	0.0096547	5	2	1.5	0.6	410.9	3727	11.381
318037591	-6.3067	0.0038874	2	2	56.1	56.1	6.2382	57	5.8968
1042314542	-0.55882	0.014978	3	3	33.1	33.1	17.321	166	15.531
1042304313	-7.4095	3.6035e-08	4	3	9	5.6	68.235	644	11.447
318065783	-2.7288	0.010223	2	2	10.1	10.1	42.442	375	3.0641
1042364803	-0.53979	0.028364	5	5	23.9	23.9	16.905	155	10.005
68161035	-9.5166	0.02688	3	2	20.3	16.4	20.821	177	26.921
1764581560	-0.86864	0.041401	9	9	22.8	22.8	37.726	347	36.227
1503185578	-1.165	0.0030615	15	1	18.3	1.2	135.37	1221	24.724
318102144	-3.4376	0.00012265	4	4	14	14	33.171	292	13.89
1764609674	-0.35148	0.0048965	3	3	8.6	8.6	38.614	372	18.384
1764630626	-0.70851	0.027428	7	3	14.5	5.7	78.052	723	51.198
1042371850	-0.29685	0.013041	12	1	22	1.8	73.618	678	75.808
1503198368	-0.29267	0.023518	21	1	41.3	2.3	61.108	574	226.44
1042352260	-0.91499	0.037621	9	5	26.6	13.4	39.328	357	24.908
1042292950	-0.47768	0.021069	4	4	25.8	25.8	23.307	213	44.209
1042325542	-0.40711	0.030289	9	9	19.6	19.6	70.854	633	80.177
1709640083	-0.45015	0.023101	6	2	23.9	8.4	39.275	356	27.331
1520500282	-2.6607	0.006405	3	2	10.9	6.6	28.844	256	7.1499
1520522351	-0.24326	0.038454	11	5	46.7	21.1	35.094	317	77.954
1709639552	-0.52079	0.0070413	3	2	38.5	18.5	15	130	16.505
82217274	-2.0791	0.03209	5	5	32.4	32.4	12.137	105	28.825
1764638019	-0.32125	0.032636	11	11	35.6	35.6	42.603	376	41.194
1520586328	-0.64829	0.032206	2	2	42.9	42.9	11.456	112	26.507
1503288287	-0.71416	0.013162	2	2	22.6	22.6	11.636	115	11.65
318056052	-0.20837	0.041199	7	7	47.6	47.6	18.853	166	41.052
1042347515	-0.20703	0.033953	8	1	25.8	7.1	34.42	326	33.93
54039448	-0.27488	0.037341	7	7	48.6	48.6	16.284	146	15.491
1520534824	-0.8154	0.0078516	8	4	23.4	12.8	57.818	538	36.951
1764586305	-0.5132	0.049293	19	4	57.5	12.7	50.149	449	41.903
1503205142	-2.3677	0.022328	5	2	18.8	10.2	47.653	451	32.463
1520569133	-0.47291	0.035937	3	3	12.8	12.8	28.532	265	19.001

Table S8: *Rhamdia quelen* male increased proteins identification exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	Gene Symbol	Gene Name	UniProt Entry Name	UniProt Name
1709638785	*agmat*	agmatine ureohydrolase (agmatinase)	F1QJZ3 (F1QJZ3_DANRE)	Agmatine ureohydrolase (agmatinase)
1503191056	*aldh9a1a.1*	aldehyde dehydrogenase 9 family, member A1a, tandem duplicate 1	Q7ZVB2 (A9A1A_DANRE)	4-trimethylaminobutyraldehyde dehydrogenase A
576888011	*npepl1*	aminopeptidase like 1	F1Q6S1 (F1Q6S1_DANRE)	Aminopeptidase-like 1
1520548750	*bcat1*	branched chain amino-acid transaminase 1, cytosolic	Q5RHB8 (Q5RHB8_DANRE)	Branched-chain-amino-acid aminotransferase
1520609711	*ctsc*	cathepsin C	Q6P2V1 (Q6P2V1_DANRE)	Cathepsin C
21552717	*ctsd*	cathepsin D	Q8AWD9 (Q8AWD9_DANRE)	Cathepsin D
1764639828	*col6a3*	collagen, type VI, alpha 3	F1QKE8 (F1QKE8_DANRE)	Collagen, type VI, alpha 3
1042324110	*eppk1*	epiplakin 1	I3ISA6 (I3ISA6_DANRE)	Epiplakin 1
1709641367	*gbe1b*	glucan (1,4-alpha-), branching enzyme 1b	A0A0R4IN76 (A0A0R4IN76_DANRE)	Glucan (1,4-alpha-),-branching enzyme 1b
1709634328	*got1*	glutamic-oxaloacetic transaminase 1, soluble	Q7ZUW8 (Q7ZUW8_DANRE)	Aspartate aminotransferase
1503277546	*gnmt*	glycine N-methyltransferase	Q6P607 (Q6P607_DANRE)	Glycine N-methyltransferase
576887855	*gnmt*	glycine N-methyltransferase	Q6P607 (Q6P607_DANRE)	Glycine N-methyltransferase
1520566949	*hbaa1*	hemoglobin, alpha adult 1	Q90487 (HBA_DANRE)	Hemoglobin subunit alpha
1764639993	*hpxa*	hemopexin a	HEMO_DANRE	Hemopexin
1764602676	*hist1h2a4*	histone cluster 1 H2A family member 4	Q4FZZ7 (Q4FZZ7_DANRE)	Histone H2A
1764617054	*hagh*	hydroxyacylglutathione hydrolase	Q6P963 (GLO2_DANRE)	Hydroxyacylglutathione hydrolase, mitochondrial
1764613484	*lgals9l1*	lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1	Q6PBV2 (Q6PBV2_DANRE)	Galectin
1042336943	*mdh1aa*	malate dehydrogenase 1Aa, NAD (soluble)	Q7T3D9 (Q7T3D9_DANRE)	Malate dehydrogenase
1520579773	*mthfd1b*	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1b	Q7ZVV5 (Q7ZVV5_DANRE)	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent)
1042363791	*mao*	monoamine oxidase	Q6NSN2 (AOF_DANRE)	Amine oxidase [flavin-containing]
1520584693	*pygl*	phosphorylase, glycogen, liver	Q5RKM9 (Q5RKM9_DANRE)	Alpha-1,4 glucan phosphorylase
1042301809	*pipox*	pipecolic acid oxidase	F6P928 (F6P928_DANRE)	Pipecolic acid oxidase
591290568	*rab8a*	RAB8A, member RAS oncogene family	A4FVK4 (A4FVK4_DANRE)	RAB8A, member RAS oncogene family
576886406	*sdr16c5b*	short chain dehydrogenase/reductase family 16C, member 5b	Q7SZ49 (Q7SZ49_DANRE)	Short chain dehydrogenase/reductase family 16C, member 5
576892999	*tpm3*	tropomyosin 3	Q6P0W3 (Q6P0W3_DANRE)	Tropomyosin 3
1709638704	*upp2*	uridine phosphorylase 2	B0S6V8 (B0S6V8_DANRE)	Uridine phosphorylase

Table S9: *Rhamdia quelen* male increased proteins information exposed to a temperature increase (from 25 to 30 ºC) for 96h.

ID	log2(FC)	raw.pval	Peptides	Unique peptides	Sequence coverage [%]	Unique sequence coverage [%]	Mol. Weight [kDa]	Sequence length	Score
1709638785	0.40772	0.010617	8	8	16.4	16.4	37.171	342	19.563
1503191056	0.40572	0.0021426	15	3	40.2	5.1	55.167	508	323.31
576888011	6.4458	2.8566e-05	2	2	4.6	4.6	55.852	524	6.6996
1520548750	0.6155	0.042075	6	2	22.2	9.9	46.527	414	34.6
1520609711	8.0593	0.0065927	2	2	4.2	4.2	50.726	452	4.6277
21552717	0.3391	0.050355	4	4	16.5	16.5	43.03	395	27.979
1764639828	9.1375	0.026105	6	1	2.7	0.4	291.83	2692	4.7972
1042324110	0.57108	0.047469	6	3	20	9.5	103.47	931	149.05
1709641367	2.4376	0.015461	10	2	22.2	7.6	73.321	632	9.5859
1709634328	0.90755	0.011957	11	0	29	0	45.899	411	210.23
1503277546	0.50049	0.023046	6	3	24.7	13.2	33.327	295	22.401
576887855	0.57523	0.0030646	5	2	18	6.4	33.118	295	12.627
1520566949	0.86677	0.038719	4	2	39.2	26.6	15.849	143	14.593
1764639993	0.81279	0.030919	6	3	13.6	7.6	56.39	500	68.691
1764602676	0.39871	0.048017	10	4	39.5	14.3	28.394	258	112.92
1764617054	1.9291	0.045573	4	4	13.7	13.7	34.159	307	4.5788
1764613484	6.8035	0.0050467	2	2	7.4	7.4	34.874	312	3.7601
1042336943	0.44066	0.046175	13	7	39.3	26.7	36.159	333	119.35
1520579773	6.6031	0.0057355	17	2	28.5	1.3	100.64	934	3.5114
1042363791	0.42783	0.015286	8	8	23	23	58.571	522	47.634
1520584693	2.5135	0.011476	28	2	35.9	2.7	98.05	853	6.3025
1042301809	1.7484	0.049973	7	5	22.9	17.6	37.992	340	43.725
591290568	2.1854	0.035073	2	2	12.6	12.6	23.582	207	23.311
576886406	0.6924	0.036592	6	6	20.3	20.3	33.721	306	73.494
576892999	6.3481	9.6046e-06	3	3	15.7	15.7	28.678	248	22.364
1709638704	0.39695	0.029442	7	2	31.2	10.4	35.618	317	18.033

CAPÍTULO 3

A temperatura pode influenciar os efeitos tóxicos causados por pelo extrato bruto de cianobactéria em um peixe neotropical?

“Can temperature influence the toxic effects caused by cyanobacteria crude extract in a Neotropical catfish?”, a ser submetido para Environmental Toxicology and Chemistry

Can temperature influence the toxic effects caused by cyanobacteria crude extract in a Neotropical catfish?

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Abstract

Potentially toxic cyanobacteria blooms have become a problem in public water supply reservoirs. Temperature rise can increase the blooms frequency and intensity, which may influence the cyanotoxins amount in the environment. The aim of study was to evaluate Neotropical catfish responses to the exposure to neurotoxins produced by *Raphidiopsis raciborskii* (T3 strain), at different temperatures scenarios. Juveniles of *Rhamdia quelen* were exposed to four treatments, based on literature data: control at 25°C (C25), control at 30°C (C30), crude extract equivalent to 10^5 cells/mL of *R. raciborskii* at 25°C (CE25) and crude extract equivalent to 10^5 cells/mL at 30°C (CE30). After 96 hours, the animals were anesthetized and blood collected. After euthanasia, the gill, posterior kidney, brain, muscle, liver and gonad were collected for biomarker analysis (hematological, biochemical, genotoxic and histopathological). Water samples were collected at the beginning and at the end of the experiment for neurotoxin quantification. Different parameters between males and females were altered by saxitoxins at 25°C, such as glucose. Different parameters were changed on this global warming scenario (30 °C), such as genotoxic damage in different female tissues, in addition to the activation of the glutathione pathway for saxitoxin metabolism in females that was not observed at 25°C. The results demonstrated that the increase in temperature is able to aggravate the toxicity of cyanotoxins produced by *R. raciborskii* in female fishes.

Key words: Global warming, saxitoxins, toxicology, *Rhamdia quelen*

1. Introduction

Degradation of environmental resources, such as water, has been intensified as a human expansion result, whether through pollution or climate change. Water pollution occurs through xenobiotics and excess nutrients, the latter being

responsible for eutrophication. This process can be aggravated by climate change intensification, since the temperature increase can accentuate the thermal stratification and the nutrients entry in water bodies (Fonseca, 2012; Wells et al., 2015).

The eutrophication and temperature increase processes in continental waters favor the cyanobacterial blooms, such as occurrence and intensity (Manning & Nobles, 2017). This is a serious problem in reservoirs for water supply, since cyanobacteria are microorganisms capable of producing toxins, called cyanotoxins (Azevedo, 1998). Saxitoxins are an example of this toxin.

Saxitoxins are neurotoxins also known as paralytic shellfish toxins (PSTs) because they are associated with seafood. They are produced by different genera of cyanobacteria and dinoflagellates and have the property of binding, among others, in the axon membranes sodium channels (Mantovani et al., 2011). There are many variants of saxitoxins, including neosaxitoxins and goniautoxins, which differ in toxicity and the sulfates presence (O'Neill et al., 2016). In reservoirs in Paraná, there are blooms reports of *Raphidiopsis raciborskii* (also called *Cylindrospermopsis raciborskii*), producer of some of these variants (Calado et al., 2020).

Brazilian law, through ordinance 888/2021, establishes the limit of 20,000 cyanobacteria cells.mL^{-1}, however concentrations 50 times above this value have already been found in reservoirs, with accumulation of toxins in the fish muscle (Calado et al, 2017). Thus, fish are organisms that can be used as models of toxicity caused by cyanotoxins because they are found in these reservoirs in large quantities.

Rhamdia quelen is a catfish species with commercial value, popularly known as jundiá, found in South America (Gomes et al., 2000). In these organisms, biomarkers can be applied, which are the quantifiable responses to environmental degradation. Examples are biochemical, genotoxic, hematological and histopathological biomarkers. The response at these lower biological levels, it is possible to infer about the effects of toxic substances at higher biological levels (Snape et al., 2004). In this way, damage at the level of a cell or tissue can be used to understand how adverse conditions can affect an organism and how it can result in further problems at the population and community levels.

Most of the toxicological data described for saxitoxins was obtained from experiments with marine organisms, having limited information about saxitoxins in freshwater organisms (Žegura et al., 2011; Li & Persson, 2021). Furthermore, studies

relating temperature to cyanotoxins effects are needed to assess how global warming can influence fish responses contaminants present in the environment. Therefore, the aim of the present study is to evaluate the catfish Neotropical species environmental contamination biomarkers responses after exposure to *Raphidiopsis raciborskii* (*Cylindrospermopsis raciborskii*) crude extract, at different temperature scenarios.

2. Material and methods

2.1. Cyanobacteria culture and extract production

Raphidiopsis raciborskii (*Cylindrospermopsis raciborskii*) T3 strain, obtained from the Federal University of Rio de Janeiro, was grown in ASM-1 medium, pH 8, under controlled conditions of light (16 h light/8 h dark) and temperature (26 ± 1 °C).

After reaching a high cell density, a culture sample was stored in acetic lugol for later cell counting. The culture remainder was centrifuged at 3500 *x*g, at 8 ºC for 10 minutes and then the culture medium was removed from the supernatant. The cyanobacteria were resuspended in milliQ water, and went through the freezing and thawing process so that the cells were lysed and the intracellular toxins released, a fact confirmed by further analysis under a microscope. This crude extract was stored in amber flasks at -20 °C, while an extract sample was lyophilized for quantification of PSTs.

2.2. Animal acclimation and bioassay

During 30 days, males and females of *Rhamdia quelen* (120 juveniles, 12.48 ± 1.04 cm e 15.75 ± 4.25 g), from Western Paraná State University, were acclimatized at the Federal University of Paraná. The project was approved for Federal University of Paraná Animal Use Ethics Committee (CEUA, n° 1140). This acclimation occurred in tanks containing filtered water, controlled photoperiod (12h) and constant aeration. After this period, fish are divided at two groups and each one were acclimated at bioassay temperature (25 and 30 °C) during 7 days.

For the experiment, two cyanobacteria conditions (0 and 10^5 cells.mL^{-1}) were tested at two temperatures (25 °C and 30 °C) totaling four exposure conditions:

filtered water at 25 °C (C25), filtered water at 30 °C (C30), exposure to cyanobacteria extract equivalent of 10^5 cells.mL^{-1} at 25 °C (CE25) and exposure to cyanobacteria extract equivalent of 10^5 cells.mL^{-1} at 30 °C (CE30). The temperatures were chosen based on the ideal temperature for the fish species (23 °C) and the cyanobacteria (29 °C), respecting the thermal amplitude of each one, as well as simulating an increase in the water temperature (Saker and Griffiths, 2000; Montanha et al., 2011; Artaxo, 2014). The cyanobacteria concentration was based on blooms occurred in regional water supply reservoirs, as Alagados Reservoir (Calado et al., 2020).

Three 50 L aquariums containing 10 fish in each were used for each treatment, totaling 30 animals per experimental group. As in acclimation, the experiment took place under controlled conditions of photoperiod, aeration and daily feeding (Laguna® Brazilian Fish 32). After 96h, the animals were anesthetized and the blood was collected through the tail vein with heparinized syringes. After euthanasia by medullary section the brain, liver, gonad, posterior kidney, gills and muscle were taken. Liver, gonad and total weights were measured to calculate somatic index. In the blood, hematological, biochemical and genotoxic biomarkers were analyzed. The plasma, obtained by blood centrifugation at 2000 xg for 5 minutes was used for biochemical biomarkers analysis. The brain was collected for biochemical biomarkers; liver for biochemical, genotoxic and histopathological biomarkers; posterior kidney and gills for biochemical, genotoxic and histopathological biomarkers and muscle for biochemical biomarkers analysis and gonad for sex identification.

Physical-chemical parameters, such as temperature, pH, dissolved oxygen, ammonia and nitrite, were monitored. For the PSTs quantification (saxitoxins and some of their variants), water samples were collected at the beginning and at the end of the experiment.

2.3. STX quantification

For STX quantification, a High Efficiency Liquid Chromatography (HPLC) is used and process followed the method proposed by Oshima (1995). The water samples of 0 and 96 h were lyophilized, resuspended in acetic acid (0.5 N), under agitation for 2 hours, purified in the C18 cartridge and lyophilized. This material was resuspended in 1 ml of acetic acid (0.5 N). These samples were prepared to quantify as saxitoxins

(STX), neosaxitoxins (neoSTX) and decarbamoilsaxitoxins (dcSTX), the analogs produced by T3 strain.

2.4. Hepatosomatic and gonadosomatic index

The equation (liver or gonad weight / body weight) x 100 was used to calculate the hepatosomatic (HSI) and gonadosomatic (GSI) index.

2.5. Hematological biomarkers

Hematological profile was obtained by erythrocyte number count using the Formol-Citrate method (Oliveira Junior et al., 2009) and the leukocyte and thrombocyte number count in a blood smear (Tavares-Dias et al., 1999, 2000).

2.6. Biochemical biomarkers

On the blood, glucose levels were measured with a portable glucometer (Accu-Check Performace). On the plasm, lactate levels was measured using a commercial Kit (Labtest, Brazil).

Muscle and brain were homogenized in potassium phosphate buffer (0.1 M, pH 7.5) in 1:10 (m/v) and centrifuged for 20 minutes, at 12000 xg, 4 °C. For muscle, the supernatant was used for acetylcholinesterase activity (AChE; Ellman et al., 1961 modified to microplate by Silva de Assis, 1998) and the brain for AChE activity, lipoperoxidation (LPO; Jiang et al., 1992) and carbonylated proteins concentrations (PCO; Levine et al., 1994). Liver, posterior kidney and gill was homogenized in potassium phosphate buffer (0.1 M, pH 7.0) in the proportion of 1:10; 1:10 and 1:5 (m/v), respectively. This material was centrifuged for 30 minutes, at 15000 xg, 4 °C and the supernatant was separate for glutathione S-transferase activity (GST; Keen et al., 1976), superoxide dismutase activity (SOD; Gao et al., 1998), catalase activity (CAT; Aebi, 1984), glutathione peroxidase activity (GPx; Hafeman et al., 1974), non-protein thiols concentration (GSH; Sedlak & Lindsay, 1968) and LPO. Carbonic anhydrase was measured in kidney and gill after homogenization phosphate buffer 10% (10 mM, pH 7.4), as Vitale et al. (1999). For all the samples, total protein was quantified using the method described by Bradford (1976).

