UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG

INSTITUTO DE OCEANOGRAFIA

PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

UNIVERSIDADE DE VIGO – UVIGO

CAMPUS DoMar

PhD PROGRAM IN MARINE SCIENCE TECHNOLY AND MANAGEMENT

INFLUÊNCIA DO pH NA PRODUÇÃO DE PEIXE-PALHAÇO (*Amphiprion percula*) E DO CAVALO-MARINHO (*Hippocampus reidi*) EM SISTEMAS DE RECIRCULAÇÃO COM BAIXA SALINIDADE

MARIO DAVI DIAS CARNEIRO

RIO GRANDE, RS - BRASIL

FEVEREIRO 2022

Universidade Federal do Rio Grande - FURG

Instituto de Oceanografia

Programa de Pós-Graduação em Aquicultura

Universidade de Vigo - UVigo

Campus DoMar

PhD Program in Marine Science Technoly and Management

INFLUÊNCIA DO pH NA PRODUÇÃO DE PEIXE-PALHAÇO (*Amphiprion percula*) E DO CAVALO-MARINHO (*Hippocampus reidi*) EM SISTEMAS DE RECIRCULAÇÃO COM BAIXA SALINIDADE

MARIO DAVI DIAS CARNEIRO

Tese apresentada como parte dos requisitos para a obtenção do grau de Doutor em Aquicultura no programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande – FURG e de PhD in Marine Science Technology and Management do Campus DoMar, Universidade de Vigo - UVigo

Orientadores:

Dr. Luís André Sampaio, Universidade Federal do Rio Grande - FURG

Dr. Miquel Planas Oliver, Instituto de Investigaciones Marinas (IIM-CSIC)

Rio Grande -RS- Brasil

Fevereiro, 2022

Pós Graduação em

AQUICULTURA

Universidade Federal do Rio Grande - FURG

ATA 01/2022

DE DEFESA DA 72ª TESE DE DOUTORADO EM AQUICULTURA

No dia onze de fevereiro de dois mil e vinte e dois, às nove horas, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, do **MARIO DAVI DIAS CARNEIRO**, orientado pelo Prof. Dr. Luís André Nassr de Sampaio e Dr. Miquel Planas, composta pelos seguintes membros: Prof. Dr. Luís André Nassr de Sampaio (Orientador – IO/FURG), Dr. Miguel Planas (Orientador – Instituto de Investigações Marinhas, CSIC - Espanha), Prof.ª Dr.ª Mônica Yumi Tsuzuki (UFSC), Prof. Dr. Ricardo Berteaux Robaldo (UFPel) e o Prof. Dr. Luis André Barbas (IFPA). A defesa de Tese de Doutorado foi realizada em acordo de cotutela com dupla titulação com a Universidade de Vigo na Espanha. Título da Tese: **"INFLUÊNCIA DO PH NA PRODUÇÃO DO PEIXE-PALHAÇO (*Amphiprion percula*) E DO CAVALO-MARINHO (*Hippocampus reidi*) EM SISTEMAS DE RECIRCULAÇÃO COM BAIXA SALINIDADE"**. Dando início à defesa, o candidato apresentou sua Tese. Após ampla discussão entre os avaliadores e o candidato, a Banca se reuniu sob a presidência do Coordenador Adjunto para deliberação do resultado. O candidato **MARIO DAVI DIAS CARNEIRO** foi considerado **APROVADO**. Nada mais havendo a tratar, foi lavrada a presente ata, que após lida e aprovada, será assinada pela Banca Examinadora, pelo candidato e pelo Coordenador Adjunto do PPGAq.

PROF. DR. LUÍS ANDRÉ NASSR DE SAMPAIO (ORIENTADOR – IO/FURG)

PLANAS OLIVER MIGUEL - DNI 05232890E

Firmado digitalmente por PLANAS OLIVER MIGUEL - DNI 05232890E
Fecha: 2022.02.14 10:21:42 +01'00'

PROF. DR. MIGUEL PLANAS (ORIENTADOR - CSIC - Espanha)

Mônica Y. Tsuzuki

PROF.ª DR.ª MÔNICA YUMI TSUZUKI (UFSC)

Date: 2022.02.11
17:41:55 -03'00'

PROF. DR. LUÍS ANDRÉ BARBAS (IFPA)

PROF. DR. RICARDO BERTEAUX ROBALDO (UFPel)

Digitally signed by Mario Davi Dias Carneiro
DN: CN=Mario Davi Dias Carneiro, E=mariolddc@gmail.com, C=BR
Reason: I am approving this document with my legally binding signature
Location: Rio de Janeiro, Brasil
Date: 2022-02-13 11:02:36
Foxit Reader Version: 9.4.1

MARIO DAVI DIAS CARNEIRO

Ricardo Vieira Rodrigues

PROF. DR. RICARDO VIEIRA RODRIGUES (Coordenador Adjunto do PPGAq)

ACTA DA SESIÓN DE PRESENTACIÓN E CUALIFICACIÓN DA TESE DE DOUTORAMENTO

Ten lugar o día 11 de febreiro de 2022 a sesión pública de defensa da Tese de doutoramento titulada: "INFLUÊNCIA DO PH NA PRODUÇÃO DO PEIXE-PALHAÇO (AMPHIPRION PERCULA) E DO CAVALO-MARINHO (HIPPOCAMPUS REIDI) EM SISTEMAS DE RECIRCULAÇÃO COM BAIXA SALINDADE"
polo/a alumno/a **MARIO DAVI DIAS CARNEIRO** con D.N.I./Pasaporte.: **FP678401**
actuando como directores: D. Miguel Planas Oliver y D. Luis A. Sampaio.
O Tribunal está composto por:

Presidente/a:	Dr./a. D./Dna. Mônica Yumi Tsuziki
Secretario/a:	Dr./a. D./Dna. Luis André Luz Barbas
Vogal:	Dr./a. D./Dna. Ricardo Berteaux Robaldo
Vogal 2:	Dr./a. D./Dna. Luís André Nassr De Sampaio
Vogal 3:	Dr./a. D./Dna. Miguel Planas Oliver

Idioma de defensa da tese: Portugués

A sesión de presentación e cualificación desenvólvese segundo a normativa da Universidade de Vigo. O Tribunal acorda cualifica-la Tese de doutoramento con

☐ NON APTO (Insuficiente) ☐ APROBADO (Regular) ☐ NOTABLE (Bom) ☒ SOBRESALIENTE (Excelente)

Tralo que se levanta a sesión.

A PRESIDENTA, O SECRETARIO, O VOGAL, O VOGAL 2, O VOGAL 3

Documento assinado digitalmente
gov.br LUIS ANDRE LUZ BARBAS
Data: 14/02/2022 13:13:39-0300
Verifique em https://verificador.iti.br

Documento assinado digitalmente
Monica Yumi Tsuzuki
Data: 14/02/2022 09:09:52-0300
CPF: 597.016.500-00
Verifique as assinaturas em https://v.ufsc.br

PLANAS OLIVER MIGUEL - DNI 05232890E
Firmado digitalmente por PLANAS OLIVER MIGUEL - DNI 05232890E
Fecha: 2022.02.15 09:56:40 +01'00'

Mônica Tsuzuki Luis A. Barbas Ricardo B. Robaldo Luis A. Sampaio Miquel Planas

Documento assinado digitalmente
gov.br LUIS ANDRE NASSR DE SAMPAIO
Data: 14/02/2022 09:35:35-0300
Verifique em https://verificador.iti.br

O DOUTORANDO

Documento assinado digitalmente
gov.br RICARDO BERTEAUX ROBALDO
Data: 14/02/2022 11:30:51-0300
Verifique em https://verificador.iti.br

Mario D. Dias Carneiro

Documento assinado digitalmente
gov.br MARIO DAVI DIAS CARNEIRO
Data: 14/02/2022 21:27:53-0300
Verifique em https://verificador.iti.br

SUMÁRIO

DEDICATÓRIA

Dedico esta Tese a meus pais e todos aqueles que se surpreendem com inicialmente impossível de se realizar, que enfrentam as dificuldades e demonstram poder superá-las.

Logo dedico aos que superaram e superam inúmeros desafios para alcançar seus objetivos e que ainda me auxiliaram para que eu cumprisse os meus. Sobretudos os que compartilham a Tecnologia em Aquicultura como profissão.

AGRADECIMENTOS

Agradeço à Deus, A força que independente de nomenclaturas possibilita acreditar e realizar o que pode, ou não, ser metodicamente comprovado.

Agradeço meus pais Maria Mirian L. D. Carneiro e Daniel F. Carneiro, exemplo e me apoio no trajeto. As minhas irmãs Dayana D. Carneiro e Laura D. Carneiro pelo incentivo e compreensão.

Agradeço a todos os meus amigos que comigo estiveram durante meu período de formação e em inúmeros outros momentos na minha vida. Como vestígios deixo inicias dos que de alguma maneira contribuíram para esta Tese: J.C.T., A.P.C; L.M., B.M, A.T.N., L.F.N, J.H., J.C.L., S.C., A.G., K.B.D.; A.F.F.M., F.M.W, J.F.F., V.F., M.S.S.

Agradeço a você leitor, creia suas inicias estão aqui!

Agradeço a cidade de Rio Grande e a EMA por ter me acolhido durante esta experiência de Mestrado e Doutorado e cidade de Vigo e ao IIM, por *rellenarme con un año increíble entre estos*. Ao RJ e a recente experiência no AquaRio. E, por que não, as minhas bicicletas!

Ao grupo de Jovens CRISTIFIQUE, sal da terra e luz do mundo em muitos momentos. A galera itinerante do ALOJAMENTO, família por 7 anos neste meu lar. Aos grupos de DANÇA Rio Grandinos e Vigueses pelos momentos de arte, aprendizado e encantamento.

Em especial aos Professores Dr. Ricardo V. Rodrigues e Dra. Ana Bernabeu pelo imenso apoio no processo de co-tutela e aos Dr. Luis André Nassr de Sampaio e Dr. Miquel Planas Oliver por aceitarem orientar este trabalho.

A todos do PPG em Aquicultura, por todos os ensinamentos específicos, lições de vida e exemplos de como agir ou não agir em especial José M. Montssera, Paulo Abreu, Lucas Maltez e Bárbara Hostins. Ao Instituto de Investigaciones Marinas IIM-CSIC, por permitir meu Sanduíche e conseguinte co-tutela. *A todos del IIM que me ayudaron siempre y sobretodo en momentos en los cuales no me imaginé capaz de hacer lo que sería necesario en especial* Lourdes Nieto Leirós, Jorge Ucera Hernándes, Patrícia Ruiz, José Pintado, Josep Rotlant, Rubens Chamorro, Alex Chamorro, Xoán Martínez, Jesús Salvador Ryes Muras, Carmen Gonzales, Susana Fernández

Por fim, obrigados aos peixes que deram a vida pelo meu progresso.

"Prefiro ter questões que não podem ser respondidas

a respostas que não que não podem ser questionadas"

Rychard Feynman

RESUMO

A aquicultura ornamental expõe grande importância econômica e para a conservação. Contudo, a produção de peixes ornamentais não é majoritariamente praticada em áreas litorâneas, tornando sistemas de recirculação de água (RAS) em baixa salinidade interessantes. Uma das consequências do RAS é a acidificação da água pelo consumo das reservas de alcalinidade através do processo de nitrificação. O peixe-palhaço *Amphiprion percula* é um peixe ornamental marinho de pequeno porte e amplamente distribuído no mercado da aquariofilia devido a sua fácil produção em cativeiro. O cavalo-marinho *Hippocampus reidi* é um peixe utilizado como ornamental e consumido pela medicina tradicional chinesa. A reprodução de ambos é conhecida e praticada em muitos lugares no mundo. Entretanto, os efeitos do ambiente ácido sobre parâmetros bioquímicos em peixes ornamentais são pouco conhecidos. Portanto o objetivo central dessa tese é o estudo da produção de cavalo-marinho (*H. reidi*) e peixe palhaço (*A. percula*) em RAS, avaliando os problemas decorrentes da acidificação aguda e crônica em água marinha (SW, salinidade 33) e salobra (BW, salinidade 11). Para tal foram realizados experimentos agudos (96 h): (i) em SW, avaliando os efeitos dos pHs 5, 6, 7 e 8 sobre os parâmetros bioquímicos em juvenis de *A. percula*; (ii) avaliando os mesmos parâmetros em fígado, brânquias, trato digestivo e músculos de *A. percula* expostos aos mesmos pHs em BW e SW; (iii) avaliando os efeitos agudos dos pHs 5, 6, 7 e 8 em BW e SW em juvenis *H. reidi*; (iv) avaliando os efeitos crônicos (3 e 4 semanas) dos pHs 6,5 e 8 em BW e SW em juvenis de *H. reidi* e (v) avaliando os efeitos crônicos (8 semanas) dos pHs 6,5 e 8 em BW e SW em *A. percula*, bem como a avaliação do retorno a condição controle (8-SW). As respostas agudas em peixe palhaços *A. percula* revelaram um maior dano proteíco em peixes expostos ao pH 5. Os órgãos desta mesma espécie respondem de distintas maneiras a exposição ácida em diferentes salinidades, sendo os órgãos mais afetados as brânquias e o fígado que demonstraram redução da atividade da GST em pH 5-BW e um maiores danos lipídicos (TBARS) nas brânquias em pH 5-SW. Em relação aos cavalos-marinhos, a exposição aguda ao ambiente ácido em baixa salinidade (pH 5-BW) trouxe maiores prejuízos nos diferentes níveis de estresse testados, evidenciando um aumento no cortisol, alterações em nível enzimático (SOD, GST, GPx) e no metabolismo da glutationa, acompanhadas por redução da capacidade antioxidante (TEAC) e aumento da peroxidação lipídica (TBARS). Também foi observado peixes moribundos em pH 5-BW.. De maneira similar, os *H. reidi* cronicamente expostos a pH 6,5-BW sofrem maior mortalidade, menor crescimento e maiores alterações bioquímicas, enquanto a mesma condição ácida em SW promove melhores resultados de crescimento e de acúmulo de GSH, nestes peixes. Também na exposição crônica, *A. percula* mostraram maiores prejuízos na condição ácida BW, com menor sobrevivência e crescimento, além de maiores danos proteicos (P-SH) e lipídicos (TBARS). Em pH 8-BW, os peixes apresentaram maior TBARS, entretanto, desenvolveram uma coloração diferenciada e mais interessante ao mercado ornamental, a variedade Onix. Porém, o retorno destes animais a condições controle (pH 8-SW) não promove mortalidade além de que os parâmetros bioquímicos podem ser considerados estabilizados após 7 dias. Assim, como conclusão geral, podemos dizer que para ambas as espécies a exposição ao ambiente ácido é mais onerosa em baixa salinidade.