2.7. Genotoxicity biomarkers

Micronuclei and nuclear abnormalities were analyzed 2000 erythrocytes for each animal after a blood smear stained with Giemsa 10% (Hooftman & De Raat, 1982; Carrasco et al., 1990). Comet assay was performing accord to Speit and Hartmann (2005) modified by Ramsdorf et al. (2009) to identified DNA breaks. The blood (10 µL) was placed into 500 µL of fetal bovine serum. Into 250 µL of fetal bovine serum, 10 mg of liver and posterior kidney and one gill arch was placed. After blades preparation, 100 nucleoids were analyzed based on undamaged to maximally damaged (Collins et al. 1995). This technique not consider the nucleoids with small (or non-existent) visible head and large diffuse tails.

2.8. Histopathogical biomarkers

Tissues fragments (gonad, liver, posterior kidney and gill) were fixed in ALFAC solution (80% alcohol, formaldehyde and glacial acetic acid). After samples dehydrated, diaphanized and included in Paraplast®, histological sections were stained with hematoxylin-eosin (HE). Gonadal slides were used to define the animal's sex. Liver, posterior kidney and gill slides were analyzed to determine the injury index, from values for different types of histological alterations, proposed by Bernet et al. (1999).

2.9. Data analysis

To test the assumption of homoscedasticity and normality, Levene and Shapiro-Wilk tests were used. According to these assumptions, data were submitted to the *t* test or Mann Whitney U-test, with $p \leq 0.05$. In this study was analyzed two thermal situations: C25 x STX25, simulating a *R. raciborskii* bloom at 25°C and C30 x STX30, simulating a *R. raciborskii* bloom at 30°C, assuming that 30°C can be a global warming scenario. The responses of these two situations were comparated. Non-Metric Multidimensional Scaling (nMDS) was performed to visualize the group distribution. These analyzes were performed in an R environment.

3. Results

3.1. Water parameters

The water physical-chemical parameters did not change during the experiment and the temperatures were close to which was initially established (Table S1).

3.2. STX quantification

In the crude extract, three analogs (STX, neoSTX and dcSTX) were analyzed. Only two of them was quantified, the neoSTX (18.61 $\mu g.L^{-1}$) and dcSTX (1.81 $\mu g.L^{-1}$). In the initial time of the experiment, the presence of the three analogs was not detected in any treatment. At the end of the experiment, only neoSTX (4.99 $\mu g.L^{-1}$) was found at group exposed to crude extract to 25 °C and 5.06 $\mu g.L^{-1}$ at group exposed to crude extract to 30 °C.

3.3. Hepatosomatic and gonadosomatic index

The hepatosomatic index did not change (Figures 1A and 1B). However, the gonasomatic index increased in females exposed to crude cyanobacteria extract at 25 °C (Figure 1C).

3.4. Hematological biomarkers

It was not observed any change in the hematological parameters, such as numbers of erythrocytes, leukocytes and thrombocytes (Table 1).

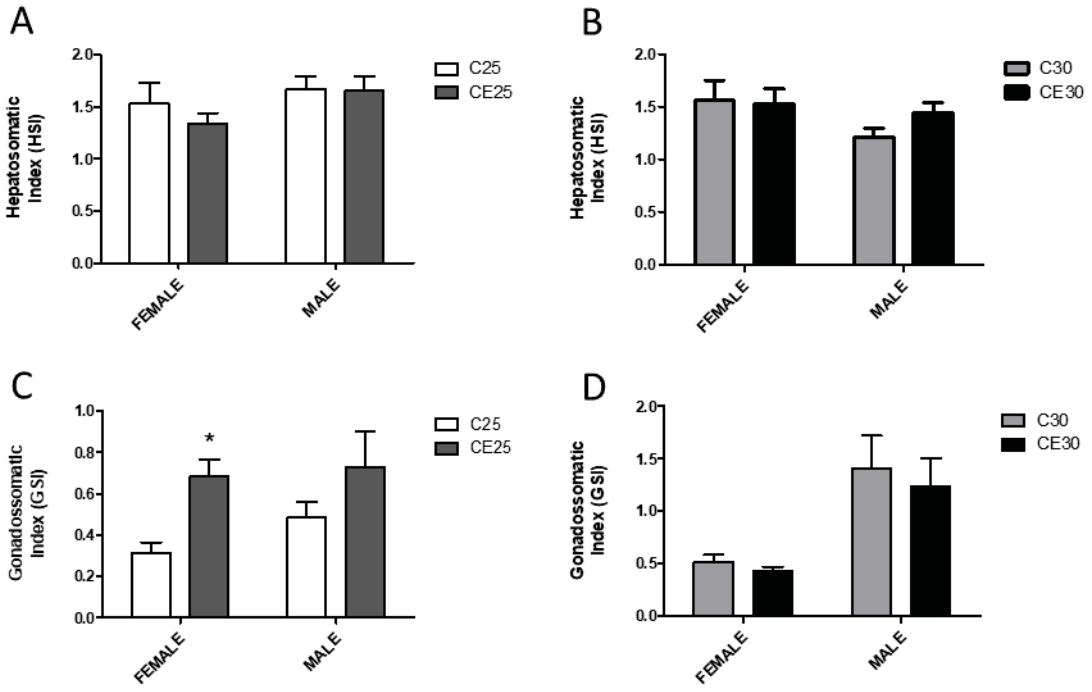

FIGURE 1: Hepatic (A and B) and gonadossomatic (C and D) indexes, in *Rhamdia quelen* males and females in exposure to crude cyanobacteria extract at 25°C (A and C) and 30°C (B and D). * representing significant difference by *t* test.

TABLE 1: Hematological parameters on *Rhamdia quelen* exposed to saxitoxins at 25 °C and 30 °C, separated by sex.

Biomarker	Sex	C25	CE25	C30	CE30
Erythrocytes	Female	2.18 ± 0.24	2.17 ± 0.61	2.17 ± 0.66	2.03 ± 0.41
	Male	2.10 ± 0.62	2.02 ± 0.75	2.17 ± 0.50	1.78 ± 0.51
Leukocytes	Female	5.23 ± 2.10	3.29 ± 1.45	2.59 ± 0.78	2.78 ± 0.52
	Male	4.29 ± 1.94	3.41 ± 1.23	3.39 ± 1.13	2.43 ± 0.89
Thrombocytes	Female	6.63 ± 1.56	7.43 ± 2.88	4.52 ± 2.57	4.82 ± 2.59
	Male	5.55 ± 2.75	4.70 ± 1.77	5.89 ± 2.26	4.30 ± 1.64

Data represented as mean ± standard deviation, with the following units: erythrocytes number.10^6.μL^{-1}; leukocytes number.μL^{-1}; thrombocytes number.μL^{-1}. There is no significant difference by *t* test.

3.5. Biochemical biomarkers

Blood glucose increased only in females exposed to crude cyanobacteria extract at 25 °C. The plasma lactate increased only in males, in both temperature conditions. The muscle acetylcholinesterase activity was not affected. In the brain, the enzyme activity was also not altered, as well as the lipid peroxidation concentration. However, the carbonylated proteins concentration in the female brain exposed to saxitoxin at 25 °C was increased, while in males it was reduced by 30 °C. The hepatic

biochemical biomarkers were not affected in male. Therefore, in females, GST activity and GSH and LPO concentrations were increased at 30 °C. In kidney, a decreased SOD activity in exposure to saxitoxins at 30 °C was observed males and females, without effects on the others enzymes activities tested or lipoperoxidation concentration. In female gills, GPx increased in exposure to crude cyanobacteria extract at 25 °C, with a reduction of lipoperoxidation. In male gills, GPx increased in exposure to saxitoxins at 25 °C, without lipoperoxitation alteration. The female gill carbonic anhydrase activity decreased after the exposure to crude cyanobacteria extract at 25°C, with no alteration at 30°C, but in males, an increase was observed (Table 2).

TABLE 2: Biochemical biomarkers of *Rhamdia quelen* exposed to crude cyanobacteria extract comparisons at 25 °C and 30 °C and separated per sex.

Tissue	Biomarker	Female		Male	
		C25 x CE25	C30 x CE30	C25 x CE25	C30 x CE30
Blood	Glucose	I	NA	NA	NA
	Lactate	NA	NA	I	I
Muscle	AChE	NA	NA	NA	NA
Brain	AChE	NA	NA	NA	NA
	LPO	NA	NA	NA	NA
	PCO	I	NA	NA	D
Liver	GST	NA	I	NA	NA
	SOD	NA	NA	NA	NA
	CAT	NA	NA	NA	NA
	GPx	NA	NA	NA	NA
	GSH	NA	I	NA	NA
	LPO	NA	I	NA	NA
Kidney	GST	NA	NA	NA	NA
	SOD	NA	D	NA	D
	CAT	NA	NA	NA	NA
	GPx	NA	NA	NA	NA
	LPO	NA	NA	NA	NA
	CA	NA	NA	NA	NA
Gill	GST	NA	NA	NA	NA
	SOD	NA	NA	NA	NA
	CAT	NA	NA	NA	NA
	GPx	I	NA	I	NA
	LPO	D	NA	NA	NA
	CA	D	NA	I	NA

I: increase; D: decreased; NA: no alteration. Results based on t test.

3.6. Genotoxicity biomarkers

In females blood nuclear morphological abnormalities increased in saxitoxins at 25 °C (Figure 2A-B). In the blood, an increase of DNA damage was also observed in all tested conditions (Figure 2C-D). Liver, kidney and gills showed the same behavior, with increased DNA damage in females only at 30 °C and in males at both temperatures (Figure 3).

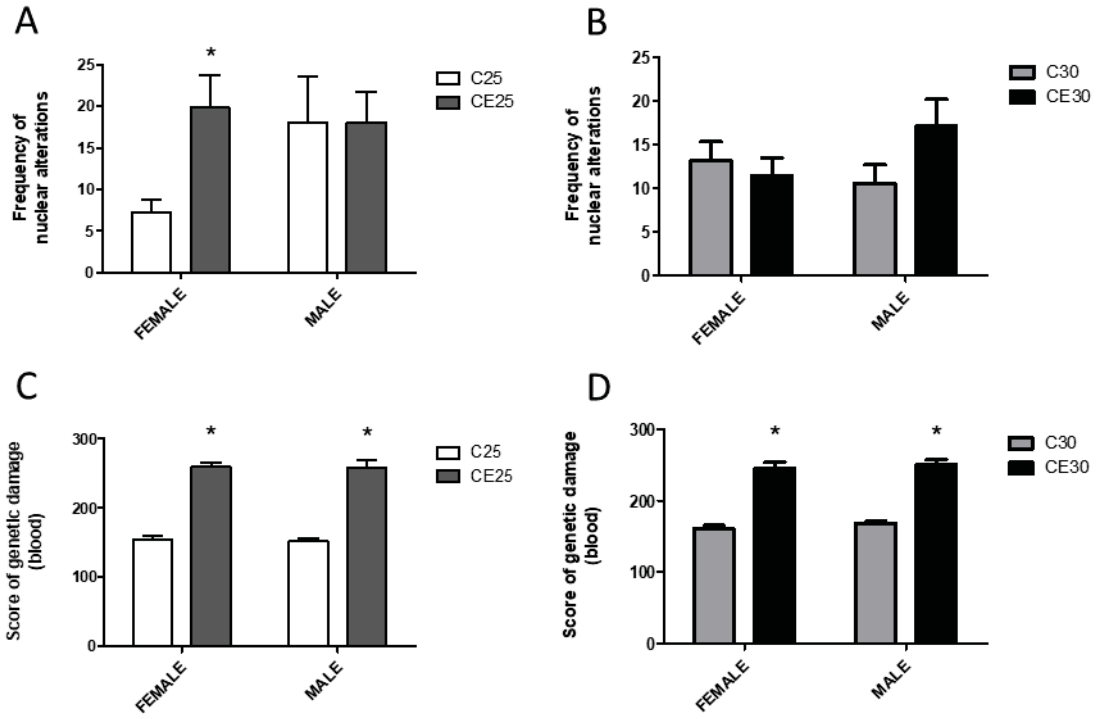

FIGURE 2: Blood genotoxic biomarkers in *Rhamdia quelen* males and females in exposure to crude cyanobacteria extract at 25 and 30 °C. * representing significant difference by *t* test. A-B: Nuclear Morphological Abnormalities, C-D: Blood DNA damage.

3.7. Histopathological biomarkers

An injury index increased in liver and gill to exposure to crude cyanobacteria extract at 25°C and 30°C (Figure S1). *R. raciborskii* crude extract caused necrosis, leukocyte infiltration and sinusoids dilation in liver, and hyperplasia of the epithelial tissue and aneurysm in gill (Figures 4). In kidney, an injury index increased in females for the both temperature conditions of crude cyanobacteria extract exposure, with some leukocyte infiltration at these conditions. In males, this occurred just at 25°C (Figure S1).

3.8. Multivariate analysis

The nMDS analysis showed differentiation between sex and toxicity differentiation with increasing temperature, especially in females (Figure 5).

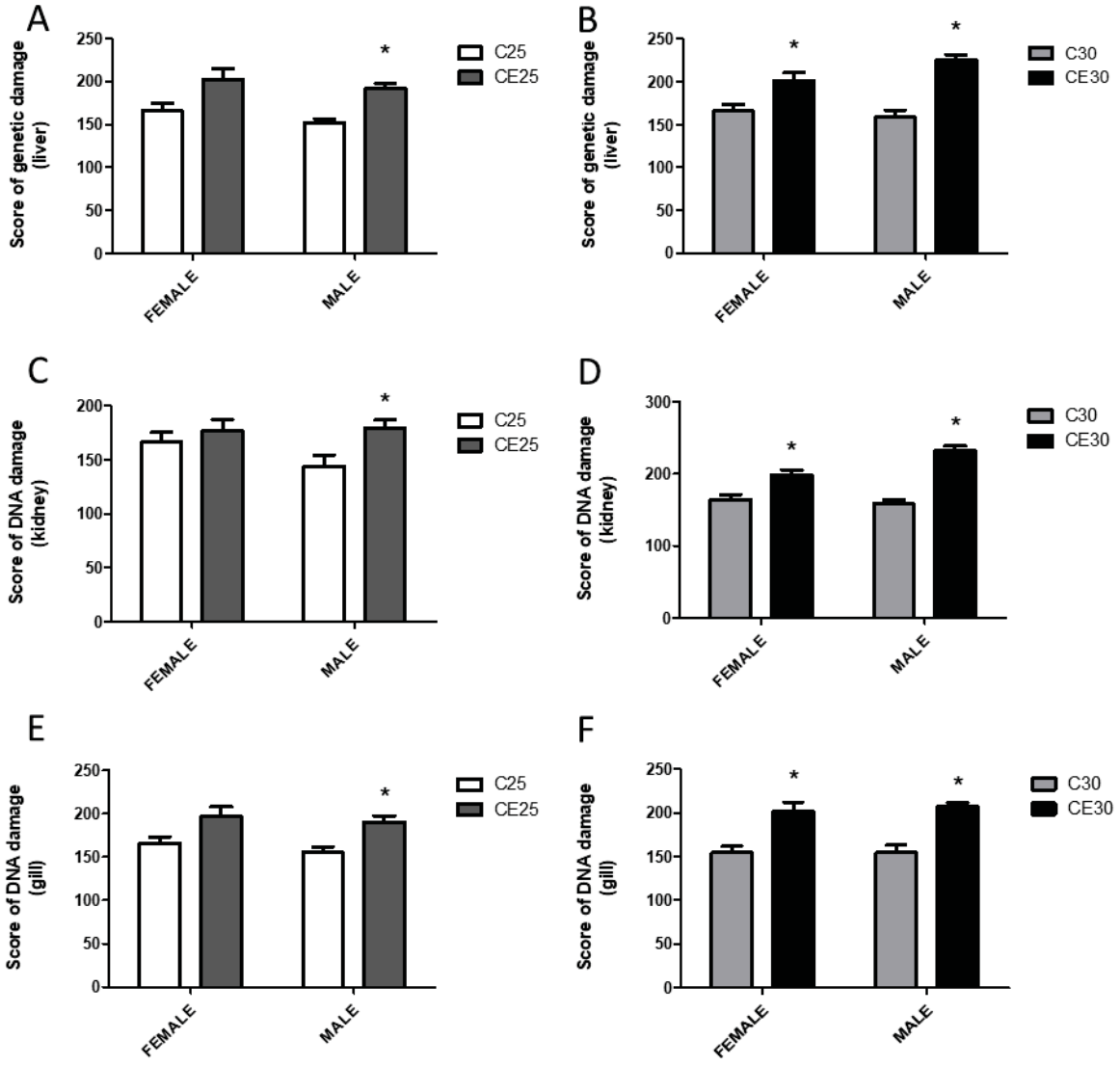

FIGURE 3: Genotoxic biomarkers in *Rhamdia quelen* males and females in exposure to crude cyanobacteria extract at 25 and 30°C. * representing significant difference by *t* test. A-B: liver, C-D: kidney, E-F: gill.

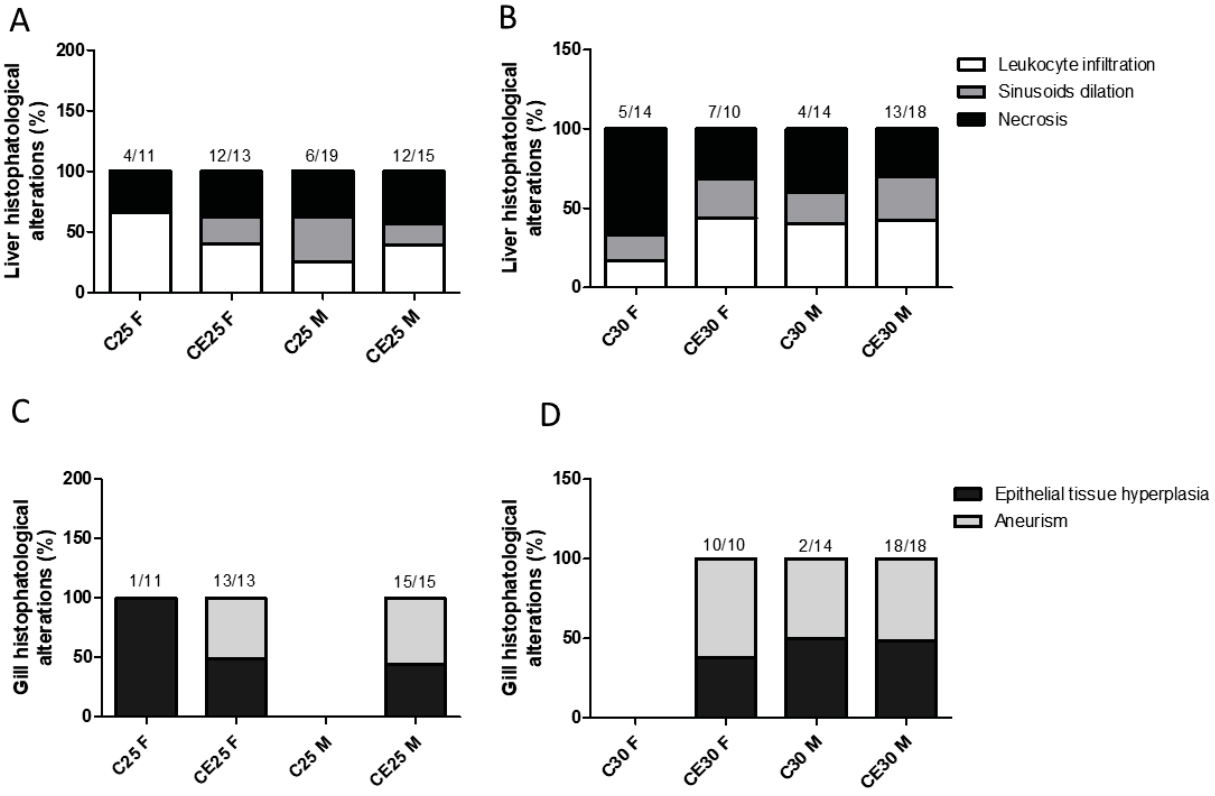

FIGURE 4: Histopathological alterations graphical representation in *Rhamdia quelen* males and females in exposure to crude cyanobacteria extract at 25 and 30°C. A: Liver histopathological types at 25°C, B: Liver histopathological types at 30°C, C: Gill histopathological types at 25°C, D: Gill histopathological types at 30°C. The number above the bars represents how many animals in the total group had some type of alteration.

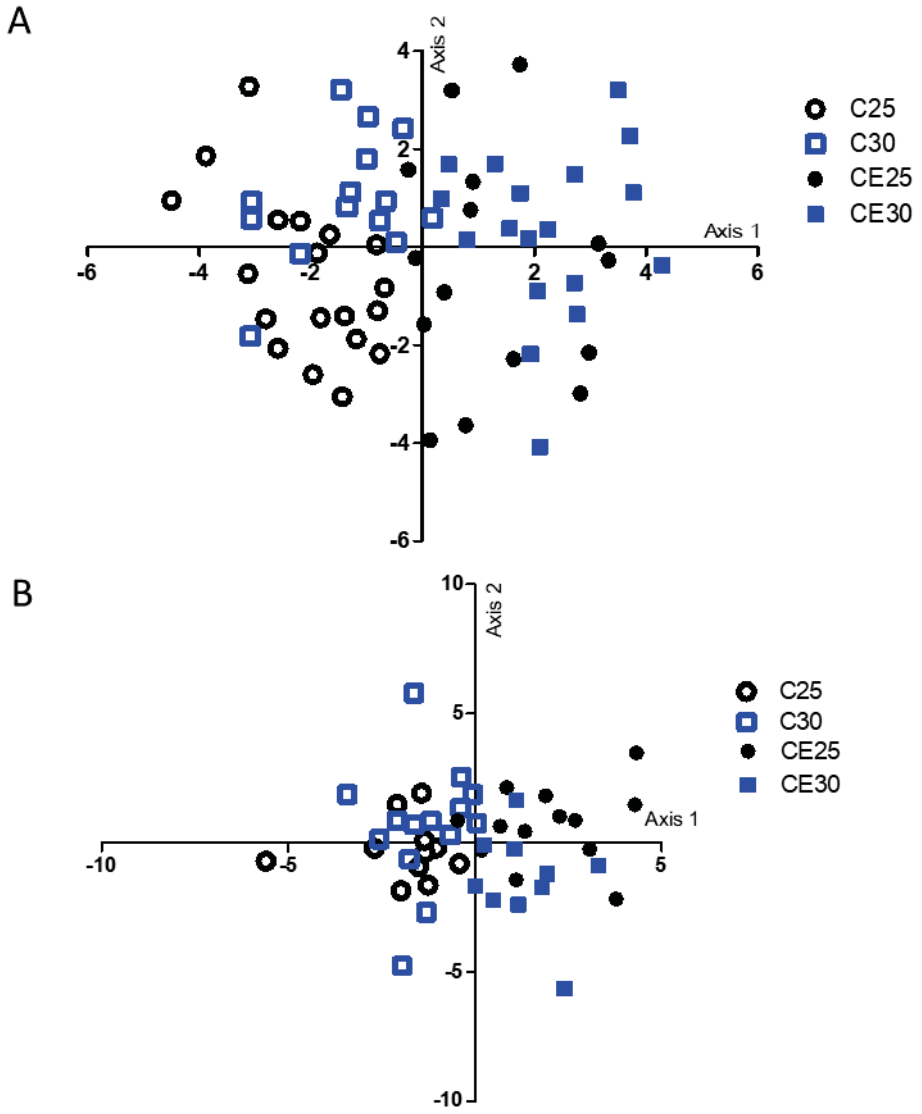

FIGURE 5: nMDS representation for female (A) and male (B) *Rhamdia quelen* exposure to crude cyanobacteria extract at 25 and 30°C, with all biomarkers compiled in two dimensions, with the coordinates representing the analysis scores.

4. Discussion

STX quantification

Raphidiopsis raciborskii (*Cylindrospermopsis raciborskii*) T3 strain, the cyanobacterium used in this study has already been described as producing the STX, NeoSTX and dcNeoSTX variants (Soto-Liebe et al., 2010; Da Costa et al., 2013), in addition to also producing other substances, such as cyanopeptides (Silva-Stenico et al., 2011). At the beginning of the experiment, the neoSTX and dcSTX variants were not found. However, NeoSTX high concentrations were found at the

end of the experiment. The detection limit for the STX variant in this method is usually high, so this variant can be converted to NeoSTX, justifying the detection only at the end of the experiment (Guéguen et al., 2011; Calado et al., 2019). The NeoSTX variant is still considered one of the most toxic, with a relative toxicity factor similar to that of STX (Munday et al., 2013).

Saxitoxins are known to be stable and remain in the aquatic environment for long-term (Malik et al., 2020). Even after the boiling of contaminated animals, they are still present (Kaur, 2019). In this study, the STX concentrations remained close in the two thermal conditions tested, showing that even with the imminent water warming, they still persist in the environment. Similar STX concentrations have already been found in the summer period in public water supply reservoirs, where temperatures were around 25°C, with toxic effects in fish (Calado et al., 2017).

Crude cyanobacteria extract effects: 25 °C

Considering the 25°C scenario, crude cyanobacteria extract were able to cause different effects on catfish, even differently between the sex of the animals. Females, for example, have an increase in GSI, commonly used to determine the organism reproductive status (DeGrasse et al., 2014). A higher GSI may indicate an earlier reproductive period. Thus, despite the few studies dealing with the cyanobacteria extract reproductive effects, this study shows signs that an alteration in the reproductive cycle can occur.

Different biochemical biomarker responses were observed in the organisms exposed to crude cyanobacteria extract. Glucose and lactate are normally used as indicators of energy metabolism in organisms, because high levels of glucose and lactate suggest a change in metabolism caused by the oxygen reduction (Coates & Soderhall, 2020). *R. raciborskii* extract seem increasing the aerobic metabolism or energy mobilization, by increasing glucose in females and lactate in males.

Saxitoxins, the cyanotoxin produced by *R. raciborskii*, are known for their neurotoxic effect (Aráoz et al., 2010). This toxin can cross the brain barrier and cause alterations in the fish brain, as observed in in vitro studies (Silva et al., 2014). Females in this study showed an increase in carbonyl proteins. They are proteins marked for proteolysis, due to the oxidative stress. However, some can escape of this degradation and form high molecular weight agglomerates, which are cytotoxic

(Nyström, 2005). This is a possible explanation for the cerebral cytotoxicity in females.

Saxitoxin toxicity were found in different fish tissues such as the liver, posterior kidney and gills (Bakke & Hosberg, 2010). We found no biochemical changes in males and females exposed to crude cyanobacteria extract contain saxitoxins at 25°C in liver and posterior kidney. However, the gills, the first organ in contact with chemical water exposure, presented some alterations. In female gills, an increase in GPx activity, a reduction in carbonic anhydrase activity and a decrease in LPO were observed. In males, increased GPx and carbonic anhydrase activity was observed. The induction of enzymes of the antioxidant system, such as GPx, indicates a defense to reactive oxygen species resulting from the STX presence (Braga et al., 2020). In this way, oxidative damage such as LPO can be reduced or not altered, as shown for female and male gills, respectively.

Differently from the antioxidant system, gill carbonic anhydrase (CA) presented different behavior between sexes when exposed to crude cyanobacteria extract at 25°C. This enzyme is important ion and acid base-balance and represents an important toxicant exposure biomarker in fish gills (Lionetto et al., 2012). Female gills presented a decrease in CA showing an enzyme downregulation by crude cyanobacteria extract. This reduction was also observed in *Geophagus brasiliensis* exposed to other cyanotoxins type, the microcystins (Calado et al., 2018). On the other hand, the higher CA activity in males exposed to STX at 25°C could be a compensation for osmoregulatory, respiration, and/or acid-base (acidosis prevention) disturbances (Henry & Swenson, 2000; Perry & Gilmour, 2006) caused by contaminants in water (Freire et al., 2015). This increase may be related to an increase in plasma lactate in these males, indicating a reduction in O_2 and an increase in CO_2, which leads to greater anhydrase activity.

In addition to causing biochemical damage, saxitoxins have also been described as genotoxic. In females, crude cyanobacteria extract contain saxitoxins were able to cause nuclear morphological abnormalities. This induction of erythrocyte nuclear morphological abnormalities has already been observed in marine fish *Diplodus sargo*, after injection of 1.60 µg equiSTX/kg purified of *Gynodimium catenatum*, a dinoflagellate (Costa el al., 2012). These abnormalities can be considered irreparable injuries, as they result from chromosomal breakage or loss, being overcome only with erythrocyte renewal (Costa et al., 2012).

Genotoxic damage can also be measured using a comet assay, where DNA breakage is analyzed. In this study, exposure to crude cyanobacteria extract contain saxitoxins at 25°C increased DNA breakage in blood from females and males and in liver, posterior kidney and gills from males. DNA fragmentation caused by saxitoxins has already been found in in vitro assays with *Crassostrea gigas* hemocytes (Abi-Khalil et al., 2017). However, as occurred in different tissues of males lipid damage was not found and other mechanism can be involved. A possible mechanism is the caspase gene activation, which activates the apoptosis pathway, as observed in *Danio rerio* embryos exposed to saxitoxins (Chen et al., 2020). The same was observed in *Mytilus galloprovincialis* fed on saxitoxin producer *Gynodimium catenatum* (Braga et al. 2020).

In addition to genotoxic damage, crude cyanobacteria extract can also lead to histopathological damage. Males and females showed increased tissue injury in the liver, posterior kidney and gills. One of the main lesions found in the liver, in addition to necrosis, was leukocyte infiltration, indicating a process of inflammation in this tissue caused by crude cyanobacteria extract, although the biotransformation system was not activated. This inflammatory effect was also observed in *Mytilus edulis* mussels exposed to the saxitoxin-producing dinoflagellate *Alexandrium fundyense* (Galimany et al., 2008).

Crude cyanobacteria extract effects: 30 °C

Temperature is one of the extremely important factors to understand the substances toxicity. Considering the global warming scenario, in which the warming waters will occur, saxitoxins were able to cause different effects in females and males of the fish species *Rhamdia quelen*.

Some biochemical biomarkers can be altered by the crude cyanobacteria extract exposure at 30°C. For example, plasmatic lactate increased in males, suggesting a change in this animals metabolism since lactate elevated were associated with anaerobiosis (Coates & Soderhall, 2020). In the liver, high saxitoxin concentrations have already been found as demonstrated in tilapia collected at fish farm (Galvão et al., 2009). In this study, effects were observed in female livers such as increase of glutathione S-transferase (GST) activity and levels of non-protein thiols (GSH) and lipid peroxidation (LPO). In the literature, the glutathione (GSH and GST)

participation in the saxitoxins metabolization has already been reported, with a possible STX role as a substrate for GST, with direct entry into phase II biotransformation (Gubbins et al., 2000; Fast et al., 2006; Costa et al., 2012). Even though these biomarkers are also part of the antioxidant system, this increase was not enough to prevent lipoperoxidation, demonstrating that saxitoxins can not only alter the levels of oxidizing agents such as GST and GSH, but can also promote oxidative stress in females liver, but not in males.

Kidney is known to be an important organ for metabolizing and retaining saxitoxins (Liu et al., 2020). The posterior kidney is an excretor organ and saxitoxin have already been found in this tissue. It has been shown to be an important biotransformation organ for scallops exposed to STX produced by dinoflagellates (Liu et al., 2020). In this study, STX decreased superoxide dismutase activity in female and male catfish kidneys. This SOD activity inhibition in exposure to STX was also found in scallops (*Patinopecten yessoensis*) and with trophic exposure to *Alexandrium tamarense*, a PST-producing dinoflagellate (Qiu et al., 2013).

In addition to biochemical changes, crude cyanobacteria extract led to DNA breakdown in blood, liver, kidney and gills of male and female catfish. Different mechanisms may be associated with this genotoxic damage, however in female liver this alteration may be consequence of an oxidative stress. Lipid damage observed in female hepatocytes can cause membrane destabilization and disintegration, leaving DNA accessible for damage (Braga et al., 2020).

Histopathological damage was also observed in these animals. In females, liver, kidney and gills showed increased injury index. For males, only liver and gill. Female and male differences in response may indicate that the kinetics of STX in both sexes may differ.

Crude cyanobacteria extract effects: temperature enhancement

As future climate changes predicted by global warming will favor the cyanobacteria growth, it is necessary to understand how aquatic biota will respond to this possible increased cyanotoxins input (O'Neill et al., 2016). Studies with *M. galloprovincialis* fed on *G. catenatum*, a saxitoxin producer, for example, indicate STX analogues retention under heating conditions compared to current temperature conditions, and thus less toxic effects can be found, but which will be prolonged (Braga et al., 2018).

In this study, some biomarkers were altered in both thermal conditions of exposure to crude cyanobacteria extract contain saxitoxins. Therefore, the blood glucose in females and histopathological damage in male kidneys increased only at 25°C, for example. The same behavior was observed in *M. galloprovincialis* gills fed on *G. catenatum*, a saxitoxin producer, showing that these effects are dominated by processes triggered only by STX (Braga et al., 2020).

In contrast, some changes only occurred when the animals were exposed to crude cyanobacteria extract at 30°C. The females, showed hepatic oxidative stress, with the alteration of the antioxidant system only at this temperature as well as the glutathione biotransformation pathway, known for its relationship with STX metabolism. DNA breakage was also observed in liver, posterior kidney and female gill only at the highest temperature. Changes in the antioxidant system under water heating conditions were observed in *M. galloprovincialis* fed with *G. catenatum* (Braga et al., 2020).

This potentiation of crude cyanobacteria extract effects/damages caused by the increase in temperature occurred in females. Data regarding this sexual differentiation and sensitivity to STX are scarce in the literature. However, saxitoxin and decarbamoylSTX (dcSTX) have already been identified as the main toxins in the ovary in the marine fish *Arothron firmamentum*, indicating, in a way, the possibility that female is more sensitive to exposure to saxitoxin, a toxin produces by *R. raciborskii* (Nakashima et al., 2004).

5. Conclusion

The present study showed the crude cyanobacteria extract contain saxitoxins effects in two thermal scenarios, but with sexual differences. At 25°C, this crude cyanobacteria extract caused alterations in gills antioxidant system at both sexes and was genotoxic in male liver, kidney and gill. At 30 °C, the crude cyanobacteria extract female metabolism was activated with increased glutathione activity. The DNA damage were observed in male liver, kidney and gill. Therefore, different of 25 °C, genotoxicity was observed in female liver, kidney and gill. At both thermal conditions, increase in blood DNA and histopathological damage were observed. At the higher temperature, the global warming scenario, the temperature rise can increase the cyanotoxins toxicity at female catfish produced by cyanobacteria blooms.

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Supplementary Data

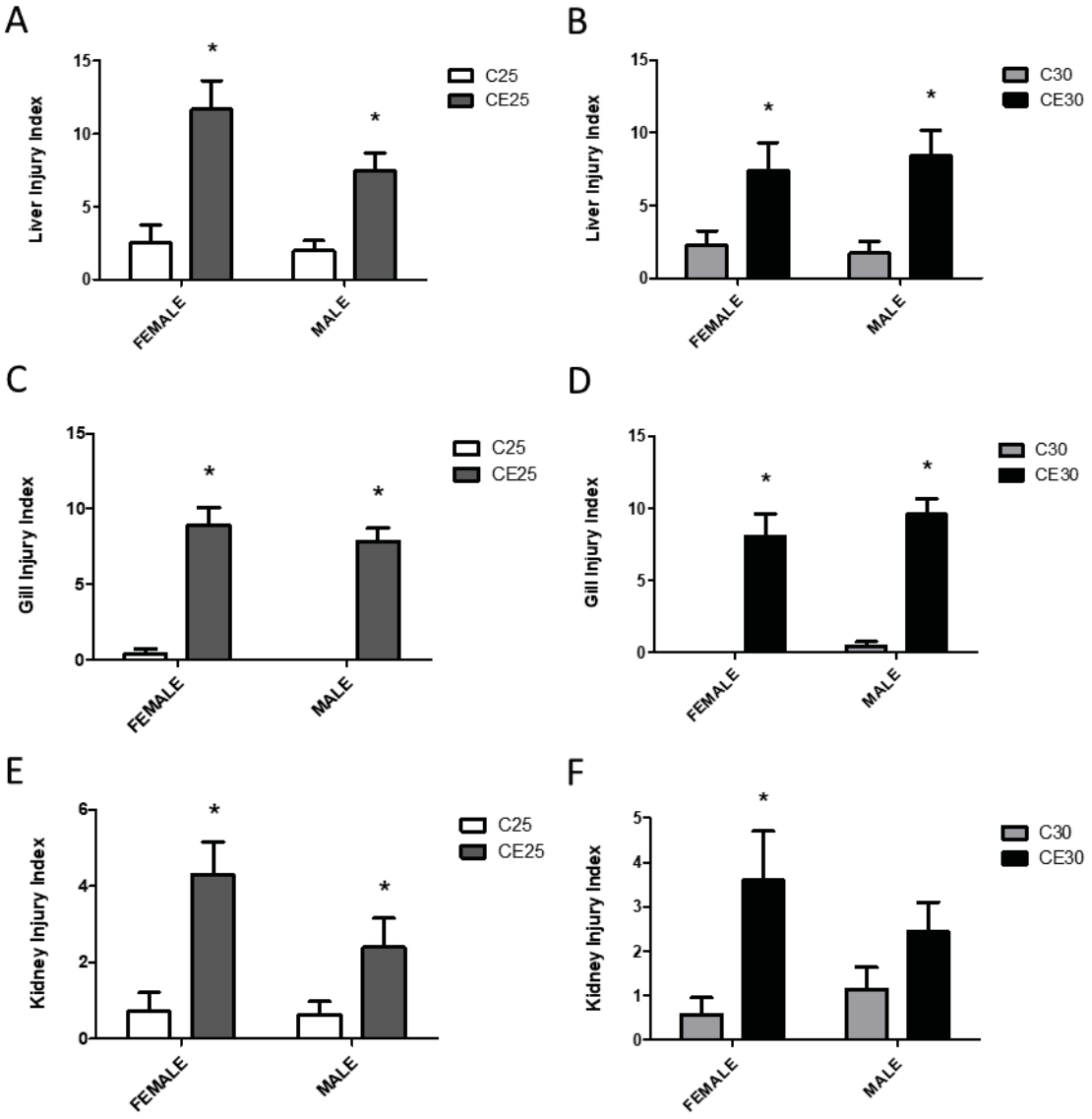

FIGURE 1: Histopathological biomarkers graphical representation. A: Liver injury index at 25°C, B: Liver injury index at 30°C, C: Gill injury index at 25°C, D: Gill injury index at 30°C, E: Kidney injury index at 25°C, F: Kidney injury index at 30°C, G: Liver histopathological types of altered animals, H: Gill histopathological types of altered animals.

CAPÍTULO 4

Abordagem proteômica para avaliar os efeitos de extrato bruto de cianobactéria sob diferentes condições de temperatura

“Proteomic approach to evaluate cyanobacteria crude extract effects under different temperature conditions”, a ser submetido para Chemosphere

Proteomic approach to evaluate cyanotoxins effects under different temperature conditions

Maiara Vicentini, Helena Cristina Silva de Assis

Abstract

The aquatic environment's eutrophication, together with the temperature rise due to the greenhouse gases, make potentially toxic cyanobacterial blooms more frequent and intensely. *Raphidiopsis raciborskii* is a cyanobacterium, whose strains in the Southern hemisphere produce neurotoxins called saxitoxins. Proteomics analysis can be a tool to evaluate these coumpound effects, showing which and how proteins in a tissue can be altered. This study aimed to evaluate how female Neotropical catfish respond to temperature rise and exposure to cyanotoxins produced by *R. raciborskii* (T3 strain), using a proteomic approach. *Rhamdia quelen* juvenile females and males were exposed to four treatments, based on literature: control at 25 °C (C25), control at 30 °C (C30), crude extract equivalent to 10^5 cells/ml of *R. raciborskii* at 25 °C (CE25) and crude extract equivalent to 10^5 cells/ml at 30 °C (CE30). After 96 hours, the animals were anesthetized and, after euthanasia, the liver, an important metabolism tissue, was collected for proteomic analysis. The proteins were extracted and the samples were analyzed at LC/MC. Three comparisons were conducted, using online software and databases (e.g. MetaboAnalyst, Gene Ontology and KEGG): C25 x CE25, to evaluate the cyanobacterial bloom in the 25 °C scenario; C30 x CE30, to evaluate cyanobacteria bloom in a 30 °C scenario, simulating a global warming scenario; and CE25 x CE30 to assess the difference between temperatures. Changes in the abundance of different proteins were found in female and male fish exposed to saxitoxins. Cell structures, energy production, metabolic process, immune system and reproduction process pathways altered by saxitoxin exposure. Comparing the responses in these two thermal conditions, the females showed more alterations and it suggests that females may be more sensitive to saxitoxin exposure in a global warming scenario.