Palavras-chave: fisiologia, bioquímica, stress oxidativo, RAS, aquariofilia, acidificação.

RESUMEN

La acuicultura ornamental tiene gran importancia económica y para conservación. Sin embargo, la producción de peces ornamentales no se practica principalmente en las zonas costeras, lo que hace que los sistemas de recirculación de agua (RAS) en baja salinidad sean interesantes. Una de las consecuencias del RAS es la acidificación del agua debido al consumo de alcalinidad por la nitrificación. El pez payaso *Amphiprion percula* es un pequeño pez ornamental marino que esta ampliamente distribuido en el mercado de acuarismo debido a su fácil producción en cautiverio. El caballito de mar *Hippocampus reidi* es un pez que tiene su uso tanto para ornamentación y consumo por medicina tradicional china. La reproducción de estos peces es conocida e platicada en muchos sitios del mundo. Sin embargo, los efectos del ambiente ácido sobre los parámetros bioquímicos en peces ornamentales son poco conocidos. Por lo tanto el objetivo principal de esta tesis es el estudio de la producción de caballito de mar (*H. reidi*) y pez payaso (*A. percula*) en RAS con baja salinidad evaluando los problemas derivados de la acidificación aguda y crónica en agua marina (SW, salinidad 33) y salobre (BW, salinidad 11). Para ello, se realizaron experimentos agudos (96 h): (i) en SW, evaluando los efectos de pHs 5, 6, 7 y 8 sobre los parámetros bioquímicos en juveniles de *A. percula*; (ii) evaluando los mismos parámetros en hígado, branquias, tracto digestivo y músculos de *A. percula* expuestos a los mismos pH en BW y SW; (iii) evaluando los efectos agudos de pHs 5, 6, 7 y 8 en BW y SW en juveniles *H. reidi*; (iv) evaluando los efectos crónicos (3 y 4 semanas) de pH 6.5 y 8 en BW y SW en juveniles *H. reidi* y (v) evaluar los efectos crónicos (8 semanas) del pH 6.5 y 8 en BW y SW en jueniles *A. percula*, así como la evaluación del retorno a la condición de control (pH 8-SW). Las respuestas agudas en el pez payaso *A. percula* revelaron un mayor daño proteico en los peces expuestos a pH 5. Los órganos de esta misma especie responden de diferentes formas a la exposición al ácido en diferentes salinidades, siendo los órganos más afectados las branquias y el hígado que demostraron reducción de la actividad de GST en pH 5-BW y un mayor daño lipídico (TBARS) en las branquias en pH 5-SW. Con respecto a los caballitos de mar, la exposición aguda a un ambiente ácido de baja salinidad (pH 5-BW) trajo mayor daño a los diferentes niveles de estrés estudiados, evidenciando un aumento de cortisol, cambios en lola actividad de enzimas (SOD, GST, GPx) y en el metabolismo del glutatión, acompañado de una capacidad antioxidante reducida (TEAC) y un aumento de la peroxidación lipídica (TBARS). Además se observó peces moribundos en pH 5-BW. De manera similar, *H. reidi* crónicamente expuesto a pH 6.5-BW sufren una mayor mortalidad, menor crecimiento y mayores cambios bioquímicos en estos peces, mientras que la misma condición ácida en SW mostró mejores resultados de crecimiento y de acumulación de GSH. También en exposición crónica, *A. percula* se demostraron más afectados en la condición ácida BW, con menor supervivencia y crecimiento, aparte de un mayor daño en proteínas (P-SH) y lípidos (TBARS). En pH 8-BW, los peces mostraron mayores TBARS, sin embargo desarrollaron un color diferente y más interesante para el mercado, la variedad Onix. Asimismo, el retorno de estos animales a las condiciones de control, pH 8-SW, no promueve la mortalidad, pudiendo considerarse que los parámetros bioquímicos están estabilizados a los 7 días. Por lo tanto, como conclusión general, podemos decir que para ambas especies la exposición al ambiente ácido es más costosa en baja salinidad.

Palavras-chaves: fisiología, bioquímica, estrés oxidativo, RAS, acuarismo, acidificación.