Key words: Global warming, saxitoxins, toxicology, *Rhamdia quelen*

Highligths

Raphidiopsis raciborskii crude extract alter the proteome of *Rhamdia quelen*.
Females and males respond differentially to cyanobacteria extract exposure and water heating.
Female liver proteome was more sensitive to thermal variation.

1. Introduction

Proteomic analysis is a technique that describes the total complement of proteins in an organism or tissue under certain conditions (Snape et al., 2004). It provides information on the conditions action mechanism and captures the post-translational proteins modifications (Rodriguez et al., 2012), and it is possible to identify potential biomarkers for these certain conditions (López-Pedrouso et al., 2020). This approach has gained attention because it is possible to identify and quantify proteins altered by certain conditions and try to predict the effects at higher biological levels, before a loss of biodiversity occurs (Liang et al., 2018).

The proteomic approach can be applied in different areas, such as ecotoxicology, the science that studies the environmental contaminants effect on organisms (Snape et al., 2004). One of the compounds studied within ecotoxicology in which proteomic analysis can be applied is cyanotoxins. Cyanotoxins are compounds produced by cyanobacteria and can be neurotoxic, hepatotoxic and dermatotoxic (Aráoz et al., 2010). Proteomic effects after exposure to cyanotoxins, such as microcystins have been found (Marie, 2020). In the case of neurotoxins, the literature showed that saxitoxins (STX) alters the growth of marine medaka embryos, affecting the metabolic rate (Tian et al., 2014). However, the proteomis studies to medaka adults are unknown.

Furthermore, when it comes to environmental stressors, it is extremely important to understand the cause/effect of temperature impacts on them, as environmental degradation has led to an exaggerated increase in global temperature (Sokolova and Lanning, 2008; IPCC, 2021). Studies of the combined temperature and cyanotoxins effects become important to understand what the scenario will be like in the not-too-distant future. Moreover, it is unknown if STX, or STX producer cyanobacteria extract, induce changes in protein expression in the freshwater fish liver, reflecting

the complex cyanobacterial blooms effects on fish. Freshwater fish have already been used as a study model for the STX effects (Silva et al., 2011; Silva et al., 2014) and isolated temperature effects (Ahmad et al., 2011; Dietrich et al., 2018), becoming a good study model for studying both conditions.

In the present study, we investigated the effects of the *Raphidiopsis racibroskii* crude extract and waters warming caused by climate change on females and males of the Neotropical fish species *Rhamdia quelen*. The hepatic proteins in the fish was studied to elucidate the molecular mechanisms underlying the fish liver damage caused by two combined stressors.

2. Material and methods

2.1. Experimental conditions and proteomic analysis

The fish were acclimated to laboratory conditions and exposed for 96h to four different conditions: water at 25 °C (C25), water at 30 °C (C30), exposure to *R. raciborskii* extract equivalent of 10^5 cells.mL^{-1} at 25 °C (CE25) and exposure to *R. raciborskii* extract equivalent of 10^5 cells.mL^{-1} at 30 °C (CE30). About 10 mg of liver was collected and stored at -80°C. The detailed bioassays procedures were described in Vicentini et al in preparation. The experiment was approved for Federal University of Paraná Animal Use Ethics Committee (CEUA), under de number 1140.

The samples were processed, the protein extracted and the material were analyzed in an Easy-nLC 1000 liquid chromatograph coupled to a hybrid mass spectrometry equipment LTQ Orbitrap XL ETD P02-004 / Carlos Chagas Institute - Fiocruz Paraná, Brazil. The MaxQuant program version 1.6.10.43 was used for mass spectra analysis and the NCBI Siluriform database for protein identification. More information about the analysis conditions can be found in the Vicentini et al. (in preparation) supplementary material.

2.2. Data analysis

For the analysis of proteomic data, it was necessary to divide the data into three large comparisons, for each sex: C25 x CE25, simulating exposure at 25°C; C30 x CE30, simulating an exposure at 30°C; and CE25 x CE30, simulating exposure at

different temperatures. LFQ intensity data were analyzed using MetaboAnalyst 5.0 (Pang et al., 2021). Significant proteins, with p-values ≤ 0.05, were identified at the gene level using the NCBI Blast protein against the *Danio rerio* database (taxid: 7955), accessed in May and June 2021. These data were used for Gene Ontology (GO) gene enrichment analysis. To identify the functions and pathways of such proteins, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and UniProtKB were used, both using the basis for *D. rerio*. The UniProt entry name data is in the Supplementary Data.

3. Results

Different proteome responses were observed depending on sex or condition. At 25°C, cyanobacteria crude extract caused an abundance decreased in 54 proteins in females and 20 in males (Table S1 and S3). An abundance increased in 7 proteins in females and 17 in males are observed at this thermal condition (Table S2 and S4). At 30°C, cyanobacteria crude extract caused an abundance decreased in 15 proteins in females and 28 in males (Table S5 and S7). An abundance increased in 26 proteins in females and 9 in males are observed at this temperature (Table S6 and S8). Cyanobacteria crude extract exposure at 30°C directly compared to exposure at 25°C caused an abundance decreased in 26 proteins in females and 33 in males (Table S9 and S11). An abundance increased in 59 proteins in females and 7 in males are also observed at this comparation (Table S10 and S12).

Cyanobacteria crude extract contains saxitoxins and at 25°C and 30°C altered proteins with molecular functions such as catalytic activity, structural molecule activity and binding (Table 1-3) that can interfere in several biological process. Some of the process are biological regulation, cellular process, localization, metabolic process, response to stimulus and signaling, that can be alter by saxitoxins independent of thermal condition. However, the exposure to cyanobacteria crude extract at 25°C was able to alter developmental process and growth, while exposure simulating a 30°C environment was able to alter proteins related to reproduction and the immune system (Figure 1).

All these altered proteins are part of different pathways (Figure 2). On total, saxitoxin altered proteins of 118 different pathways. Regardless of the animal's thermal condition or sex, cyanobacteria crude extract altered proteins in pathways

involved in energy metabolism (such as glucose-related pathways), purine metabolism and different amino acids metabolism. In females, changes in the proteins involved in pathways of autophagy, apoptosis, necroptosis, endocytosis, protein processing, MAPK signaling, cell junctions, progesterone-mediated oocyte maturation, and also in the other drug metabolism were observed at all saxitoxin exposure groups. At the environment of 30°C, saxitoxin activated the glutathione pathway, the main pathway for these cyanotoxins metabolism, and the RNA transport. In males, all the cyanobacteria crude extract conditions altered proteins from pathways of focal adhesion, PPAR signaling and ribosome. Therefore, only at 30°C, the cyanobacteria crude extract altered amicoacyl-tRNA biosynthesis, mitophagy, phagosome, protein processing and ubiquitin mediated proteolysis both compared to control and cyanobacteria crude extract at 25°C.

Table 1: Protein molecular functions of C25xCE25.

GO Term	GO Description	D/U	Females		Male	
			Count	Genes	Count	Genes
GO:0005488	Binding	D	8	*gpia, anxa6, actr2a ,adprh, gnai2a, tktb, shmt1, aco1*	0	
		U	3	*pleca, rps10, prpf8*	5	*prps1b, capza1b, ap2a1, tktb, rpl19*
GO:0003824	Catalytic activity	D	21	*mthfd1b, gpia, fbp1b, hgd, prdx5, npsn, gapdh, pdhb, adprh, eno1a, gnai2a, f2, map2k1, aldh1l1, tktb, shmt1, acat2, capn1, aco1, aldh4a1, tpi1b*	1	*akt2*
		U	2	*srpk1b, cyp1a*	7	*dpys, prps1b, ctsc, bhmt, gbe1b, tktb, aldh9a1a*
GO:0060090	Molecular Adaptor Activity	D	0		0	
		U	0		1	*ap2a1*
GO:0098772	Molecular Function Regulator	D	1	*adprh*	0	
		U	0		0	
GO:0005198	Structural molecule activity	D	0		0	
		U	2	*pleca, rps10*	1	*rpl19*
GO:0005215	Transport activity	D	2	*anxa6, mttp*	0	
		U	0		0	

Table 2: Protein molecular functions of C30xCE30.

GO Term	GO Description	D/U	Females		Male	
			Count	Genes	Count	Genes
GO:0005488	Binding	D	6	*pgk1, gdi2, atp5fa1, ddx19, u2af2b, cbsb*	9	*kars1, lman2, pygb, ubc, hnrnpdl, cyp2p9, rpl13, sec61b, lgals9l1*
		U	8	*capza1a, eef2b, eef1a, tubb2b, hsp90aa1, gak, rpl11, hspa8*	3	*histh1l1, gldc, shmt1*
GO:0003824	Catalytic activity	D	8	*pgk1, gdi2, atp5fa1, pitrm1, ddx19, dgki, ppat, agla*	8	*kars1, pygb, comta, cyp2p9, gbe1b, rab5c, uchl3, nsdhl*
		U	8	*ak2, tkfc, gpt2, eef2b, gstt1b, eef1a, hspa8, gpt2*	6	*pdhb, gldc, shmt1, nt5c2b, gars1, msrb2*
GO:0098772	Molecular Function Regulator	D	1	*gdi2*	0	
		U	0		0	
GO:0005198	Structural molecule activity	D	0		1	*rpl13*
		U	2	*tubb2b, rpl11*	0	
GO:0045182	Translation regulator activity	D	0		1	*sec61b*
		U	2	*eef2b, eef1a*	0	

Table 3: Protein molecular functions of CE25xCE30.

GO Term	GO Description	D/U	Females		Male	
			Count	Genes	Count	Genes
GO:0005488	Binding	D	12	*eppk1, ncl, snrpd3l, ddx39ab, hnrnpdl, fubp3, cyb5r3, hnrnpl, prpf8, eftud2, serbp1a, nop58*	11	*kars1, alad, snd1, lcp1, uba52, hdlbpa, hnrnpdl, eif3k, srsf5a, hnrnpl, serpinc1*
		U	2	*hspa5, ralaa*	3	*abat, gldc, pgd*
GO:0003824	Catalytic activity	D	4	*papss2b, ddx39ab, gbe1b, eftud2*	8	*kars1, alad, agxta, snd1, crata, gbe1b, chdh, serpinc1*
		U	2	*hspa5, ralaa*	6	*mthfd1b, abat, papss2b, gldc, sdr16c5b, pgd*
GO:0098772	Molecular Function Regulator	D	0		1	*serpinc1*
		U	0		0	
GO:0005198	Structural molecule activity	D	0		1	*col1a1a*
		U	0		0	
GO:0045182	Translation regulator activity	D	1	*eftud2*	1	*eif3k*
		U	0		0	
GO:0005215	Transport activity	D	0		0	
		U	0		1	*slc25a10*

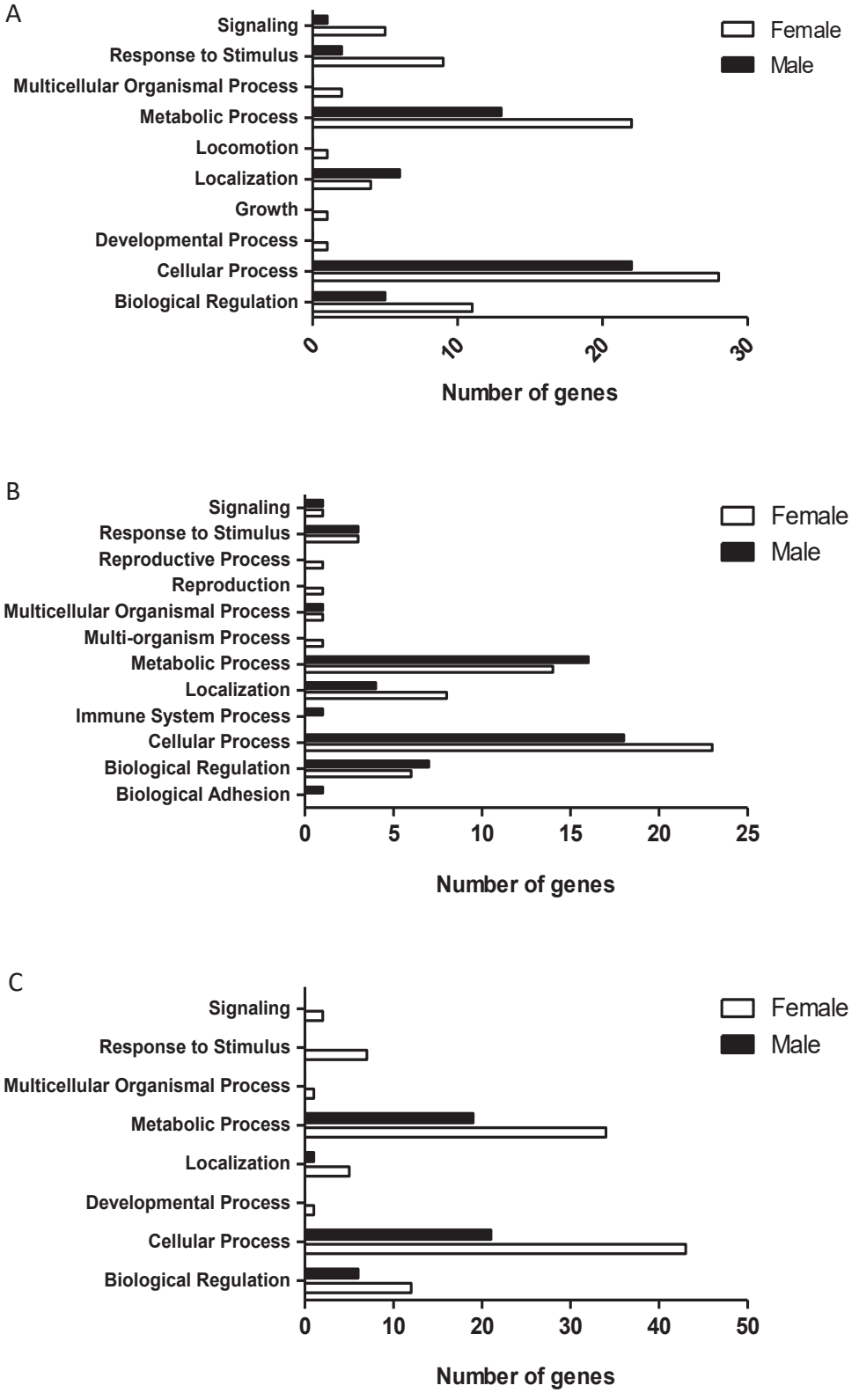

Figure 1: Biological process of altered proteins during different saxitoxin thermal conditions exposure according to Gene Onthology. A: C25 x CE25; B: C30 x CE30; C: CE25 x CE30.

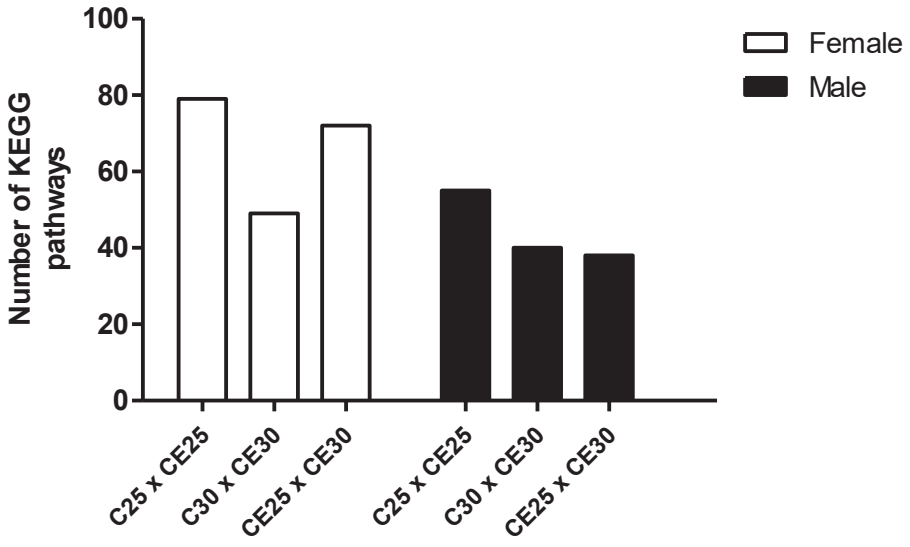

Figure 2: Pathways of altered proteins during different crude cyanobacteria extract thermal conditions exposure according to KEGG.

4. Discussion

At in vivo studies, saxitoxins produced by *R. raciborskii* are capable of causing biochemical effects on the biotransformation pathways and antioxidant system of freshwater fish, as well as causing damage at the DNA and tissue level (Silva et al., 2011; Calado et al., 2017). In the present study, we observed alterations in proteins pathways according to the experiment conditions, such as alterations on cellular process, energy production, metabolic process, immune system and reproduction process. It is possible to complement the effects in the biochemical level observed in others studies with STX. In this study, organism's proteome alteration was observed in a cyanobacterial bloom simulation in water at 25 and 30°C, a temperature normally higher than the current one.

Cyanobacteria extract effects at 25°C

In water reservoirs where the temperature is close to 25°C, blooms of *Raphidiopsis raciborskii* have already been found, with STX fish accumulation (Calado et al., 2017). Based on the results of the protein changes of this study, we suggest that these changes can occur in the field fish from the reservoir and can

cause consequences in the physiological process. At 25°C, the extract of *R. raciborskii* content saxitoxins alter proteins of different cellular process.

In female and males, proteins related to apoptosis and cellular senescence were decreased, such as mitogen-activated protein kinase 1 (Map2k1). Cell death by apoptosis is an extremely important and regulated process, which allows the deletion of cells from a tissue, avoiding tissue damage and is a process influenced by environmental contaminants (AnvariFar et al., 2018). In this study, apoptosis pathway was down-regulated by some proteins. Signaling pathways have also been down-regulated such as ERBB (KEGG, dre04012), FOXO (KEGG, dre04068), VEGF (KEGG, dre04370) and MAPK (KEEG, dre04010), all of which have the potential to regulate cell proliferation. In females, the apelin signaling pathway was also negatively affected, which is also involved in cell proliferation (KEGG, dre04371).

Another form of cellular homeostasis was also affected: an autophagy. This mechanism is important for maintaining cellular health through subcellular degradation of pathogens or other substances (Xia et al., 2019). Under these conditions, crude cyanotoxin extract was also able to down-regulated this process in females and males, by reducing the proteins abundance: Map2k1, serine/threonine-protein phosphatase (Ppp2cb) and non-specific serine/threonine protein kinase (Akt2).

Changes in pathways involved in cell structuring were also observed. Cell junction proteins reduction was observed in males and females and actin cytoskeleton regulation proteins reduction in females. The cytoskeleton is extremely important in several important processes, such as the immune response (Mylvaganam et al., 2021). Thus, crude cyanotoxin extract can cause the cells cytoskeleton rearrangement as a response to stress.

In addition to structural rearrangements, metabolic changes also occurred as a result of exposure to crude cyanotoxin extract at 25 °C. In females, several pathways related to amino acid metabolism were down-regulated with the reduction of related proteins. One of these routes was that of arginine. This amino acid is indispensable for fish and influences nutrient metabolism and insulin release, in addition to being involved in immune responses (Wang et al., 2021). The female liver can be compromised by the down-regulated amino acid pathways that can reduce the amino acids amount, such as arginine. In males, many metabolic pathways were also affected, including amino acids. On the contrary, some of these pathways was up-

regulated in females. Glutathione is normally triggered to detoxify saxitoxins, the toxins produces by *R. raciborskii*, and it was induced only in males, showing a sexual difference in response. Cytochrome P450 pathway induction, normally activated for other organic compounds, was observed in males. Increased activity of glutathione S-transferase (GST) and ethoxyresorufin-O-deethylase (EROD, cytochrome P450 complex enzyme) has been observed in Atlantic salmon with intraperitoneal STX injection (Gubbins et al., 2000).

For all reactions to take place, it is also necessary for the cell to have enough energy. This energy is in the cell at the adenosine triphosphate (ATP) form produced by cellular respiration in different stages: glycolysis, the Krebs cycle and oxidative phosphorylation (Hill et al., 2012). Crude cyanotoxin extract altered some of these stages at 25°C. In females, the glycolysis and Krebs cycle processes were down regulated, with many proteins decreasing in abundance. The only up-regulated process was the oxidative phosphorylation, with an increase in ubiquinol-cytochrome c reductase core protein 2b (Uqcrc2b). One way for these females to get more energy would be through beta fatty acid oxidation (Hill et al., 2012). However, this process was also full of proteins with a decreased abundance, demonstrating that these organisms could be in energy deficit from the crude cyanotoxin extract exposure. Males, however, had an increase in the abundance of some proteins related to cellular respiration processes, thus showing that the aforementioned metabolic increase induced an increase in energy production.

Processes related to the immune system were also altered by crude cyanotoxin extract. In females, the toll-like receptor signaling pathway (KEGG, dre04620) was affected, with a reduction in the abundance of the Map2k1 protein, thus demonstrating that the recognition of these females to possible pathogens can be compromised by exposure to *R. raciborskii* extract (Fitzgerald and Kagan, 2020). Males also had this pathway affected, with the Akt2 decreased, and the C-Type lectine receptor-signaling pathway affected. The last one it is responsible for the C-type lectin receptors (CLRs), which trigger signaling pathways that induce the cells recruitment to fight pathogens. Thus, crude *R. raciborskii* extract may be able to influence the fish immunity at a temperature of 25°C.

Different proteome responses were found between the both sex. Many changes in reproductive processes were found in these animals, and more reproductive-related pathways were affected in females. The synthesis of estrogen hormones was

affected by the protein hydroxysteroid (17-beta) dehydrogenase 12b (hsd17b12b) reduction. Females need this hormone for their ovaries maintenance (Li et al., 2019), so crude cyanotoxin extract has been shown to alter the reproductive process of these females when they are exposure at 25°C.

Cyanobacteria extract effects at 30°C

The effects of cyanobacterial blooms on the proteomic changes in liver was also observed at 30 °C. With regard to cellular processes, many of them have already been presented in exposure to 25°C. Females showed an increase in lamin A (Lmna) protein belonging to the apoptosis pathway (KEGG, dre04210), while males showed a reduction in cathepsin D, a protein of this same pathway, thus showing the apoptosis induction in females and negative regulation in males. Another pathway related to the cell cycle is the proteosome pathway. In females, this pathway was down regulated, with the protease regulatory subunit S10B (Psmc6) protein decreased. In contrast, another related pathway, the rig-1-like receptor-signaling pathway, was induced by the increase in protein ATP-dependent dihydroxyacetone kinase (Tkfc). Both act on ubiquitin mediated proteolysis. This pathway, which was also reduced in males, plays a role in cell cycle progression and signal transduction, consequently in cellular processes (Ilieş et al., 2012).

Many metabolic pathways were altered, as well as to crude cyanobacteria extract exposure at 25°C. However, unlike what occurred at 25°C, the glutathione and cytochrome P450 pathway was activated in females and not in males. It shows that for females the detoxification of saxitoxin present in the extract was activated at a higher temperature. Furthermore, at 30°C there was a greater variation in responses between sex and more up-regulated pathways than at 25 °C. The increased demand for amino acid synthesis may be a way for the body to offset the increased energy demand, common at higher temperatures (Rocha et al., 2001). However, the protein synthesis process itself is highly costly, requiring 5 to 9 moles of ATP (Bombardelli et al., 2003).

Therefore, it was necessary for these organisms to increase their energy metabolism (Little et al., 2020). Males showed an increase in proteins involved in the main cellular respiration processes, such as glycolysis and the Krebs cycle. However, females had a balance between increasing and decreasing protein abundance, with

only decreased proteins in the phosphorylation process. These results demonstrate that, even not activating STX detoxification pathways at this temperature, males still had a high energy demand.

As in the 25°C scenario, in 30°C the crude extract also alter the immune system. Mainly in females, by the activation of the NOD-like receptor-signaling pathway, with the increase of the heat shock protein 90 protein (Hsp90aa1.2). These intracellular cytoplasmic receptors recognize microorganism ligands (Sahoo, 2020). It showed the *Raphidiopsis raciborskii* extract was able to activate the immune system at 30 °C. In addition to this difference, males and females showed alterations in the phagosome pathway at 30 °C. In females this process was up regulated, while in males it was down regulated. This is a process that leads to the possible pathogens elimination. In males, damage to the immune system may be occurring (Xia et al., 2019).

The reproductive processes pathway were also altered as at 25 °C, but in a different way. While in females exposed to crude *R. raciborskii* extract at 25°C it was down regulated by the progesterone-mediated oocyte maturation pathway, at 30 °C this pathway was up regulated. In males, via steroids it was down regulated by the catechol O-methyltransferase A (Comta) protein decreased. These hormones are extremely important for spermatogenesis (Hatef and Unniappan, 2019), for example, so *R. raciborskii* extract in an environmental scenario at 30°C can compromise reproduction.

Temperature influence

Directly comparing the effects on the proteome found in exposure to crude cyanobacteria extract at 25° and at 30°C, proteins from various pathways were altered, with the majority in females.

In females, the *R. raciborskii* blooms simulation at 30°C was able to cause alterations in apoptosis and autophagy pathways, with proteins with increase and decrease in abundance compare to 25°C. The main alteration in females related to cellular processes was related to genetic material. The transcribed genes contain intervening sequences (introns) and expressed sequences (exons), and the introns must be removed for the correct mRNA production only with exons. The cell uses machinery called a spliceosome (Matera and Wang, 2014). Altogether, nine proteins decreased in the females spliceosome pathway, showing that with increasing

temperature, effects on genetic material levels can occur in exposure to *R. raciborskii* extract.

Regarding metabolic processes, at 30°C several pathways of amino acid metabolism were affected, with reduced or increased proteins. The P450 pathway was down regulated in terms of exposure to higher temperature saxitoxins. Although no general increase in metabolic processes was observed, energy production was highly requested by females. Fatty acid metabolism was up regulated, the same responsible for energy production through beta oxidation. The same happened with the glycolysis pathways, pyruvate metabolism and the Krebs cycle. As temperature alone increases energy demand (Campos et al., 2018), its combined effect with *R. raciborskii* blooms may be the cause of these processes up-regulation.

Effects on reproductive pathways were also found, with protein reduction in the steroid biosynthesis pathway, mainly UDP-glucuronosyltransferase (Ugt2a1), which appears at 8 different points of this pathway (KEGG, dre00140). This protein has already been described as one of the saxitoxin metabolization pathways, because through this glucuronidation process it can conjugate these toxins and help in their elimination (García et al., 2010). Although the expected were the protein increase or remain close to that found at 25°C, it reduced. It showed that the temperature somehow can compromise not only reproductive processes, but also saxitoxin detoxification processes in females.

In males, fewer differences were found between crude cyanobacteria extract exposures at 25 and 30°C. Cell processing-related pathways were down-regulated, such as different cell junctions and cell signaling pathways, thus affecting cell homeostasis. Amino acid metabolism pathways have also been altered, either with increases or with decreases in proteins. However, contrary to expectations, energy demand was reduced by exposure to crude cyanobacteria extract at higher temperature, both with down regulation of phosphorylative oxidation and beta oxidation in fatty acid metabolism.

Differences in responses between genders were observed in the present work. In exposure to saxitoxins, STX accumulation in the ovaries of fish *Arothron firmamentum* and delay in spawning of the mollusc *Acanthina monodon* have been observed (Nakashima et al., 2004; Andrade-Villagrán et al., 2019). The proteins alteration of this study showed that females were more sensitive to temperature variation in exposure to crude cyanobacteria extract contain saxitoxins.

5. Conclusion

In the present study, we present the first analysis of the protein profiles in the liver of *Rhamdia quelen* exposed to crude *R. raciborskii* extract in two different temperatures. The data provided evidence of the molecular mechanisms involved in different metabolic pathways of male and female catfish. Alterations in the proteins abundance, such as changes in cell structures, energy production and in the immune system were found in female and male fish exposed to crude cyanobacteria extract. In the simulation of most current temperature conditions (25°C) and in water warming conditions (30°C), many protein pathways were changed. Comparing the effect of exposure to crude *R. raciborskii* extract in these two thermal conditions, the female fish might be more sensitive to this exposure in a global warming scenario.

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Supplementary Data

Table S1 (Part 1): Decreased female proteins of C25 x CE25.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1042282557	-10.343	0.0091754	*aass*	aminoadipate-semialdehyde synthase	F8W3V5 (F8W3V5_DANRE)	Aminoadipate-semialdehyde synthase
1764603005	-12.561	0.044765	*acat2*	acetyl-CoA acetyltransferase 2	Q5SPA3 (Q5SPA3_DANRE)	Acetyl-CoA acetyltransferase 2
1709638277	-0.77371	0.038073	*aco1*	aconitase 1, soluble	F1QM42 (F1QM42_DANRE)	Citrate hydro-lyase
1764598791	-10.319	0.0056778	*acss2l*	acyl-CoA synthetase short chain family member 2 like	F1QQH3 (F1QQH3_DANRE)	Acetyl-coenzyme A synthetase
1709609573	-0.38922	0.00061096	*actr2a*	actin related protein 2a	Q7SXW6 (ARP2A_DANRE)	Actin-related protein 2-A
1042276359	-0.57213	0.050013	*adprh*	ADP-ribosylarginine hydrolase	A0A2R8Q1N4 (A0A2R8Q1N4_DANRE)	ADP-ribosylarginine hydrolase
1764647261	-0.66604	0.049684	*aldh1l1*	aldehyde dehydrogenase 1 family, member L1	E3NZ06 (E3NZ06_DANRE)	10-formyltetrahydrofolate dehydrogenase
1520628439	-0.6742	0.0055744	*aldh1l1*	aldehyde dehydrogenase 1 family, member L1	E3NZ06 (E3NZ06_DANRE)	10-formyltetrahydrofolate dehydrogenase
1764600230	-0.64167	0.050059	*aldh4a1*	aldehyde dehydrogenase 4 family, member A1		
1709657983	-0.58737	0.014861	aldh7a1	aldehyde dehydrogenase 7 family, member A1	F1QR17 (F1QR17_DANRE)	Aldehyde dehydrogenase 7 family, member A1
1764606686	-0.86838	0.0043253	aldh7a1	aldehyde dehydrogenase 7 family, member A1	F1QR17 (F1QR17_DANRE)	Aldehyde dehydrogenase 7 family, member A1
317637913	-0.60968	0.045411	*anxa6*	annexin A6	F1R6N3 (F1R6N3_DANRE)	Annexin
1764638154	-0.50931	0.0096298	*blvrb*	biliverdin reductase B	B8JMC1 (B8JMC1_DANRE)	Biliverdin reductase B
1764642220	-0.43361	0.038223	*capn1*	calpain 1	F1QI24 (F1QI24_DANRE)	Calcium-activated neutral proteinase 1
1764618472	-11.059	0.0059247	*crym*	crystallin, mu	R4GET2 (R4GET2_DANRE)	Ketimine reductase mu-crystallin
1764611993	-14.247	0.030641	*dlat*	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	B3DIV6 (B3DIV6_DANRE)	Acetyltransferase component of pyruvate dehydrogenase complex
1764638212	-0.94321	0.028921	*eif3m*	eukaryotic translation initiation factor 3, subunit M	Q6AXJ2 (EI3HA_DANRE)	Eukaryotic translation initiation factor 3 subunit H-A
1503217506	-0.36263	0.030168	*eno1a*	enolase 1a, (alpha)	A0A2R8Q1X2 (A0A2R8Q1X2_DANRE)	2-phospho-D-glycerate hydro-lyase
1042342345	-28.625	0.0062422	*f2*	coagulation factor II (thrombin)	A0A1D5NSK6 (A0A1D5NSK6_DANRE)	Coagulation factor II (thrombin)
1709608665	-17.069	0.028061	*fasn*	fatty acid synthase	A0A2R8Q644 (A0A2R8Q644_DANRE)	Fatty acid synthase
1520590588	-1.561	0.0058016	*fasn*	fatty acid synthase	A0A2R8Q644 (A0A2R8Q644_DANRE)	Fatty acid synthase
1042276269	-1.466	0.032771	*fasn*	fatty acid synthase	A0A2R8Q644 (A0A2R8Q644_DANRE)	Fatty acid synthase
576888482	-0.69211	0.0073668	*fbp1b*	fructose-1,6-bisphosphatase 1b	Q6PFT1 (Q6PFT1_DANRE)	Fructose-bisphosphatase
1764589250	-0.71889	0.0237	*ftcd*	formimidoyltransferase cyclodeaminase	F1QZ40 (F1QZ40_DANRE)	Formimidoyltetrahydrofolate cyclodeaminase
308321716	-0.5841	0.032392	*gapdh*	glyceraldehyde-3-phosphate dehydrogenase	Q5XJ10 (G3P_DANRE)	Glyceraldehyde-3-phosphate dehydrogenase
1520539234	-0.4595	0.023318	*gapdhs*	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	F1R3D3 (F1R3D3_DANRE)	Glyceraldehyde-3-phosphate dehydrogenase
1764600198	-0.35305	0.029544	*gmppb*	GDP-mannose pyrophosphorylase B	Q6DBU5 (GMPPB_DANRE)	Mannose-1-phosphate guanyltransferase beta
1764599862	-0.6263	0.049356	*gnai2a*	guanine nucleotide binding protein (G protein), α inhibiting activity polypeptide 2a	Q6TNT8 (Q6TNT8_DANRE)	Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2
576893146	-0.87858	0.048618	*gpia*	glucose-6-phosphate isomerase a	Q8QFU2 (Q8QFU2_DANRE)	Glucose-6-phosphate isomerase
1764596346	-0.81636	0.042034	*hgd*	homogentisate 1,2-dioxygenase	Q6P2V4 (Q6P2V4_DANRE)	Homogentisate 1,2-dioxygenase
1764589008	-12.706	0.016794	*hsd17b12b*	hydroxysteroid (17-beta) dehydrogenase 12b	A7MCK2 (A7MCK2_DANRE)	Hsd17b12b protein
1042363791	-0.8605	0.037775	*mao*	monoamine oxidase	Q6NSN2 (AOF_DANRE)	Amine oxidase [flavin-containing]
1764638018	-13.421	0.011987	*map2k1*	mitogen-activated protein kinase kinase 1	A5WWB4 (A5WWB4_DANRE)	Mitogen-activated protein kinase kinase 1
1764625561	-0.8138	0.022133	*mthfd1b*	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1b	K7DYA3 (K7DYA3_DANRE)	Methylenetetrahydrofolate dehydrogenase (NADP+-dependent) 1b

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Table S1 (Part 2): Decreased female proteins of C25 x CE25.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1520599409	-0.93914	0.039442	*mttp*	microsomal triglyceride transfer protein	A0A0R4IVV0 (MTP_DANRE)	Microsomal triglyceride transfer protein large subunit
1520535532	-0.7584	0.040031	*myh10*	myosin, heavy chain 10, non-muscle	F1R3G4 (F1R3G4_DANRE)	Myosin, heavy chain 10, non-muscle
1520595889	-18.117	0.021442	*npsn*	nephrosin	Q7SYH7 (Q7SYH7_DANRE)	Metalloendopeptidase
1503278067	-12.156	0.048163	*paics*	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	A0A0A0MPG9 (A0A0A0MPG9_DANRE)	AIR carboxylase
1042322387	-14.701	0.011317	*pdha1a*	pyruvate dehydrogenase E1 subunit alpha 1a	A0A2R8QAA8 (A0A2R8QAA8_DANRE)	Pyruvate dehydrogenase E1 component subunit alpha
1520613516	-12.149	0.012993	*pdhb*	pyruvate dehydrogenase E1 subunit beta	Q7T368 (Q7T368_DANRE)	Pyruvate dehydrogenase E1 component subunit beta
1764600156	-11.949	0.029455	*pdhb*	pyruvate dehydrogenase E1 subunit beta	Q7T368 (Q7T368_DANRE)	Pyruvate dehydrogenase E1 component subunit beta
1520554561	-0.31019	0.010551	*ppm1bb*	protein phosphatase, Mg2+/Mn2+ dependent, 1Bb	Q5U386 (Q5U386_DANRE)	Protein-serine/threonine phosphatase
317574221	-0.68344	0.006904	*ppp2cb*	protein phosphatase 2, catalytic subunit, beta isozyme	Q803G3 (Q803G3_DANRE)	Serine/threonine-protein phosphatase
1764611849	-16.573	0.016104	*prdx5*	peroxiredoxin 5	F1QCE3 (F1QCE3_DANRE)	Peroxiredoxin-5
1503264914	-0.78844	0.043219	*psmd13*	proteasome 26S subunit, non-ATPase 13	Q6TGV4 (Q6TGV4_DANRE)	26S proteasome non-ATPase regulatory subunit 13
1503239884	-1.229	0.029603	*shmt1*	serine hydroxymethyltransferase 1 (soluble)	Q2TL58 (Q2TL58_DANRE)	Serine hydroxymethyltransferase
1042358820	-11.723	0.0506	*sult1st2*	sulfotransferase family 1, cytosolic sulfotransferase 2	Q7ZUS4 (ST2S2_DANRE)	Cytosolic sulfotransferase 2
1520569604	-19.608	0.00025322	*taldo1*	transaldolase 1	Q6P6Y0 (Q6P6Y0_DANRE)	Transaldolase
1764638333	-17.113	0.037061	*taldo1*	transaldolase 1	Q6P6Y0 (Q6P6Y0_DANRE)	Transaldolase
1503198938	-0.42827	0.025474	*tfg*	trafficking from ER to golgi regulator	Q6P0G5 (Q6P0G5_DANRE)	Tfg protein
1709639585	-10.666	0.015507	*tktb*	transketolase b	F1Q8Q6 (F1Q8Q6_DANRE)	ATP-dependent dihydroxyacetone kinase
1520508268	-0.27355	0.025294	*tpi1b*	triosephosphate isomerase 1b	Q90XG0 (TPISB_DANRE)	Triosephosphate isomerase B
576887698	-0.51367	0.048445	*ugp2a*	UDP-glucose pyrophosphorylase 2a	B8JMZ1 (B8JMZ1_DANRE)	UTP--glucose-1-phosphate uridylyltransferase
1520598290	-1.356	0.046145	*usp14*	ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)	Q803B3 (Q803B3_DANRE)	Ubiquitin carboxyl-terminal hydrolase

Table S2: Increased female proteins of C25 x CE25.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
987638706	1.383	0.011045	*cyp1a*	cytochrome P450, family 1, subfamily A	Q8UW07 (Q8UW07_DANRE)	Unspecific monooxygenase
1520517410	23.405	0.017657	*pds5b*	PDS5 cohesin associated factor B	A0A0R4IP12 (A0A0R4IP12_DANRE)	PDS5 cohesin-associated factor B
1042324108	0.44197	0.038112	*pleca*	plectin a	A0A2R8QKW7 (A0A2R8QKW7_DANRE)	Plectin a
1709641385	0.75888	0.04746	*prpf8*	pre-mRNA processing factor 8	A0A0R4J8E6 (A0A0R4J8E6_DANRE)	Pre-mRNA-processing factor 8
318056052	0.37674	0.038935	*rps10*	ribosomal protein S10	Q7T1J9 (Q7T1J9_DANRE)	Ribosomal protein S10
1709660023	25.447	0.018253	*srpk1b*	SRSF protein kinase 1b	F1REN8 (F1REN8_DANRE)	SRSF protein kinase 1b
1520591273	27.106	0.04458	*uqcrc2b*	ubiquinol-cytochrome c reductase core protein 2b	Q6IQ59 (Q6IQ59_DANRE)	Ubiquinol-cytochrome c reductase core protein 2b