ABSTRACT

Ornamental aquaculture shows economic and conservation importance. However, the production of ornamental fish is not mandatory practiced in coastal areas, making water recirculation systems (RAS) in low salinity an interesting alternative. One consequence of RAS is the acidification of water due to the consumption of alkalinity through nitrifying process. The clownfish *Amphiprion percula* is a small marine ornamental fish widely distributed in the aquarium trade because of its easy production in captivity. The seahorse *Hippocampus reidi* is a fish used in the ornamental fish trade and consumed by traditional Chinese medicine. The reproduction of these both fishes are known being practiced around the world. However, effects of the acidic environment on biochemical parameters for ornamental fish are little known. Thus, the main objective of this thesis is the study of the production of seahorse (*H. reidi*) and clownfish (*A. percula*) in RAS with low salinity, assessing problems arising from acutely and chronically acidification in marine water (SW, salinity 33) and brackish (BW, salinity 11). For this purpose, acute experiments were carried out (96 h): (i) in SW, evaluating the effects of pHs 5, 6, 7 and 8 on biochemical parameters A. whole perculas, (ii) evaluating the same parameters in liver, gills, digestive tract and muscles of *A.percula* exposed to the same pHs in BW and SW, (iii) evaluating the acute effects of pHs 5, 6, 7 and 8 in BW and SW in whole *H. reidi*, (iv) evaluating the chronic effects (3 and 4 weeks) of pH 6.5 and 8 in BW and SW in *H. reidi* and (v) evaluating the chronic effects (8 weeks) of pH 6.5 and 8 in BW and SW in *A. percula*, as well as the evaluation of the return to the control condition (8-SW). The acute responses in clownfish *A. percula* revealed greater protein damage in fish exposed to pH 5. Organs of this same species respond in different ways to acid exposure in different salinities, with the most affected organs being the gills and liver that exposed reduction of the activity of GST pH 5 in BW and a greater lipid damage (TBARS) in the gills in pH 5 SW. With respect to seahorses, acute exposure to an acidic environment at low salinity (pH 5-BW) caused greater damage at the different stress levels tested, showing an increase in cortisol, changes in enzyme levels (SOD, GST, GPx) and metabolism of glutathione, accompanied by reduced antioxidant capacity (TEAC) and increased lipid peroxidation (TBARS), in addition moribund fish was observed in pH 5-BW. Similarly, H. reidi chronically exposed to acidic condition in SW showed better growth results and GSH accumulation trends, while the same acidic condition in BW promotes higher mortality, lower growth and greater biochemical changes in these fish. Also in chronic exposure, *A. percula* showed losses in the acidic condition BW, with lower growth survival, higher protein (P-SH) and lipid (TBARS) damage. In the control condition in BW, the fish showed higher TBARS, however they presented a different color and more interesting to the market called the Onix variety. Also, the return of these animals to control conditions, regarding pH and salinity, does not promote mortality, and the biochemical parameters can be considered stabilized after 7 days. As a general conclusion, we can say that for both species exposure to the acidic environment is more costly in low salinity.

Keywords: physiology, biochemistry, oxidative stress, RAS, aquarium hobby, acidification

1. INTRODUÇÃO GERAL

O conhecimento de uma espécie quanto a sua resiliência pode trazer possibilidades e traçar táticas para sua conservação, produção e na integração destas em favor dos indivíduos estudados. Na aquicultura fatores ambientais relacionados a qualidade de água, oxigênio dissolvido, temperatura, nitrogenados, dureza, alcalinidade, pH e salinidade (dentre outros) têm sido considerados como indicadores críticos para a produção de diversas espécies de peixes (Kim et al., 2017). Assim, avaliar e compreender como estes fatores ambientais, dentre eles pH e salinidade, afetam os organismos aquáticos se faz importante, tanto pelo conhecimento biológico, como para a aquicultura e conservação de qualquer espécie.

1.1 pH

O pH, potencial hidrogeniônico, traduz a relação entre o próton H^+ e o ânion OH^-, também chamados de hidrônio e hidroxila, em meios líquidos. Esta concentração molar foi transformada em uma escala logarítmica numeral de 0-14, sendo considerado 7 o pH neutro e portando abaixo e acima dele são considerados ácidos e básicos/alcalinos, respectivamente (Boyd, 2015). O pH afeta a vida dos animais aquáticos marinhos de maneira direta uma vez que, ainda que dependa da temperatura, o pH dos meios extracelulares (pH_e) dos peixes marinhos deve ser mantido em uma pequena faixa 7,7 -8,1, e entre 7,0 -7,4 no meio intracelular (pH_i), pelo chamada regulação ácido-base (Claiborne et al., 2002; Heuer and Grosell, 2014). Ainda que os pHs internos (pH_e e pH_i) sejam espécie específicos, alterações muito além das faixas citadas podem levar a distúrbios no funcionamento biológico uma vez que importantes macromoléculas como as enzimas, são sensíveis ao pH, e uma mudança deste pode gerar alterações nas funções de proteínas, lipídeos, carbohidratos e ácidos nucleicos (Burggren e Bautista, 2019; Shartau et al., 2019).

Ainda que a acidificação tenha sido estudado em peixes por diversas questões fisiológicas (Heuer and Grosell, 2014), nos últimos anos a preocupação com a acidificação dos oceanos é o que estimula o estudo das respostas dos organismos aquáticos frente a diferentes pHs. Segundo Gagliano et al. (2010) a oscilação causada pelo aumento da concentração de CO_2, levará a uma acidificação de 0,5 no pH dos oceanos até 2100, entretanto uma oscilação de 0,4 a 1 unidade de pH ocorre entre diferentes ambientes recifais, demonstrando que peixes deste ambiente, geralmente territorialistas e sem comportamento migratório, estão sujeitos a variações de pH inclusive em ambiente natural, e portanto podem não se demonstrar tão sensíveis a estas alterações.

Esta preocupação também afeta a Aquicultura como pode ser visto em documentos da FAO, que utilizam-se de um somatório de estudos de caso de alteração de pH para ressaltar a preocupação com a acidificação dos corpos hídricos, tendo em vista que apesar de a variabilidade de pH ser maior em ambiente dulcícola, existem ambientes muito específicos como os lagos alcalinos africanos onde a acidificação pode apresentar efeitos bastante intensos para os peixes, e consequentemente para pesca e aquicultura (Barange et al., 2018; Johnson et al., 2019).

Diversas substâncias têm sido utilizadas para avaliar os impactos da acidificação do pH em peixes, ressaltando que como introduzido, uma boa parte destas está associada a acidificação dos oceanos portando utilizam CO_2, substancia que promove a hipercapnia, toxicidade e, portanto, tem efeitos mais prejudiciais para os peixes (Gattuso et al., 2011; Kikkawa et al., 2004). Ainda que, nos mesmo níveis de pH, a acidificação por CO_2 cause mais mortalidade que o HCl (Kikkawa et al., 2004) a última está muito mais relacionada com a realidade de produção através do consumo de alcalinidade e redução do pH (Ebeling et al., 2006).

1.2 Salinidade

A salinidade é um fator determinante que age através de receptores aumentando ou diminuindo o crescimento (Bœuf and Payan, 2001). Esses autores concluem em sua revisão, que peixes eurihalinos marinhos apresentam maiores taxas de crescimento/desenvolvimento em salinidades intermediárias, optando muitas vezes os juvenis por ambientes com salinidade entre 8 e 20‰ para um ótimo desenvolvimento. Segundo Schmidt-Nilsen (2002), os fluídos corporais dos peixes teleósteos se mantêm em uma concentração em torno de 1/3 da água do mar que corresponde a uma salinidade de 11-12‰. Sendo assim, os teleósteos têm um gasto energético para manter seus fluídos nesta concentração.