Table S3: Decreased male proteins of C25 x CE25.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
318066051	-14.011	0.041513	*acaa1*	acetyl-CoA acyltransferase 1	Q6GQN6 (Q6GQN6_DANRE)	Acaa1 protein
1552122390	-0.6255	0.028757	*acox1*	acyl-CoA oxidase 1, palmitoyl	F1R071 (F1R071_DANRE)	Acyl-coenzyme A oxidase
1709657866	-14.748	0.038953	*akt2*	v-akt murine thymoma viral oncogene homolog 2	A0A0F6PK88 (A0A0F6PK88_DANRE)	Adenylate kinase 2, mitochondrial
1503241789	-0.22773	0.026178	*copb1*	COPI coat complex subunit beta 1	Q66HV4 (COPB_DANRE)	Coatomer subunit beta
318065783	-13.216	0.039202	*eif3m*	eukaryotic translation initiation factor 3, subunit M	Q7T3B0 (EIF3M_DANRE)	Eukaryotic translation initiation factor 3 subunit M
1503265234	-0.28572	0.035284	*eif3s10*	eukaryotic translation initiation factor 3, subunit 10 (theta)	Q6PCR7 (EIF3A_DANRE)	Eukaryotic translation initiation factor 3 subunit A
1042316209	-29.528	0.020218	*flnb*	filamin B	F1QGL5 (F1QGL5_DANRE)	Filamin B, beta (actin-binding protein 278)
68161035	-33.337	0.038894	*fth1a*	ferritin, heavy polypeptide 1a	Q9DDT0 (Q9DDT0_DANRE)	Ferritin
318102144	-21.643	0.0059478	*hnmt*	histamine N-methyltransferase	Q6DC37 (HNMT_DANRE)	Histamine N-methyltransferase
1764600264	-25.943	0.0081365	*hp1bp3*	heterochromatin protein 1, binding protein 3	A0A140LGU1 (A0A140LGU1_DANRE)	Heterochromatin protein 1-binding protein 3
1042292950	-0.41484	0.019237	*naca*	nascent polypeptide associated complex subunit alpha	Q8JIU7 (NACA_DANRE)	Nascent polypeptide-associated complex subunit alpha
317777987	-0.27714	0.0043903	*rpl10a*	ribosomal protein L10a	Q6PC69 (RL10A_DANRE)	60S ribosomal protein L10a
52783261	-0.19599	0.012615	*rpl24*	ribosomal protein L24	Q8JGR4 (RL24_DANRE)	60S ribosomal protein L24
1764638019	-0.3176	0.02144	*rpl4*	ribosomal protein L4	Q7ZW95 (Q7ZW95_DANRE)	Ribosomal protein L4
1764621875	-0.47941	0.045159	*rps13*	ribosomal protein S13	Q6IMW6 (Q6IMW6_DANRE)	40S ribosomal protein S13
54039448	-0.3682	0.012148	*rps16*	ribosomal protein S16	Q1LWH1 (Q1LWH1_DANRE)	Ribosomal protein S16
1503279041	-0.11785	0.022472	*rps19*	ribosomal protein S19	A0A0R4IJ49 (A0A0R4IJ49_DANRE)	40S ribosomal protein S19
1764602998	-0.28986	0.018873	*rps7*	ribosomal protein S7	P62084 (RS7_DANRE)	40S ribosomal protein S7
1520534824	-0.39179	0.028593	*scp2a*	sterol carrier protein 2a	Q6P4V5 (Q6P4V5_DANRE)	Acetyl-CoA C-myristoyltransferase
1520580704	-0.2793	0.047157	*slc25a3b*	solute carrier family 25 member 3b	Q7SZ49 (Q7SZ49_DANRE)	Short chain dehydrogenase/reductase family 16C, member 5

Table S4: Increased male proteins of C25 x CE25.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1503191056	0.49815	0.0081934	*aldh9a1a.1*	aldehyde dehydrogenase 9 family, member A1a, tandem duplicate 1	Q7ZVB2 (A9A1A_DANRE)	4-trimethylaminobutyraldehyde dehydrogenase A
576889580	25.497	0.0021743	*ap1b1*	adaptor related protein complex 1 subunit beta 1	A5PMS9 (A5PMS9_DANRE)	AP complex subunit beta
1709657115	0.29993	0.05016	*ap2a1*	adaptor related protein complex 2 subunit alpha 1	X1WD71 (X1WD71_DANRE)	AP-2 complex subunit alpha
1520523601	0.84341	0.022823	*bhmt*	betaine-homocysteine methyltransferase	Q32LQ4 (BHMT1_DANRE)	Betaine–homocysteine S-methyltransferase 1
1764630452	24.216	9,16E-01	*capza1b*	capping actin protein of muscle Z-line subunit alpha 1b	Q6NWK1 (Q6NWK1_DANRE)	F-actin-capping protein subunit alpha
1764618472	28.032	0.027911	*crym*	crystallin, mu	R4GET2 (R4GET2_DANRE)	Ketimine reductase mu-crystallin
1520609711	27.378	0.0015857	*ctsc*	cathepsin C	A0A0R4IK18 (A0A0R4IK18_DANRE)	Cathepsin C
1042322793	0.58625	0.034228	*dpys*	dihydropyrimidinase	B1H1J6 (B1H1J6_DANRE)	Dihydropyrimidinase
1709641367	18.845	0.01883	*gbe1b*	glucan (1,4-alpha-), branching enzyme 1b	A0A0R4IN76 (A0A0R4IN76_DANRE)	1,4-alpha-glucan branching enzyme
1520564697	0.85321	0.0085193	*gstm.2*	glutathione S-transferase mu tandem duplicate 2	A8KBR8 (A8KBR8_DANRE)	Glutathione transferase
1520623410	24.176	0.00014822	*lrp1ab*	low density lipoprotein receptor-related protein 1Ab	F1QY34 (F1QY34_DANRE)	Low density lipoprotein receptor-related protein 1Ab
317621901	0.51471	0.013027	*pebp1*	phosphatidylethanolamine binding protein 1	Q6NYS4 (Q6NYS4_DANRE)	Phosphatidylethanolamine binding protein
1764642143	24.524	0.0017155	*prdx6*	peroxiredoxin 6	A0A2R8QA71 (A0A2R8QA71_DANRE)	Peroxiredoxin 6
1764586426	0.66848	0.015548	*prps1b*	phosphoribosyl pyrophosphate synthetase 1B	Q08CA5 (Q08CA5_DANRE)	Ribose-phosphate diphosphokinase
51316504	0.23867	0.02221	*rpl19*	ribosomal protein L19	Q6P5L3 (RL19_DANRE)	60S ribosomal protein L19
1042249995	25.393	0.0063035	*selenbp1*	selenium binding protein 1	A0A286Y9S7 (A0A286Y9S7_DANRE)	Methanethiol oxidase
1520574377	0.47891	0.0075745	*tktb*	transketolase b	Q7T2Q9 (Q7T2Q9_DANRE)	Transketolase

Table S5: Decreased female proteins of C30 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
0.42915	-12.204	0.015347	*agla*	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase a	A0A0R4IV67 (A0A0R4IV67_DANRE)	4-alpha-glucanotransferase
0.79903	-0.32368	0.011163	*atp5fa1*	ATP synthase F1 subunit alpha	Q08BA1 (Q08BA1_DANRE)	ATP synthase subunit alpha
0.66427	-0.59017	0.02868	*atp5pd*	ATP synthase peripheral stalk subunit d	Q6PC77 (Q6PC77_DANRE)	ATP synthase subunit d, mitochondrial
0.38515	-13.765	0.035454	*cbsb*	cystathionine beta-synthase b	F1QIC0 (F1QIC0_DANRE)	Cystathionine beta-synthase
0.74665	-0.4215	0.031885	*ddx19b*	DEAD-box helicase 19b		
0.18304	-24.497	0.00051551	*dgki*	diacylglycerol kinase, iota	E7F577 (E7F577_DANRE)	Diacylglycerol kinase
0.14434	-27.925	0.0066713	*gdi2*	GDP dissociation inhibitor 2	Q7ZVL9 (Q7ZVL9_DANRE)	Rab GDP dissociation inhibitor
0.76101	-0.39401	0.031283	*krt4*	keratin 4	A9JRS6 (A9JRS6_DANRE)	Keratin 4
0.7714	-0.37445	0.035064	*pgk1*	phosphoglycerate kinase 1	F1QXV8 (F1QXV8_DANRE)	Phosphoglycerate kinase
0.74792	-0.41904	0.031502	*pitrm1*	pitrilysin metallopeptidase 1	F1QNA0 (F1QNA0_DANRE)	Pitrilysin metalloproteinase 1
0.46799	-10.955	0.031971	*ppat*	phosphoribosyl pyrophosphate amidotransferase	Q5RG54 (Q5RG54_DANRE)	Amidophosphoribosyltransferase
0.71168	-0.4907	0.0038107	*ppp2cb*	protein phosphatase 2, catalytic subunit, beta isozyme	Q803G3 (Q803G3_DANRE)	Serine/threonine-protein phosphatase
0.80529	-0.31243	0.037728	*psmc6*	proteasome 26S subunit, ATPase 6	Q6DRD2 (Q6DRD2_DANRE)	26S protease regulatory subunit S10B
0.86741	-0.20521	0.039856	*si:ch73-265h17.1*	si:ch73-265h17.1		
0.86621	-0.20722	0.014819	*u2af2b*	U2 small nuclear RNA auxiliary factor 2b	A0A0R4IMP0 (A0A0R4IMP0_DANRE)	U2 snRNP auxiliary factor large subunit

Table S6: Increased female proteins of C30 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1764647109	0.2206	0.048118	*abhd14b*	abhydrolase domain containing 14B	Q7T387 (Q7T387_DANRE)	Abhydrolase domain-containing 14B
1520629504	0.4914	0.040207	*abhd14b*	abhydrolase domain containing 14B	Q7T387 (Q7T387_DANRE)	Abhydrolase domain-containing 14B
1764625327	0.93572	0.035202	*adpgk2*	ADP-dependent glucokinase 2	E7F6M4 (E7F6M4_DANRE)	ADP-dependent glucokinase 2
1520585839	0.65305	0.032801	*ak2*	adenylate kinase 2	A0A0F6PK88 (A0A0F6PK88_DANRE)	Adenylate kinase 2, mitochondrial
1042294676	0.95625	0.007531	*aldh8a1*	aldehyde dehydrogenase 8 family, member A1	Q66I21 (AL8A1_DANRE)	2-aminomuconic semialdehyde dehydrogenase
1764647220	0.3636	0.024629	*capza1a*	capping actin protein of muscle Z-line subunit alpha 1a	Q6NWK1 (Q6NWK1_DANRE)	F-actin-capping protein subunit alpha
1465188361	10.625	0.040241	*eef1a1l1*	eukaryotic translation elongation factor 1 alpha 1, like 1	Q92005 (EF1A_DANRE)	Elongation factor 1-alpha
1520571406	0.6229	0.033589	*eef2b*	eukaryotic translation elongation factor 2b	A0A286Y8X9 (A0A286Y8X9_DANRE)	Eukaryotic translation elongation factor 2b
576892467	0.2327	0.011036	*fam114a2*	family with sequence similarity 114 member A2	E9QJA6 (E9QJA6_DANRE)	Family with sequence similarity 114 member A2
1503207150	0.71717	0.041536	*gak*	cyclin G associated kinase	F1QH49 (F1QH49_DANRE)	Cyclin G-associated kinase
1520611216	0.5715	0.036742	*git2b*	G protein-coupled receptor kinase interacting ArfGAP 2b	F1QCL0 (F1QCL0_DANRE)	G protein-coupled receptor kinase-interacting ArfGAP 2b
1764603242	0.37739	0.039417	*glrx5*	glutaredoxin 5 homolog (S. cerevisiae)	Q6PBM1 (GLRX5_DANRE)	Glutaredoxin-related protein 5, mitochondrial
807066251	0.60535	0.020967	*gpt*	glutamic--pyruvic transaminase	A2BII1 (A2BII1_DANRE)	Alanine aminotransferase 2-like
470011943	24.091	0.0031896	*gstt1b*	glutathione S-transferase theta 1b	Q6PH41 (Q6PH41_DANRE)	Glutathione transferase
1503225243	0.29968	0.021214	*hsp90aa1.2*	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	Q5RG12 (Q5RG12_DANRE)	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2
1709608926	0.30968	0.0059029	*hspa8*	heat shock protein 8	Q90473 (HSP7C_DANRE)	Heat shock cognate 71 kDa protein
1764650030	13.786	0.050201	*lmna*	lamin A	A0A2R8QPG3 (A0A2R8QPG3_DANRE)	Lamin A
1520583194	0.69427	0.0073163	*mpc1*	mitochondrial pyruvate carrier 1	F1Q6Z3 (F1Q6Z3_DANRE)	Mitochondrial pyruvate carrier
1503283962	0.44736	0.006908	*mtch2*	mitochondrial carrier homolog 2	Q6NW52 (MSRB2_DANRE)	Metionina-R-sulfóxido redutase B2, mitocondrial
1764588846	0.34971	0.0045347	*phkb*	phosphorylase kinase, beta	F1Q6F0 (F1Q6F0_DANRE)	Phosphorylase b kinase regulatory subunit
1520495810	0.61073	0.016245	*pudp*	pseudouridine 5'-phosphatase	F1RE99 (F1RE99_DANRE)	Pseudouridine 5'-phosphatase
1709634865	0.70193	0.035652	*rpl11*	ribosomal protein L11	Q6IQI6 (Q6IQI6_DANRE)	60S ribosomal protein L11
1764599932	0.73705	0.037198	*rpn1*	ribophorin I	Q8AYB9 (Q8AYB9_DANRE)	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1
1042267969	0.37019	0.032567	*spire1a*	spire-type actin nucleation factor 1a	A0A0R4IHM3 (A0A0R4IHM3_DANRE)	Spire-type actin nucleation factor 1a
1764642428	0.54451	0.029894	*tkfc*	triokinase/FMN cyclase	F1Q8Q6 (F1Q8Q6_DANRE)	ATP-dependent dihydroxyacetone kinase
1042349427	31.339	0.010709	*tubb2b*	tubulin, beta 2b	A0A0R4IPH2 (A0A0R4IPH2_DANRE)	Tubulin beta chain

Table S7: Decreased male proteins of C30 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1520548750	-0.69378	0.014804	*bcat1*	branched chain amino-acid transaminase 1, cytosolic	Q5RHB8 (Q5RHB8_DANRE)	Branched-chain-amino-acid aminotransferase
1042314542	-11.336	0.050726	*cirbpb*	cold inducible RNA binding protein b	F1R6L3 (F1R6L3_DANRE)	Cold-inducible RNA-binding protein b
1764639828	-34.227	0.049456	*col6a3*	collagen, type VI, alpha 3	F1Q4X1 (F1Q4X1_DANRE)	Collagen, type VI, alpha 3
1520519603	-14.496	0.012455	*comta*	catechol-O-methyltransferase a	F4ZGF2 (COMTA_DANRE)	Catechol O-methyltransferase A
21552717	-15.908	0.038882	*ctsd*	cathepsin D	Q8AWD9 (Q8AWD9_DANRE)	Cathepsin D
1042327048	-20.469	0.047657	*cyp2p9*	cytochrome P450, family 2, subfamily P, polypeptide 9	Q5TZ85 (Q5TZ85_DANRE)	Cytochrome P450, family 2, subfamily P, polypeptide 9
1764649887	-0.84152	0.032417	*cyp4t8*	cytochrome P450, family 4, subfamily T, polypeptide 8	E7FB64 (E7FB64_DANRE)	Cytochrome P450, family 4, subfamily T, polypeptide 8
1520581893	-20.699	0.0030745	*dync1h1*	dynein, cytoplasmic 1, heavy chain 1	Q2LEK1 (Q2LEK1_DANRE)	Dynein cytoplasmic 1 heavy chain 1
1709641367	-16.349	0.018372	*gbe1b*	glucan (1,4-alpha-), branching enzyme 1b	A0A0R4IN76 (A0A0R4IN76_DANRE)	1,4-alpha-glucan branching enzyme
1042303721	-17.774	0.044595	*hnrnpdl*	heterogeneous nuclear ribonucleoprotein D-like	Q7SXN2 (Q7SXN2_DANRE)	Heterogeneous nuclear ribonucleoprotein D-like
1709663889	-18.059	0.027882	*kars1*	lysyl-tRNA synthetase 1	Q6DHE6 (Q6DHE6_DANRE)	Lysine--tRNA ligase
1764613484	-29.694	0.016227	*lgals9l1*	lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1	Q6TGN3 (Q6TGN3_DANRE)	Galectin
1764634499	-19.343	0.019781	*lman2*	lectin, mannose-binding 2	A5PMC4 (A5PMC4_DANRE)	Lectin, mannose-binding 2
1503274858	-0.53089	0.0049639	*ndrg2*	NDRG family member 2	Q5PR98 (NDRG2_DANRE)	Protein NDRG2
576888011	-0.63525	0.033762	*npepl1*	aminopeptidase like 1	F1Q6S1 (F1Q6S1_DANRE)	Aminopeptidase-like 1
1764634190	-30.406	0.0020352	*nsdhl*	NAD(P) dependent steroid dehydrogenase-like	Q566U2 (Q566U2_DANRE)	NAD(P) dependent steroid dehydrogenase-like
1764644722	-0.43799	0.030684	*pygb*	phosphorylase, glycogen; brain	F1R5X0 (F1R5X0_DANRE)	Alpha-1,4 glucan phosphorylase
1520550440	-10.699	0.046068	*rab5c*	RAB5C, member RAS oncogene family	Q7ZVP2 (Q7ZVP2_DANRE)	RAB5C, member RAS oncogene family
591290568	-21.914	0.018667	*rab8a*	RAB8A, member RAS oncogene family	A4FVK4 (A4FVK4_DANRE)	RAB8A, member RAS oncogene family
1042243878	-0.54741	0.039049	*rbp5*	retinol binding protein 1a, cellular	A0A2R8QHL7 (A0A2R8QHL7_DANRE)	Retinol-binding protein 1a, cellular
1503240411	-0.72364	0.042777	*rpl13*	ribosomal protein L13	Q90Z10 (RL13_DANRE)	60S ribosomal protein L13
1520523952	-26.847	0.0014058	*rpl28*	ribosomal protein L28	A0A286YBP4 (A0A286YBP4_DANRE)	60S ribosomal protein L28
1503285744	-13.456	0.032894	*rrbp1b*	ribosome binding protein 1b	A0A0R4IGW7 (A0A0R4IGW7_DANRE)	Ribosome-binding protein 1b
1764596072	-0.71916	0.040254	*sec61b*	SEC61 translocon subunit beta	Q6DGH3 (Q6DGH3_DANRE)	Protein transport protein Sec61 subunit beta
1503222480	-0.60472	0.045237	*ubc*	ubiquitin C	F1QHN6 (F1QHN6_DANRE)	Ubiquitin C
1764640226	-0.35623	0.019411	*uchl3*	ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	Q504C0 (Q504C0_DANRE)	Ubiquitin carboxyl-terminal hydrolase
1520566949	-11.542	0.046906	*zgc:163057*	zgc:163057	A3KP86 (A3KP86_DANRE)	Zgc:163057
1709636714	-25.994	0.0025438	*zgc:64106*	zgc:64106	F1Q911 (F1Q911_DANRE)	Zgc:64106

Table S8: Increased male proteins of C30 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1042304313	23.655	0.00041984	*dlat*	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	B3DIV6 (B3DIV6_DANRE)	Acetyltransferase component of pyruvate dehydrogenase complex
1709637461	15.438	0.03505	*gars1*	glycyl-tRNA synthetase 1	F1QUV7 (F1QUV7_DANRE)	Diadenosine tetraphosphate synthetase
1042319535	11.315	0.037141	*gldc*	glycine dehydrogenase (decarboxylating)	Q6PFN9 (Q6PFN9_DANRE)	Glycine dehydrogenase (aminomethyl-transferring)
1764591559	1.702	0.049554	*gldc*	glycine dehydrogenase (decarboxylating)	Q6PFN9 (Q6PFN9_DANRE)	Glycine dehydrogenase (aminomethyl-transferring)
1709657502	0.20602	0.0098952	*histh1l1*	histone H1 like1	A3KPR3 (A3KPR3_DANRE)	Histone H1 like
1520498115	26.423	0.0016795	*msrb2*	methionine sulfoxide reductase B2	Q6NW52 (MSRB2_DANRE)	Metionina-R-sulfóxido redutase B2, mitocondrial
1520594148	27.591	0.036198	*nt5c2b*	5'-nucleotidase, cytosolic IIb	F1QAK5 (F1QAK5_DANRE)	5'-nucleotidase, cytosolic IIb
1709640083	0.51759	0.0031898	*pdhb*	pyruvate dehydrogenase E1 subunit beta	Q7T368 (Q7T368_DANRE)	Pyruvate dehydrogenase E1 component subunit beta
1764594065	0.78468	0.025803	*shmt1*	serine hydroxymethyltransferase 1 (soluble)	Q2TL58 (Q2TL58_DANRE)	Serine hydroxymethyltransferase

Table S9: Decreased female proteins of CE25 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1503187710	-0.82711	0.019537	*cirbpb*	cold inducible RNA binding protein b	F1R6L3 (F1R6L3_DANRE)	Cold-inducible RNA-binding protein b
1561967988	-0.96881	0.014149	*ctsd*	cathepsin D	Q8AWD9 (Q8AWD9_DANRE)	Cathepsin D
1709607954	-22.648	0.0019277	*cyb5r3*	cytochrome b5 reductase 3	Q6NYE6 (Q6NYE6_DANRE)	NADH-cytochrome b5 reductase
1764580684	-0.38189	0.024039	*ddx39ab*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39Ab	F6NVB9 (F6NVB9_DANRE)	RNA helicase
576886061	-0.44269	0.021102	*eftud2*	elongation factor Tu GTP binding domain containing 2	F1Q6N0 (F1Q6N0_DANRE)	116 kDa U5 small nuclear ribonucleoprotein component
1042324110	-0.63904	0.040014	*eppk1*	epiplakin 1	I3ISA6 (I3ISA6_DANRE)	Epiplakin 1
1764595867	-0.67418	0.025484	*fbl*	fibrillarin	Q7ZTZ4 (Q7ZTZ4_DANRE)	Fibrillarin
1520603684	-0.59928	0.039563	*fubp3*	far upstream element (FUSE) binding protein 3	E7EYA8 (E7EYA8_DANRE)	Far upstream element (FUSE)-binding protein 3
1042300172	-0.16153	0.045307	*gbe1b*	glucan (1,4-alpha-), branching enzyme 1b	A0A0R4IN76 (A0A0R4IN76_DANRE)	1,4-alpha-glucan branching enzyme
1764609674	-0.45854	0.044961	*hnrnpa1b*	heterogeneous nuclear ribonucleoprotein A1b	Q803K3 (Q803K3_DANRE)	Heterogeneous nuclear ribonucleoprotein A1
1520614198	-11.363	0.00065898	*hnrnpdl*	heterogeneous nuclear ribonucleoprotein D-like	Q7SXN2 (Q7SXN2_DANRE)	Heterogeneous nuclear ribonucleoprotein D-like
1503220341	-0.45141	0.043846	*hnrnpl*	heterogeneous nuclear ribonucleoprotein L	A0A2R8RN29 (A0A2R8RN29_DANRE)	Heterogeneous nuclear ribonucleoprotein L
1764646905	-23.306	0.007264	*krt4*	keratin 4	A9JRS6 (A9JRS6_DANRE)	Keratin 4
1709636008	-0.84764	0.010109	*ncl*	nucleolin	F1R6L6 (F1R6L6_DANRE)	Nucleolin
1520547465	-0.62267	0.032579	*nop58*	NOP58 ribonucleoprotein homolog (yeast)	Q6P6X6 (Q6P6X6_DANRE)	NOP58 ribonucleoprotein homolog (yeast)
1503204954	-0.5484	0.011755	*papss2b*	3'-phosphoadenosine 5'-phosphosulfate synthase 2b	Q802U9 (Q802U9_DANRE)	3'-phosphoadenosine-5'-phosphosulfate synthase
1520500282	-1.912	0.0077705	*phyhd1*	phytanoyl-CoA dioxygenase domain containing 1	Q5U3U0 (PHYD1_DANRE)	Phytanoyl-CoA dioxygenase domain-containing protein 1
1709641385	-0.66432	0.018912	*prpf8*	pre-mRNA processing factor 8	A0A0R4J8E6 (A0A0R4J8E6_DANRE)	Pre-mRNA-processing factor 8
1520528282	-0.95912	0.028257	*serbp1a*	SERPINE1 mRNA binding protein 1a	F1Q5Q3 (F1Q5Q3_DANRE)	SERPINE1 mRNA-binding protein 1a
308321147	-0.28084	0.030102	*snrpd3l*	small nuclear ribonucleoprotein D3 polypeptide, like	Q7ZVB5 (Q7ZVB5_DANRE)	Small nuclear ribonucleoprotein Sm D3
1042348127	-0.98801	0.031249	*ugdh*	UDP-glucose 6-dehydrogenase	A8WGP7 (A8WGP7_DANRE)	UDP-glucose 6-dehydrogenase
1503220843	-1.374	0.013491	*ugt2a1*	UDP glucuronosyltransferase 2 family, polypeptide A1	Q6DHD1 (Q6DHD1_DANRE)	UDP-glucuronosyltransferase
1520554656	-1.614	0.030334	*zgc:92040*	zgc:92040	F1QCH4 (F1QCH4_DANRE)	Proline dehydrogenase

Table S10 (Part 1): Increased female proteins of CE25 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1764603005	14.349	0.0075388	*acat2*	acetyl-CoA acetyltransferase 2	Q5SPA3 (Q5SPA3_DANRE)	Acetyl-CoA acetyltransferase 2
576892078	0.44536	0.0089713	*aco1*	aconitase 1, soluble	F1QM42 (F1QM42_DANRE)	Citrate hydro-lyase
1709638277	0.60555	0.020326	*aco1*	aconitase 1, soluble	F1QM42 (F1QM42_DANRE)	Citrate hydro-lyase
1764598791	13.423	0.0089761	*acss2l*	acyl-CoA synthetase short chain family member 2 like	F1QQH3 (F1QQH3_DANRE)	Acetyl-coenzyme A synthetase
1709638785	1.059	0.013557	*agmat*	agmatine ureohydrolase (agmatinase)	F1QJZ3 (F1QJZ3_DANRE)	Agmatine ureohydrolase (agmatinase)
1764606686	0.50208	0.018653	*aldh7a1*	aldehyde dehydrogenase 7 family, member A1	F1QR17 (F1QR17_DANRE)	Aldehyde dehydrogenase 7 family, member A1
1764616630	0.2441	0.0024213	*ap2a1*	adaptor related protein complex 2 subunit alpha 1	X1WD71 (X1WD71_DANRE)	AP-2 complex subunit alpha
1520523601	10.589	0.03794	*bhmt*	betaine-homocysteine methyltransferase	Q32LQ4 (BHMT1_DANRE)	Betaine--homocysteine S-methyltransferase 1
1764642385	0.24058	0.03371	*cct7*	chaperonin containing TCP1, subunit 7 (eta)	B3DKJ0 (B3DKJ0_DANRE)	T-complex protein 1 subunit eta
1764618472	0.76365	0.0026825	*crym*	crystallin, mu	R4GET2 (R4GET2_DANRE)	Ketimine reductase mu-crystallin
1764646950	0.6786	0.041293	*cth*	cystathionase (cystathionine gamma-lyase)	F1QPF7 (F1QPF7_DANRE)	Cystathionine gamma-lyase
1520510047	0.69425	0.036764	*dbt*	dihydrolipoamide branched chain transacylase E2	A0A0R4INZ8 (A0A0R4INZ8_DANRE)	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
1520603632	0.27626	0.012468	*ddrgk1*	DDRGK domain containing 1	Q6P0E5 (DDRGK_DANRE)	DDRGK domain-containing protein 1
1709637612	16.172	0.029293	*ddx46*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46	Q4TVV3 (DDX46_DANRE)	Probable ATP-dependent RNA helicase DDX46
1764611993	10.367	0.045568	*dlat*	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	B3DIV6 (B3DIV6_DANRE)	Acetyltransferase component of pyruvate dehydrogenase complex
318048367	0.67018	0.036023	*echs1*	enoyl CoA hydratase, short chain, 1, mitochondrial	Q7ZZ04 (Q7ZZ04_DANRE)	Enoyl CoA hydratase, short chain, 1, mitochondrial
1042339744	0.24898	0.0224	*eif3f*	eukaryotic translation initiation factor 3, subunit F	Q6AXJ2 (EI3HA_DANRE)	Eukaryotic translation initiation factor 3 subunit H-A
1042290513	0.48963	0.041706	*eno3*	enolase 3, (beta, muscle)	Q568G3 (Q568G3_DANRE)	2-phospho-D-glycerate hydro-lyase
318102079	0.44243	0.009591	*etfa*	electron transfer flavoprotein subunit alpha	Q7ZUS9 (Q7ZUS9_DANRE)	Electron transfer flavoprotein subunit alpha
1520609153	0.70263	0.047488	*fbp1a*	fructose-1,6-bisphosphatase 1a	Q6PFT1 (Q6PFT1_DANRE)	Fructose-bisphosphatase
1764589250	0.55922	0.013215	*ftcd*	formimidoyltransferase cyclodeaminase	F1QZ40 (F1QZ40_DANRE)	Formimidoyltetrahydrofolate cyclodeaminase
1764630929	0.35644	0.016905	*gfpt1*	glutamine--fructose-6-phosphate transaminase 1	Q3S344 (Q3S344_DANRE)	Glutamine--fructose-6-phosphate transaminase (isomerizing)
1764618858	0.31301	0.039442	*glrx3*	glutaredoxin 3	Q5XJ54 (GLRX3_DANRE)	Glutaredoxin 3
1764600198	0.22885	0.023721	*gmppb*	GDP-mannose pyrophosphorylase B	Q6DBU5 (GMPPB_DANRE)	Mannose-1-phosphate guanyltransferase beta
1042352596	10.123	0.022972	*got1*	glutamic-oxaloacetic transaminase 1, soluble	Q7ZUW8 (Q7ZUW8_DANRE)	Aspartate aminotransferase
576887209	0.63838	0.047824	*gpd1b*	glycerol-3-phosphate dehydrogenase 1b		
576893146	0.65445	0.0039006	*gpia*	glucose-6-phosphate isomerase a	Q8QFU2 (Q8QFU2_DANRE)	Glucose-6-phosphate isomerase
507182879	15.729	0.034939	*gpia*	glucose-6-phosphate isomerase a	Q8QFU2 (Q8QFU2_DANRE)	Glucose-6-phosphate isomerase
1764596346	0.77451	0.017314	*hgd*	homogentisate 1,2-dioxygenase	Q6P2V4 (Q6P2V4_DANRE)	Homogentisate 1,2-dioxygenase
1764639993	0.6539	0.0024349	*hpxa*	hemopexin a	Q6PHG2 (HEMO_DANRE)	Hemopexin
1503225243	31.174	0.042652	*hsp90aa1.2*	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	Q5RG12 (Q5RG12_DANRE)	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2
1465648784	0.5957	0.032864	*hsp90ab1*	heat shock protein 90, alpha (cytosolic), class B member 1	O57521 (HS90B_DANRE)	Heat shock protein HSP 90-beta
1023043046	0.81589	0.027272	*hsp90b1*	heat shock protein 90, beta (grp94), member 1	A0A286YAP4 (A0A286YAP4_DANRE)	Heat shock protein 90, beta (grp94), member 1
1520603690	0.73864	0.0096655	*hspa5*	heat shock protein 5	Q6P3L3 (Q6P3L3_DANRE)	78 kDa glucose-regulated protein

Table S10 (Part 2): Increased female proteins of CE25 x CE30

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
576891777	0.81692	0.016212	*hspa5*	heat shock protein 5	Q6P3L3 (Q6P3L3_DANRE)	78 kDa glucose-regulated protein
1503271866	0.38466	0.029678	*hspa8*	heat shock protein 8	Q90473 (HSP7C_DANRE)	Heat shock cognate 71 kDa protein
730915996	0.52333	0.034903	*hspa8*	heat shock protein 8	Q90473 (HSP7C_DANRE)	Heat shock cognate 71 kDa protein
1520562597	0.54116	0.0069879	*hspd1*	heat shock 60 protein 1	Q803B0 (Q803B0_DANRE)	60 kDa chaperonin
576886900	0.53124	0.031977	*hspd1*	heat shock 60 protein 1	Q803B0 (Q803B0_DANRE)	60 kDa chaperonin
1764600189	0.81243	0.043717	*manf*	mesencephalic astrocyte-derived neurotrophic factor	F1QDQ5 (F1QDQ5_DANRE)	Mesencephalic astrocyte-derived neurotrophic factor
1520608997	0.7055	0.042791	*mccc2*	methylcrotonyl-CoA carboxylase subunit 2	A2BIN5 (A2BIN5_DANRE)	Methylcrotonoyl-CoA carboxylase 2 (beta)
1764625561	11.792	0.018063	*mthfd1b*	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1b	K7DYA3 (K7DYA3_DANRE)	Methylenetetrahydrofolate dehydrogenase (NADP+-dependent) 1b
576888917	14.427	0.026171	*mthfd1b*	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1b	K7DYA3 (K7DYA3_DANRE)	Methylenetetrahydrofolate dehydrogenase (NADP+-dependent) 1b
1520599409	0.49374	0.0090733	*mttp*	microsomal triglyceride transfer protein	A0A0R4IVV0 (MTP_DANRE)	Microsomal triglyceride transfer protein large subunit
1520613516	0.971	0.046749	*pdhb*	pyruvate dehydrogenase E1 subunit beta	Q7T368 (Q7T368_DANRE)	Pyruvate dehydrogenase E1 component subunit beta
1764596175	0.59489	0.0095639	*pdia4*	protein disulfide isomerase family A, member 4	Q6P3I1 (Q6P3I1_DANRE)	Protein disulfide-isomerase A4
1042323120	0.60824	0.021873	*pdia4*	protein disulfide isomerase family A, member 4	Q6P3I1 (Q6P3I1_DANRE)	Protein disulfide-isomerase A4
1764644875	0.83232	0.0024343	*pgam1a*	phosphoglycerate mutase 1a	Q7SZR4 (Q7SZR4_DANRE)	Phosphoglycerate mutase
1709638388	28.614	0.00070003	*plaa*	phospholipase A2-activating protein	F1QCT6 (F1QCT6_DANRE)	Phospholipase A2-activating protein
1503215387	0.89002	0.00042515	*pmt*	phosphoethanolamine methyltransferase	A0A2R8QIW3 (A0A2R8QIW3_DANRE)	Phosphoethanolamine methyltransferase
1709635732	19.016	0.048743	*pmt*	phosphoethanolamine methyltransferase	A0A2R8QIW3 (A0A2R8QIW3_DANRE)	Phosphoethanolamine methyltransferase
317574221	0.46604	0.025794	*ppp2cb*	protein phosphatase 2, catalytic subunit, beta isozyme	Q803G3 (Q803G3_DANRE)	Serine/threonine-protein phosphatase
318103394	0.38531	0.041775	*psmb2*	proteasome 20S subunit beta 2	Q6DHI9 (Q6DHI9_DANRE)	Proteasome subunit beta
1764598521	0.30163	0.013491	*ralaa*	v-ral simian leukemia viral oncogene homolog Aa (ras related)	Q7T383 (Q7T383_DANRE)	Small monomeric GTPase
1503239884	1.1	0.0054297	*shmt1*	serine hydroxymethyltransferase 1 (soluble)	Q2TL58 (Q2TL58_DANRE)	Serine hydroxymethyltransferase
576887288	0.49253	0.0077999	*snx12*	sorting nexin 12	F1RDJ8 (F1RDJ8_DANRE)	Sorting nexin 12
1764630441	0.71948	0.015898	*tuba5*	tubulin alpha 5	Q6TNP9 (Q6TNP9_DANRE)	Tubulin alpha chain
1764586664	0.38458	0.030382	*ugp2a*	UDP-glucose pyrophosphorylase 2a	B8JMZ1 (B8JMZ1_DANRE)	UTP--glucose-1-phosphate uridylyltransferase
1503258009	0.46124	0.042269	*unm_hu7910*	un-named hu7910	A0A2R8QJF6 (A0A2R8QJF6_DANRE)	Un-named hu7910