A manutenção da concentração iônica dos teleósteos é feita por vários órgãos, e de diferentes maneiras ao longo da vida. Nas fases iniciais ela é muito dependente dos tegumentos e regiões de contato, passando ao longo do desenvolvimento a ser desempenhada principalmente por brânquias e pelo rim, e órgãos não ionorregulatórios associados a manutenção energética para a ionorregulação (Burggren and Bautista, 2019; Tseng and Hwang, 2008). “Osmorregulation first” é uma hipótese recente que revela que as brânquias tem como primeira função a regulação iônica (Burggren and Bautista, 2019). Para realização da ionorregulação um conjunto de ações é promovido através de trocadores iônicos específicos presentes no ionócitos, anteriormente também chamados de células ricas em mitocôndrias ou células de cloreto. Existem comprovadamente em modelos fisiológicos marinhos quatro tipos de ionócitos e suas predominâncias mudam de acordo com a salinidade, e portanto, da necessidade do organismo em se adaptar a ela (Kang et al., 2010; Yang et al., 2014). Esses ionócitos têm uma fase de aclimatação aguda de 24 a 48 h e uma fase de estabilização que pode durar até 14 dias (Kang et al., 2013). Assim sendo, mudanças de salinidade em peixes podem desencadear efeitos e oscilações durante este período. Portanto, os peixes podem apresentar diferentes padrões de alterações dependendo do tempo de exposição a mudanças de qualidade de água, além da própria salinidade na qual esta alteração está ocorrendo.

1.3 Efeito combinado pH x Salinidade

Assim como está a iono – osmorregulação para a compensação da salinidade, para a compensação do pH está o equilíbrio ácido-base. Assim como para a salinidade, distúrbios no equilíbrio ácido-base podem reduzir o desempenho e a sobrevivência dos peixes (Shartau et al., 2019). Desafortunadamente, ainda não é totalmente conhecida a capacidade real dos ionócitos para regular o pH do sangue. Ainda assim, é provável que a hipótese de "osmorregulação primeiro" deva ser expandida para "osmorregulação e equilíbrio ácido-base primeiro"(Burggren e Bautista, 2019).

Alguns estudos revelam essa interação entre osmorregulação e equilíbrio ácido-base, logo a relação entre salinidade e pH. Por exemplo, o número de células de cloreto aumentou progressivamente do pH 8 ao pH 5, em *Rachycentrum canadum* após 24 h de exposição ao ambiente ácido (Rodrigues et al., 2015). Apesar da atividade de Na^+/K^+-ATPase ter se mantido, o número e densidade de ionócitos epiteliais foi significativamente maior em larvas de seabass (*Atractoscion nobilis*) expostas a pCO_2 (1.971±55 μatm com pH de 7,42± 0,03) (Kwan et al., 2021). A Na^+/K^+-ATPase e o cotransportador $Na^+/K^+/2Cl^-$ tiveram sua expressão gênica aumentada principalmente em alta pCO_2 (1.000 μatm com pH de 7,7; 7,4 e 7.0, para a salinidade de 32; 10 e 2,5), provavelmente para aumentar o gradiente de Na, consequentemente no equilíbrio acido-base via o cotransportador $Na^+/HCO3^-$, sendo que somente em na salinidade 2,5 os peixes tiveram diminuição da expressão da Na^+/K^+-ATPase (Shrivastava et al., 2019). Estes dados reiteram a interação fisiológica da salinidade e do pH. Portanto alterações conjuntas de pH e salinidade podem trazer importantes respostas para a biologia, conservação e aquicultura de peixes marinhos e estuarinos.

1.4 Aquariofilia e Aquicultura ornamental

A manutenção de animais aquáticos para consumo fresco data de muito tempo antes de Cristo, entretanto na China e no Egito antigo é que estes animais começaram a tomar importância decorativa e sinal de status social, prática seguida pelos romanos ricos. Porém, o comercio de peixes ornamentais foi iniciado pelos chineses entre 265 e 316 DC, quando começaram a selecionar fenótipos de *Carassius auratus*, atualmente conhecido como peixe dourado (Palmtag, 2017).

Em meados de 1840, naturalistas britânicos coletaram vida marinha nativa mantendo-a em containers de vidro para observação no jardim zoológico Regent's Park em Londres. Assim surgiu o primeiro aquário público que expunha a vida marinha. Acompanhando a expansão do aquarismo dulcícola, nasce o aquarismo marinho que começou a se estruturar em meados 1950-60, quando peixes marinhos começaram a ser importados do Siri-Lanka, seguido das Filipinas e Havaí. Esta indústria/comércio cresceu no final dos anos 1960-70 incentivada pelo desenvolvimento do sal marinho sintético, que permitiu o aquarismo distante de locais com acesso a água marinha (Palmtag, 2017).

Peixes ornamentais são um componente significativo do comércio internacional, sendo que a importância dessa indústria pode contribuir para o desenvolvimento sustentável de recursos aquáticos, através da produção deste organismos. A criação de peixes ornamentais é uma atividade lucrativa, pois a atividade representa uma opção com custo de implantação relativamente baixo, e elevada rentabilidade para piscicultores familiares servindo como possibilidade de melhoria da situação econômica uma vez que os coletores destes animais devem ser considerados como potencias aquicultores, nessa indústria crescente que cada vez mais se conscientiza para um senso de troca de espécimes coletadas por espécimes produzidas (Calado, 2017a).

A maioria das espécies ornamentais comercializadas no mundo tem os Estados Unidos como destino, seguido pela Europa e Japão (Rhyne et al., 2012; Smith et al., 2008). Ainda segundo esses autores, mais de um bilhão de animais vivos foram importados pelos Estados Unidos entre os anos 2000 e 2005, dos quais 60% eram aquáticos majoritariamente representados por peixes tropicais (87,5% no ano de 2005) sendo 45,4% dos representantes de água doce e 42,1% marinhos, dos quais 97,6% eram selvagens.

Foi registrado o ingresso de mais de 11 milhões de peixes marinhos nos Estados Unidos entre maio de 2004 e maio de 2005, representantes de 1802 espécies de 125 famílias, sendo o

Sudeste Asiático, Oceania, Norte da América do Sul e Trinidad e Tobago os locais de origem mais comuns das importações para aquarismo (Rhyne et al., 2012).

É importante ressaltar que a aquariofilia marinha se divida em duas culturas básicas, o "fish only" e os "Reefs", que respectivamente se dedicam a manutenção somente de peixes ou de comunidades diversificadas de organismos como ocorre em um recife de coral, sendo que apenas 8% da aquariofilia marinha está representada pela cultura "fish only"(Pouil et al., 2019), assim a maioria das espécies utilizadas provem de ambientes diversificados como os recifes de coral, ainda que existam os aquaristas de "tanques de especialidade", que cuidam de indivíduos ou grupo de organismos que requerem condições especiais, como cavalos-marinhos ou águas-vivas (Palmtag, 2017).