Table S11: Decreased male proteins of CE25 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
576888699	-0.87445	0.01733	*agxta*	alanine--glyoxylate and serine--pyruvate aminotransferase a	F1QY24 (F1QY24_DANRE)	Serine--pyruvate aminotransferase
308321640	-10.108	0.048417	*alad*	aminolevulinate dehydratase	A0A0R4IPV1 (A0A0R4IPV1_DANRE)	Delta-aminolevulinic acid dehydratase
1709639416	-19.756	0.038318	*chdh*	choline dehydrogenase	A0A2R8QDT4 (A0A2R8QDT4_DANRE)	Choline dehydrogenase
1042314542	-15.991	0.0058734	*cirbpa*	cold inducible RNA binding protein a	Q566W6 (Q566W6_DANRE)	Cold-inducible RNA-binding protein a
1503187710	-25.682	0.00099622	*cirbpb*	cold inducible RNA binding protein b	F1R6L3 (F1R6L3_DANRE)	Cold-inducible RNA-binding protein b
1520610086	-27.564	0.026037	*col1a1a*	collagen, type I, alpha 1a	Q6P4U1 (Q6P4U1_DANRE)	Collagen, type I, alpha 1
1042346791	-27.584	0.015916	*crata*	carnitine O-acetyltransferase a	A2CF48 (A2CF48_DANRE)	Carnitine O-acetyltransferase a
1520556062	-23.234	0.0025896	*dhtkd1*	dehydrogenase E1 and transketolase domain containing 1	Q5PRA2 (DHTK1_DANRE)	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial
1520581893	-16.073	0.018737	*dync1h1*	dynein, cytoplasmic 1, heavy chain 1	Q2LEK1 (Q2LEK1_DANRE)	Dynein cytoplasmic 1 heavy chain 1
1709608238	-31.812	0.0079646	*eif3k*	eukaryotic translation initiation factor 3, subunit K	Q567V6 (EIF3K_DANRE)	Eukaryotic translation initiation factor 3 subunit K
1764645225	-0.67468	0.0013749	*eif3s6ip*	eukaryotic translation initiation factor 3, subunit 6 interacting protein	Q7T2A5 (EIF3L_DANRE)	Eukaryotic translation initiation factor 3 subunit L
1709641367	-15.635	0.031331	*gbe1b*	glucan (1,4-alpha-), branching enzyme 1b	A0A0R4IN76 (A0A0R4IN76_DANRE)	1,4-alpha-glucan branching enzyme
1503252416	-14.503	0.038413	*hao1*	hydroxyacid oxidase (glycolate oxidase) 1	K7DY73 (K7DY73_DANRE)	Hydroxyacid oxidase (glycolate oxidase) 1
1503185578	-12.473	0.037342	*hdlbpa*	high density lipoprotein binding protein a	F1R9Y8 (F1R9Y8_DANRE)	High density lipoprotein-binding protein a
1503273576	-2.394	0.00017041	*hnmpdl*	heterogeneous nuclear ribonucleoprotein D-like	Q7SXN2 (Q7SXN2_DANRE)	Heterogeneous nuclear ribonucleoprotein D-like
1042346064	-26.132	8,30E-02	*hnmpl*	heterogeneous nuclear ribonucleoprotein L	A0A2R8RN29 (A0A2R8RN29_DANRE)	Heterogeneous nuclear ribonucleoprotein L
1764630626	-0.56374	0.012052	*hsd17b4*	hydroxysteroid (17-beta) dehydrogenase 4	Q8AYH1 (Q8AYH1_DANRE)	17-beta-hydroxysteroid dehydrogenase type 4
1709663889	-16.621	0.03083	*kars1*	lysyl-tRNA synthetase 1	Q6DHE6 (Q6DHE6_DANRE)	Lysine--tRNA ligase
1764639811	-0.35394	0.003156	*lcp1*	lymphocyte cytosolic protein 1 (L-plastin)	Q6P698 (PLSL_DANRE)	Plastin-2
1520623410	-24.542	0.00038418	*lrp1ab*	low density lipoprotein receptor-related protein 1Ab	F1QY34 (F1QY34_DANRE)	Low density lipoprotein receptor-related protein 1Ab
1520595933	-19.417	0.04277	*mtch2*	mitochondrial carrier homolog 2	Q9PUL9 (Q9PUL9_DANRE)	Mitochondrial carrier homolog 2
1520596518	-16.348	0.01332	*ndufs1*	NADH:ubiquinone oxidoreductase core subunit S1	Q5RKM2 (Q5RKM2_DANRE)	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial
1520500282	-32.205	0.032406	*phyhd1*	phytanoyl-CoA dioxygenase domain containing 1	Q5U3U0 (PHYD1_DANRE)	Phytanoyl-CoA dioxygenase domain-containing protein 1
1520603424	-22.523	0.029091	*psmd7*	proteasome 26S subunit, non-ATPase 7	A0A2R8Q5H4 (A0A2R8Q5H4_DANRE)	Proteasome 26S subunit, non-ATPase 7
1709661503	-16.099	0.017665	*pter*	phosphotriesterase related	Q0P3Z2 (PTER_DANRE)	Phosphotriesterase-related protein
1520523952	-2.554	0.00034143	*rpl28*	ribosomal protein L28	A0A286YBP4 (A0A286YBP4_DANRE)	60S ribosomal protein L28
1503288287	-11.256	0.033344	*rplp2*	ribosomal protein, large P2	Q6PBJ9 (Q6PBJ9_DANRE)	60S acidic ribosomal protein P2
1503285744	-14.073	0.024851	*rrbp1b*	ribosome binding protein 1b	A0A0R4IGW7 (A0A0R4IGW7_DANRE)	Ribosome-binding protein 1b
1520534824	-0.84039	0.024325	*scp2a*	sterol carrier protein 2a	Q6P4V5 (Q6P4V5_DANRE)	Acetyl-CoA C-myristoyltransferase
1709635973	-24.196	8,65E-01	*serpinc1*	serpin peptidase inhibitor, clade C (antithrombin), member 1	Q8AYE3 (Q8AYE3_DANRE)	Antithrombin
1764605424	-0.64502	0.036752	*snd1*	staphylococcal nuclease and tudor domain containing 1	Q5RGK8 (Q5RGK8_DANRE)	Staphylococcal nuclease domain-containing protein
1709672503	-26.668	0.015669	*srsf5a*	serine and arginine rich splicing factor 5a	A0A2R8Q2Y4 (A0A2R8Q2Y4_DANRE)	Serine and arginine-rich-splicing factor 5a
1503222480	-0.47215	0.02732	*uba52*	ubiquitin A-52 residue ribosomal protein fusion product 1	Q3B7P7 (Q3B7P7_DANRE)	60S ribosomal protein L40

Table S12: Increased male proteins of CE25 x CE30.

ID	log2(FC)	raw.pval	Gene Symbol	Gene Name	UniProt Code	UniProt Name
1520608198	25.729	0.00028396	*abat*	4-aminobutyrate aminotransferase	I3IRW7 (I3IRW7_DANRE)	(S)-3-amino-2-methylpropionate transaminase
1764591559	13.986	0.047596	*gldc*	glycine dehydrogenase (decarboxylating)	Q6PFN9 (Q6PFN9_DANRE)	Glycine dehydrogenase (aminomethyl-transferring)
1503252510	0.67342	0.033211	*mthfd1b*	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1b	K7DYA3 (K7DYA3_DANRE)	Methylenetetrahydrofolate dehydrogenase (NADP+-dependent) 1b
1709608478	23.494	0.0021823	*papss2b*	3'-phosphoadenosine 5'-phosphosulfate synthase 2b	Q802U9 (Q802U9_DANRE)	3'-phosphoadenosine-5'-phosphosulfate synthase
1503217510	0.79781	0.035544	*pgd*	phosphogluconate dehydrogenase	Q803T9 (Q803T9_DANRE)	6-phosphogluconate dehydrogenase, decarboxylating
576886406	0.33954	0.048921	*sdr16c5b*	short chain dehydrogenase/reductase family 16C, member 5b	Q7SZ49 (Q7SZ49_DANRE)	Short chain dehydrogenase/reductase family 16C, member 5
1042337552	2.539	0.0090711	*slc25a10b*	solute carrier family 25 member 10b	E7F238 (E7F238_DANRE)	Solute carrier family 25 member 10

4 DISCUSSÃO GERAL

Neste estudo, o aumento da temperatura, a exposição às saxitoxinas produzidas pela *Raphidiopsis raciborskii* e os efeitos combinados desses dois estressores causaram diversos efeitos adversos em peixes *Rhamdia quelen*. O aumento da temperatura isoladamente, por exemplo, foi capaz de alterar diferentes biomarcadores e o proteoma dos organismos. Diferentemente da maioria dos trabalhos que testam os efeitos da temperatura, no atual estudo os parâmetros foram avaliados separadamente para fêmeas e machos.

Mudanças no período e magnitude da atividade reprodutiva podem ocorrer com o aumento da temperatura, visto que a mesma pode atuar no controle neuroendócrino da reprodução em peixes, agindo em vários níveis do eixo cérebro-pituitária-gônada (MIRANDA et a., 2013; SERVILI et al., 2020). Em fêmeas, o aumento da temperatura aumentou os valores do índice gonadossomático (IGS), um indicativo de período reprodutivo (LOUIZ; BEM-ATTIA; BEM-HASSINE, 2009), mostrando que o aumento da temperatura das águas pode alterar os ciclos reprodutivos para esta espécie neotropical.

Em fêmeas também foi observada a leucopenia, indicando uma alteração de imunidade desses organismos (FISHER et al., 2006). Além dessa redução do numero de leucócitos no sangue, foi observada a redução de proteínas no fígado envolvidas com fagocitose, outro indicativo de alteração no sistema imune em decorrência do aumento da temperatura. Em peixes marinhos da espécie *Dicentrarchus labrax*, leucopenia também foi encontrada, mas em exposição subcrônica de 30 dias à 32°C (ISLAM et al., 2020).

Alterações em proteínas relacionadas ao citoesqueleto, aumento de chaperonas responsáveis pela reparação de danos em proteínas, bem como alterações no processo de produção de energia foram observados em fêmeas. Reorganização do citoesqueleto pode ser uma resposta ao estresse térmico, como observado em fígado de *Platichthys flesus*, um peixe marinho de ambientes temperados, exposto à 12 °C acima do que a sua temperatura ambiente (PÉDRON et al., 2017). Estudos de metabolômica demonstraram, em peixes temperados *Brachymystax lenok* submetidos a 24°C, 8° C acima de seu controle, múltiplas alterações causadas pelo aquecimento das águas, incluindo diversas alterações no

metabolismo de aminoácidos (LIU et al., 2019), como observamos em fêmeas de *Rhamdia quelen*.

Em machos, assim como em fêmeas, as gônadas também se mostraram maiores, apresentando o mesmo indicativo de alteração do ciclo reprodutivo, sugerindo que o aquecimento global pode interferir no desempenho reprodutivo deste peixe, como demonstrado para outros teleósteos (ALIX; KESBU; ANDERSON, 2020). Em machos também foi observado um aumento de glicose, algo descrito para essa espécie por Lermen et al. (2004). No entanto, os autores não diferenciaram o sexo dos animais e no presente trabalho mostramos essa diferenciação de resposta. O aumento de glicose pode indicar um aumento do metabolismo por aumentar a demanda energética, visto que a mesma é considerada um dos substratos para produção de energia (KANG et al., 2021). Outro indicativo desse aumento metabólico é o aumento da atividade hepática da enzima glutationa S-transferase (GST), uma enzima responsável pela metabolização de diferentes compostos (VAN DER OOST; BEYER; VERMEULEN, 2003).

Apesar de requisitar mais glicose, no fígado desses peixes machos foi encontrada uma redução do processo de produção de energia, tanto pela via de respiração celular quanto por meio da beta oxidação. Este resultado levaria a pensar que o organismo estaria em metabolismo anaeróbico pelo uso do lactato, indicador este deste tipo de metabolismo (COATES; SODERHALL, 2020), que não aumentou no plasma desses peixes. Liu et al. (2019) em estudo com peixe de ambiente temperado *Brachymystax lenok* também observou uma redução da produção de energia no fígado dos animais em decorrência do aumento da temperatura. Contudo, os autores também analisaram os metabólitos no plasma, encontrando um aumento de lipídios, que também pode ser considerado uma fonte de energia (TOCHER, 2003). No fígado de machos de *Rhamdia quelen*, foi encontrada redução de proteínas relacionadas à lipídios, principalmente colesterol. Porém, como há mobilização de glicose e outras fontes de energia de um tecido para outro, esses machos podem estar encontrando sua fonte de energia em outros tecidos (SUN et al. 2019).

Neste trabalho, observamos diferença de resposta entre os sexos de peixes *Rhamdia quelen*, corroborando assim com estudos que encontraram mudanças no eixo de regulação da reprodução em peixes causadas pelo aumento da temperatura (MIRANDA et al., 2013) e indicando ser importante tratar os sexos de forma

separada para melhor compreender como as mudanças climáticas irão afetar a vida da espécie.

Além da temperatura, outro fator que pode causar danos às espécies aquáticas são as cianotoxinas. Florações de *Raphidiopsis raciborskii*, produtora de PSTs ou saxitonas, já foram encontradas em reservatórios de abastecimento público de água, com danos observados nos peixes da região. Reservatórios esses que se encontravam em torno de 25°C na estação mais quente (CLEMENTE et al., 2010; CALADO et al., 2017; CALADO et al., 2020).

Em exposição ao extrato bruto de *Raphidiopsis raciborskii* contendo essas toxinas, à 25°C, fêmeas apresentaram um aumento de IGS. Apesar dos poucos estudos tratando dos efeitos reprodutivos de saxitoxinas, o presente trabalho indica que a mesma pode causar danos no ciclo reprodutivo. Efeitos de genotoxicidade também foram observados em fêmeas, como o aumento de anormalidades morfológicas nucleares no sangue. Este efeito também foi observado no peixe marinho *Diplodus sargo* após injeção de 1,6 µg equiSTX/kg, sendo este tipo de dano irreversível (COSTA et al., 2011).

As saxitoxinas são consideradas neurotoxinas, tendo a capacidade de ultrapassar a barreira hematoencefálica em alguns organismos, como ratos (CIANCA et al., 2007). No presente estudo, o extrato de cianobactéria contendo essas toxinas causou dano cerebral pelo aumento de proteínas carboniladas. Essas proteínas recebem a carbonilação como marcação para sua morte programada. O acúmulo dessas proteínas marcadas pode ser tóxico para a célula (MØLLER; ROGOWSKA-WRZESINSKA; RAO, 2011). O aumento das mesmas do tecido cerebral de fêmeas indica citotoxicidade por parte do extrato da cianobactéria. Esse aumento de proteínas carboniladas também foi encontrado em *Hoplias malabaricus*, um peixe dulcícola, em exposição trófica ao extrato bruto de *R. raciborskii* por 20 dias (SILVA et al., 2011).

Em fêmeas, a exposição ao extrato bruto da cianobactéria à 25°C levou ao aumento da glicose sanguínea, indicando um aumento da demanda energética. No entanto, esse aumento não desencadeou aumento do metabolismo. A via das glutationas, principal via de metabolização de saxitoxinas (GUBBINS et al., 2000), não foi ativada à 25°C. Não foi possível observar nem alteração da atividade de enzimas envolvidas e nem das proteínas identificadas no fígado. Foi observada redução de proteínas relacionadas ao citoesqueleto, metabolismo de aminoácidos e

produção de energia. Respostas essas diferentes das que ocorreram em machos expostos as mesmas condições. Em fêmeas, a via de síntese de hormônios estrogênicos, relacionada a reprodução (LI et al., 2019), foi regulada negativamente, ressaltando o efeito da *R. raciborskii* e seus compostos em parâmetros reprodutivos de fêmeas de *Rhamdia quelen*.

Em machos, a exposição ao extrato bruto da cianobactéria à 25°C aumentou os níveis de lactato plasmático. O lactato, além de indicar o aumento metabólico, também está relacionado ao metabolismo anaeróbico, isto é, sem presença de oxigênio (COATES; SODERHALL, 2020). Nas brânquias de machos foi observada o aumento da atividade da anidrase carbônica, responsável pela osmorregulação, respiração e regulação ácido-base (HENRY; SWENSON, 2000). A maior atividade desta enzima nas brânquias, primeiro tecido de contato dos animais com o meio externo, pode compensar o aumento do lactato, diminuição de oxigênio e consequente aumento de gás carbônico, visto que essas alterações podem levar à acidez do tecido que é controlada pela anidrase carbônica (GILMOUR, 2012).

O possível aumento da demanda de energia pode ser comprovado pelo aumento de diferentes vias de metabolização observadas no fígado dos machos à 25°C, como o aumento de vias de aminoácidos e também a ativação das glutationas. Não foi observada o aumento da atividade da enzima GST, como para salmão do Atlântico tratado com injeção intraperitoneal (GUBBINS et al., 2000), mas sim o aumento de proteínas relacionadas a metabolização e sistema antioxidante.

Sabe-se que a temperatura é um dos fatores importantes para controlar florações de cianobactérias. As florações de *Raphidiosis raciborskii* encontradas em reservatórios da região aconteceram justamente nas estações mais quentes, como verão e primavera (CLEMENTE et al., 2010; CALADO et al., 2017). Assumindo que as florações irão aumentar devido ao aquecimento global (O'NEILL; MUSGRAVE; HUMPAGE, 2016), é importante compreender como os organismos irão responder as saxitoxinas quando em temperaturas mais altas. Para animais marinhos já se sabe que o aumento da temperatura das águas pode levar a uma maior toxicidade de saxitoxina (BRAGA et al., 2018; BRAGA et al., 2020), mas para animais dulcícolas essa literatura é escassa.

Fêmeas de *Rhamdia quelen* expostas à 30°C, ao contrário do que foi observado em 25°C, apresentaram aumento da atividade da GST e seu cofator GSH. As glutationas pertencem à principal via de metabolização de saxitoxinas

(FAST; CEMBELLA; ROSS, 2006), que foi ativada apenas em 30°C em fêmeas. O mesmo foi observado também por meio da análise proteômica, a qual demonstrou a o aumento das glutationas e do complexo citocromo P450.

Danos de lípideos também foram observados no fígado dessas fêmeas, algo não visto em 25°C. A peroxidação lipídica observada pode causar desestabilização e desintegração de membrana, facilitando o acesso de compostos ao DNA e causando danos ao mesmo (BRAGA et al., 2020). No fígado, e em todos os demais tecidos, foi observado dano de quebra ao DNA. Saxitoxinas podem causar esse tipo de dano por meio da ativação gênica da caspase, ativando a via de apoptose (CHEN et al., 2020). Danos de quebra ao DNA já foram observados em cérebro de *H. malabaricus* em exposição trófica ao extrato bruto de *R. raciborskii* contendo saxitoxinas (SILVA et al., 2011).

Além desses danos lipídicos, de genotoxicidade e da ativação da via de metabolização de saxitoxinas, vias relacionadas à reprodução também foram diferentes em 30°C se comparados à 25°C. Apesar de não alterar o IGS, em 30°C o extrato bruto de R. raciborskii ativou a via de maturação de oócitos mediada por progesterona, podendo afetar assim o ciclo reprodutivo de fêmeas.

Em machos, os efeitos em 30 °C não foram tão diferentes dos encontrados em 25 °C. Aumento metabólico foi observado por meio do aumento dos níveis de lactato, bem como por diferentes vias identificadas na análise proteômica, como glicólise e ciclo de Krebs. Todos esses relacionados à produção de energia (HILL et al., 2012). No entanto, diferente do que ocorreu em 25°C, a via de esteroides foi induzida em 30°C. Hormônios esteroides são importantes para a espermatogênese (HATEF; UNNIAPPAN, 2019). Sendo assim, em 30°C o extrato de *R. raciborskii* podem comprometer também a reprodução de machos.

Nossos resultados demonstram que as fêmeas são mais sensíveis quando expostas ao extrato dessa cianotoxina em uma temperatura mais alta. Estudos envolvendo reprodução e sensibilidade às saxitoxinas produzidas pela *R. raciborskii* são escassos. Mas sabe-se que o peixe marinho *Arothron firmamentum* é capaz de acumular saxitoxinas em seus ovários (NAKASHIMA et al., 2004), dando indícios dessa maior sensibilidade em fêmeas.

Além disso, maiores efeitos foram encontrados em temperaturas mais altas. Saxitoxinas são estáveis e persistentes (MALIK et al, 2020), como mostrado com nossos resultados, onde as concentrações de toxinas se mantiveram semelhantes

nas duas condições térmicas testadas. No entanto, a toxicidade da mesma, juntamente com as demais possíveis substâncias contidas no extrato bruto de *R. raciborskii*, se mostrou diferente entre ambas.

5 CONSIDERAÇOES FINAIS

O aumento da temperatura afetou o peixe *Rhamdia quelen,* que respondeu à diferentes biomarcadores de contaminação ambiental. O aumento do índice gonadossomático foi observado em fêmeas e machos, mostrando a capacidade da temperatura de alterar o ciclo reprodutivo dos mesmos. Leucopenia foi observada em fêmeas, indicando imunossupressão. Em machos, a temperatura mais elevada ocasionou o aumento de glicose sanguínea e indução do sistema de biotransformação no fígado, além de induzir o sistema antioxidante e a atividade da anidrase carbônica em brânquias. Genotoxicidade no sangue de fêmeas e machos foi observada, porém sem danos genotóxicos e histopatológicos nos demais tecidos. O aumento da temperatura também foi capaz de causar diferentes alterações no proteoma do fígado desses animais, demonstrando, dentre tantos efeitos, um possível déficit energético em machos e a diferenciação de respostas entre os sexos.

Em águas com temperatura de 25°C, a presença do extrato bruto de cianobactéria levou ao aumento de glicose sanguínea em fêmeas e lactato plasmático em machos. Efeitos neurotóxicos eram esperados pela presença de neurotoxinas no extrato e o aumento de carbonilação de proteínas foi observado no cérebro de fêmeas. A genotoxicidade foi induzida no sangue para ambos os sexos. Porém, apenas o fígado, rim posterior e brânquias dos machos apresentaram genotoxicidade.

Já em 30°C, fêmeas apresentaram aumento das glutationas, tanto pela análise enzimática da glutationa S-transferase quanto pelo aumento de proteínas relacionadas no fígado. Esse mecanismo de metabolização de saxitoxinas, cianotoxina presente na *R. raciborskii*, não foi ativado pelas mesmas em 25°C. Além disso, dano lipídico e de DNA foram observados em fêmeas em 30°C que não foram observados à 25 °C, indicando assim que a temperatura pode ser capaz de induzir mais efeitos adversos em fêmeas em casos de florações com cianobactérias produtoras de saxitoxinas.

Sendo assim, este estudo demonstrou que o aumento da temperatura é capaz de alterar diferentes parâmetros em peixes, principalmente em machos. Quando mais um estressor é adicionado, neste caso o extrato de *R. raciborskii* contendo saxitoxinas, aumento de danos são observados em fêmeas. Dessa forma, ainda é necessário compreender agora as razões pelas quais essa diferença de respostas entre os sexos é observada, como por exemplo por meio de estudos de desregulação endócrina.

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