A produção de peixes ornamentais marinhos pode ser realizada em diversos ambientes, entretanto a mesma tem benefícios quando exercida distante do litoral, , como menores custos com a terra, possibilidade de escolher o local considerando mercados alvo e facilidades para o escoamento da produção, além de uma maior facilidade de licenciamento do empreendimento (Calado, 2017b). Para isso, além da salinização, é importante a utilização de sistemas de recirculação de água. A utilização de sistemas de recirculação tem crescido nos últimos 30 anos graças a pesquisas e produções comerciais que utilizam esse sistema em suas atividades (Timmons e Ebeling, 2010). Segundo os mesmo autores, sistemas de recirculação de água possuem uma série de vantagens frente a outros regimes de produção aquícolas como a economia de água graças a filtragem biológica, maior controle das variáveis abióticas, menor risco de escape de espécies exóticas ou geneticamente modificadas, menor vulnerabilidade a doenças, maior produtividade além de permitir a produção em locais de clima inicialmente inadequado, ou no caso específico da produção de peixes ornamentais marinhos longe do litoral, distante da fonte de água utilizada na produção.

1.5 A produção *versus* o extrativismo de peixes marinhos ornamentais

Os ambientes de recife, além de conterem grande diversidade biológica, servem para os oceanos como fonte de alimento, abrigo e local de reprodução, e embora ele ocupe menos de 1% da área dos oceanos é responsável por mais que 25% da diversidade das espécies marinhas (Thornhill, 2012) sendo extraídos desse habitat a maioria dos organismos ornamentais. Estima-se que aproximadamente 11 milhões de peixes por ano são retirados dos recifes para fins de ornamentação (Rhyne et al., 2012). Este dado é bastante alarmante sabendo-se, por exemplo, que embora se conheça a reprodução de peixes-palhaço se observa uma diminuição de seus estoques naturais devido a explotação dos espécimes (Wabnitz et al., 2003), e que a aquicultura ornamental marinha representa apenas 5% do total de organismos usados na aquariofilia (Thornhill, 2012). Esse fato, somado a que a indústria movimenta milhões de dólares, cria uma atmosfera bastante favorável à aquicultura. Uma das questões que podem ser levantadas quanto a produção, principalmente de espécies exóticas, é o risco de fuga e estabelecimento da espécie no ambiente. Episódios problemáticos com espécies exóticas podem ser vistos na Costa do Atlântico com a presença dos peixes Leão (*Pterois volitans* e *P. miles*) (Luiz et al., 2013). Porém, este risco é menor para produção em sistemas de recirculação e, sobretudo em região distante do litoral.

Ao longo dos anos, a partir de cruzamentos e manejos foram obtidas uma série de variedades criadas em empresas específicas de produção ornamental. Dentre as empresas de produção de peixes palhaço pode-se citar a Ora, nos Estados Unidos, que detém uma serie de linhagens e variedades que diversificam a quantidade de peixes-palhaço comerciais. Como afirmado para as espécies melânicas, sem entrar em méritos de endogamia e hibridações para produção destas variedades, é inegável salientar que elas possuem um maior valor de mercado, e chamam a atenção do consumidor pela peculiaridade, desestimulando a explotação e consequentemente sendo vantajosas ao produtor.

1.6 Pomacentrideos, Amphyprioninae: *Amphiprion percula*

Peixes-palhaço pertencem a subfamília Amphiprioninae, que possui 30 espécies (Thornihil, 2012). Esta subfamília está na família Pomacentridae, a mais explorada no mundo, sendo desta a maior representação em peixes ornamentais marinhos, que somam 76% do número de peixes ornamentais importados para os Estados Unidos (Rhyne et al., 2012). Peixes-palhaço estão divididos em dois gêneros, *Amphiprion* e *Premnas*, respectivamente com 29 e uma espécie que habitam recifes da região oeste dos oceanos Índico e Pacífico (Thornihil, 2012; Fautin & Allen, 1992). Dos representantes da subfamília Amphiprioninae os mais conhecidos são os peixe-palhaço: *Amphiprion ocellaris* e *Amphiprion percula*, que juntos ocupam o quinto lugar em número de peixes ornamentais importados pelos EUA (Rhyne et al., 2012), apesar de ter sua reprodução conhecida desde 1900 e amplamente praticada desde 1950 (Wittenrich, 2007), demonstrando sua importância no mercado ornamental.

Alguns trabalhos foram realizados para avaliar interações abióticas, como salinidade, para peixes-palhaço quanto a reprodução (Dhaneesh et al., 2012a; Dhaneesh et al., 2012b), larvicultura (Evangelista et al., 2020) e outros inclusive para pH. Estes estudos demonstram que uma diminuição do pH de 8,4 para 7,8 causa, contraditoriamente, uma melhora em parâmetros reprodutivos como maior fecundidade para *Amphiprion melanopus* (Miller et al., 2013) e para *Amphiprion percula,* quando há uma diminuição no pH de 8,0 para 7,5 (Welch and Munday, 2015).

1.7 Sygnatídeos, Hippocampus: *Hippocampus reidi*

Cavalos-marinhos são peixes da família Syngnathidae. A essa família pertence a subfamília Hippocampinae quem compreende um número de aproximadamente 50 espécies do gênero *Hippocampus* (Planas et al., 2017a), sendo que menos de 10 delas são encontradas na aquariofilia (Wittenrich, 2007). São encontrados no Brasil três espécies de cavalo-marinho: *Hippocampus erectus*, *Hippocampus reidi* e *Hippocampus patagonicus*, que recentemente foi encontrado no litoral brasileiro (Silveira et al., 2014).

Os cavalos-marinhos são considerados uma espécie bandeira de conservação, sendo que todas as espécies do gênero *Hippocampus* estão incluídas na CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II in 2002, decisão que se tornou efetiva em 2004 (Cohen et al., 2017).

A importância econômica dos cavalos-marinhos encontra-se, além da aquariofilia, na medicina oriental chinesa, sendo que o volume de organismos vivos comercializados foi de até 120 mil animais/ano contra mais de 10 milhões/ano de organismos secos entre os anos 1997-2008 (Koldewey & Martin-Smith, 2010), demonstrando o quão importante é essa espécie para a aquariofilia e consequentemente para a aquacultura ornamental, salientando que a reprodução e larvicultura dos cavalos-marinhos é conhecida (Wittenrich, 2007).

Cavalos-marinhos são distribuídos em regiões tropicais e temperadas de todos os oceanos vivendo em ambientes desde mangues, bancos de macroalgas e gramas marinhas até encostas de recifes (Koldewey and Martin-Smith, 2010). Os cavalos-marinhos *H. reidi* são eurialinos encontrados próximo a costa e em estuários (Hora et al., 2016), circunstâncias que levam a crer que esta espécie possua uma boa tolerância não só a baixas salinidades mas também a oscilações de pH, como ocorre para linguado *Paralichthys orbignyanus*, peixe estuarino, que apresenta CL_{50} (96h) de pH em 4,4 (Wasielesky et al., 1997).

Alguns estudos foram realizados avaliando variáveis abióticas sobre o desempenho do cavalo-marinho como a transferência de *H. abdominalis* para baixas salinidades (Martinez-

Cardenas and Purser, 2016). Hora et al (2016) observaram uma boa tolerância a baixa salinidade, melhor sobrevivência e crescimento de *H. reidi* entre 10 e 20 ‰. Para cavalos-marinhos *H. abdominalis*, o aumento da temperatura de 18 para 30 °C causa incremento no consumo de oxigênio, em normocapnia ou hipercapnia, pH 8 e 7,5 respectivamente, não causando aumento na ventilação branquial em hipercapnia (Faleiro et al., 2015). Segundo os mesmos autores na hipercapnia há uma diminuição no consumo de alimento, no tempo de alimentação e de natação, e consequentemente maior tempo de descanso dos animais.

1.8 Bioquímica: Status oxidativo para peixes, porque utilizar?

Em organismos aeróbios existe uma relação entre espécies pró e anti-oxidantes, que, quando em favor das pro-oxidantes, pode danificar macromoléculas de composição celular. Esta relação, em um organismo em situação fisiológica ou ambientalmente inadequada, faz com que seu sistema antioxidante não consiga neutralizar ROS geradas naturalmente na respiração mitocondrial e seus consequentes danos (Banerjee, 2008). Para evitar os danos os organismos lançam mão de processos enzimáticos e diversos fatores não enzimáticos que servem como moléculas antioxidantes (ascorbato, tocoferol, carotenoides, glutationa etc) (Amado et al., 2009; Regoli et al., 1999). Ou seja, a ação de enzimas e moléculas antioxidantes *versus* um ambiente ou molécula pro oxidante constitui o balanço redox do organismo, que pode estar sofrendo estresse oxidativo se a ação pro oxidante superar a antioxidante evidenciando dano lipídico, protéico ou no DNA (Van der Oost et al., 2003).

Desde a descoberta dos radicais livres em sistemas biológicos em meados de 1950, sua ação foi relacionada a processos patológicos. Entretanto, diversas alterações físico químicas ambientais induzem a produção de radicais livres (Lushchak, 2016). Por exemplo, alterações bioquímicas relacionadas ao stress oxidativo foram relatadas em peixes decorrente da salinidade (Zeng et al., 2017); temperatura e salinidade (Kim et al., 2017; Tseng et al., 2020); pH e temperatura (Carney Almroth et al., 2019; Sampaio et al., 2018); somente pH (Silva et al., 2016); e pH e dureza (Copatti et al., 2019), mas nenhum relato com pH e salinidade foi encontrado.

Muitas dessas pesquisas sobre radicais livres se concentram em radicais de oxigênio, que, conjuntamente com algumas moléculas que não são propriamente radicais, são chamadas de espécies reativas de oxigênio (do inglês, ROS). Com o conhecimento do ROS em sistemas biológicos, surgiu a grande pergunta: se os organismos teriam um sistema de defesa enzimático contra estes ROS, que foi então evidenciado em 1969 com o descobrimento da superóxido dismutase (SOD) em organismos vivos (Lushchak, 2016).

Atualmente está descrita uma grande variedade de enzimas que estão envolvidas no metabolismo oxidativo, e estas podem ser ativadas dependendo da necessidade ou fator de estresse ao qual o peixe é submetido (Lushchak, 2011). Por exemplo, para evitar e resolver desequilíbrios em moléculas oxidativas, os organismos constroem suas defesas usando mecanismos como a função DT-diaforase (DTD) que transforma quinonas em hidroquinonas, evitando a oxidação das primeiras e a produção de ROS. A superóxido dismutase (SOD) e a catalase (CAT) trabalham juntas para reduzir o O_2^- prejudicial, primeiro em H_2O_2 e por último em H_2O e O_2. Concomitantemente, a glutationa peroxidase (GPx), e a glutationa-S transferase (GST) podem ajudar a eliminar as ROS e seus produtos, utilizando da glutationa (GSH) que após emprego está oxidada (GSSG), e pode ser recuperada a GSH pela ação da glutationa redutase (GR). Em relação à contribuição de algumas fontes energéticas nas vias de proteção, a glicose 6-P desidrogenase (G6PDH) produz o NADPH, que é utilizado por enzimas relacionadas ao estresse oxidativo para eliminar ROS. O estresse oxidativo também pode ser avaliado considerando a proporção de GSSG para GSH, denominada índice de estresse oxidativo (OSI), considerando a capacidade antioxidante equivalente do Trolox (TEAC), contra radicais peroxyl (ACAP), dentre outras, que representa a capacidade antioxidante total de um organismo em comparação com um antioxidante ou frente a um agente pro oxidante. Bem como os thiois-proteicos (P-SH) e as substâncias reativas ao ácido tiobarbitúrico (TBARS), que indicam dano real às biomoléculas como a peroxidação proteica e lipídica (Halliwell and Gutteridge, 2015; Lushchak, 2016; Sanz et al., 2017).

Por fim é importante lembrar que o conceito de estresse oxidativo não pode ser, como incialmente citado, associado sempre e incisivamente como consequência “problema”. Uma vez que alterações do estado redox são sinalizadores celulares relacionados ao ROS, mas em muitas funções como fertilização, crescimento e diferenciação celular promovendo mudanças necessárias a vida, ou respostas adaptativas dos organismos para condições as quais estão sendo submetidos (Lushchak, 2016; Ray et al., 2012; Y. Wang et al., 2018). Um reforço a importância de estudar o status oxidativo dos peixes, se nota no fato de que o acumulo de eventos deletérios e o consequente “envelhecimento fisiológico” está relacionado ao estresse oxidativo (Almroth et al., 2010), sendo esta uma importante questão para organismos ornamentais, que após produção, devem ter assegurado que sua expectativa de vida não foi comprometida pelos manejos aplicados na produção.

Estes parâmetros somados as avaliações pertinentes da produção aquícola permitem avaliar qual o efeito da acidificação e da salinidade sobre peixes marinhos e estuarinos, criando um cenário de avanço para o conhecimento biológico, produção e conservação destas espécies. Reiterando que as duas espécies do presente estudo são naturalmente de ambientes diferentes, uma marinha, *Amphiprion percula* e outra estuarina, *Hippocampus reidi*.

1.9 Referências

Almroth, B.C., Johansson, A., Förlin, L., Sturve, J., 2010. Early-age changes in oxidative stress in brown trout , *Salmo trutta*. Comp. Biochem. Physiol. Part B 155, 442–448. https://doi.org/10.1016/j.cbpb.2010.01.012

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123. https://doi.org/10.1016/j.scitotenv.2008.11.038

Banerjee, R., 2008. Redox Metabolism and Life, in: Redox Biochemistry. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 1–9. https://doi.org/10.1002/9780470177334.ch1

Barange, M., Bahri, T., Beveridge, M.C.M., Cochrane, K.L., Funge-Smith, S., Poulain, F., 2018. Impacts of climate change on fisheries and aquaculture: synthesis of currrent knowledge, adaptation and mitigation options. FAO, Rome.

Bœuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 130, 411–423. https://doi.org/10.1016/S1532-0456(01)00268-X

Boyd, C.E., 2015. Water Quality, Second Edi. ed. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-17446-4

Burggren, W., Bautista, N., 2019. Invited review: Development of acid-base regulation in vertebrates. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 236, 110518. https://doi.org/10.1016/j.cbpa.2019.06.018

Calado, R., 2017a. The Need for Cultured Specimens, in: Marine Ornamental Species Aquaculture. Wiley, pp. 15–22. https://doi.org/10.1002/9781119169147.ch2

Calado, R., 2017b. Location, in: Marine Ornamental Species Aquaculture. Wiley, pp. 75–79. https://doi.org/10.1002/9781119169147.ch5

Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002. Acid – Base Regulation in Fishes : Cellular and Molecular Mechanisms. J. Exp. Zool. 293, 302–319. https://doi.org/10.1002/jez.10125

Cohen, F.P.A., Valenti, W.C., Planas, M., Calado, R., 2017. Seahorse Aquaculture, Biology and Conservation: Knowledge Gaps and Research Opportunities. Rev. Fish. Sci. Aquac. 25, 100–111. https://doi.org/10.1080/23308249.2016.1237469

Cristofori-Armstrong, B., Rash, L.D., 2017. Acid-sensing ion channel (ASIC) structure and function: Insights from spider, snake and sea anemone venoms. Neuropharmacology 1–12. https://doi.org/10.1016/j.neuropharm.2017.04.042

Dhaneesh, K.V., Nanthini Devi, K., Ajith Kumar, T.T., Balasubramanian, T., Tissera, K., 2012b. Breeding, embryonic development and salinity tolerance of Skunk clownfish *Amphiprion akallopisos*. J. King Saud Univ. - Sci. 24, 201–209. https://doi.org/10.1016/j.jksus.2011.03.005

Dhaneesh, Kottila Veettil, Ajith Kumar, T.T., Swagat, G., Balasubramanian, T., 2012a. Breeding and mass scale rearing of clownfish *Amphiprion percula*: feeding and rearing in brackishwater. Chinese J. Oceanol. Limnol. 30, 528–534. https://doi.org/10.1007/s00343-012-1184-x

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358. https://doi.org/10.1016/j.aquaculture.2006.03.019

Evangelista, I.R., dos Santos, L.N., dos Santos, A.F.G.N., 2020. Influence of salinity, temperature and photoperiod on eye asymmetry of *Amphirion ocellaris* larvae. Aquaculture 521, 734976. https://doi.org/10.1016/j.aquaculture.2020.734976

Faleiro, F., Baptista, M., Santos, C., Aurélio, M.L., Pimentel, M., Pegado, M.R., Paula, J.R., Calado, R., Repolho, T., Rosa, R., S, S., D, Q., M, Manning, Z, C., M, Marquis, KB, A., M, T., HL, M., 2015. Seahorses under a changing ocean: the impact of warming and acidification on the behaviour and physiology of a poor-swimming bony-armoured fish. Conserv. Physiol. 3, 1–7. https://doi.org/10.1093/conphys/cov009

Frommel, A.Y., Maneja, R., Lowe, D., Malzahn, A.M., Geffen, A.J., Folkvord, A., Piatkowski, U., Reusch, T.B.H., Clemmesen, C., 2012. Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nat. Clim. Chang. 2, 42–46.

https://doi.org/10.1038/nclimate1324

Frommel, A.Y., Margulies, D., Wexler, J.B., Stein, M.S., Scholey, V.P., Williamson, J.E., Bromhead, D., Nicol, S., Havenhand, J., 2016. Ocean acidification has lethal and sub-lethal effects on larval development of yellowfin tuna, *Thunnus albacares*. J. Exp. Mar. Bio. Ecol. 482, 18–24. https://doi.org/10.1016/j.jembe.2016.04.008

Gagliano, M., McCormick, M.I., Moore, J.A., Depczynski, M., 2010. The basics of acidification: Baseline variability of pH on Australian coral reefs. Mar. Biol. 157, 1849–1856. https://doi.org/10.1007/s00227-010-1456-y

Gattuso, J.-P., Gao, K., Lee, K., Rost, B., Schulz, K.G., 2011. Approaches and tools to manipulate the carbonate chemistry, in: Riebesell, U., Fabry, V.J., Hansson, L., Gattuso, J.-P. (Eds.), Guide to Best Practices for Ocean Acidification Research and Data Reporting. Luxembourg, pp. 41–51. https://doi.org/10.4324/9781315164298-12

Halliwell, B., Gutteridge, J.M.C., 2015. Free Radicals in Biology and Medicine. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198717478.001.0001

Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am. J. Physiol. Integr. Comp. Physiol. 307, R1061–R1084. https://doi.org/10.1152/ajpregu.00064.2014

Hora, M. dos S.C. da, Joyeux, J.-C., Rodrigues, R.V., Sousa-Santos, L.P. de, Gomes, L.C., Tsuzuki, M.Y., 2016. Tolerance and growth of the longsnout seahorse *Hippocampus reidi* at different salinities. Aquaculture 463, 1–6. https://doi.org/10.1016/j.aquaculture.2016.05.003

Ishimatsu, A., Hayashi, M., Kikkawa, T., 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. 373, 295–302. https://doi.org/10.3354/meps07823

Johnson, J., De Young, C., Bahri, T., Soto, D., Virapat, C., Eds, 2019. Proceedings of FishAdapt: The Global Conference on Climate Change Adaptation for Fisheries and Aquaculture, Bangkok, Thailand, 8-10 August 2016. FAO, Bangkok, p. 242.

Kang, C., Tsai, H., Liu, C., Lee, T., Hwang, P., 2010. Comparative Biochemistry and Physiology , Part A Salinity-dependent expression of a Na^+, K^+, $2Cl^-$ cotransporter in gills of the brackish medaka *Oryzias dancena* : A molecular correlate for hyposmoregulatory endurance. Comp. Biochem. Physiol. Part A 157, 7–18. https://doi.org/10.1016/j.cbpa.2010.05.013

Kang, C.-K., Yang, W.-K., Lin, S.-T., Liu, C.-C., Lin, H.-M., Chen, H.-H., Cheng, C.-W., Lee, T.-H., Hwang, P.-P., 2013. The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 181–91. https://doi.org/10.1016/j.cbpa.2012.08.010

Kikkawa, T., Kita, J., Ishimatsu, A., 2004. Comparison of the lethal effect of CO_2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Mar. Pollut. Bull. 48, 108–110. https://doi.org/10.1016/S0025-326X(03)00367-9

Kim, J., Park, H., Kim, K., 2017. Growth performance , oxidative stress , and non-specific immune responses in juvenile sablefish , *Anoplopoma fimbria* , by changes of water temperature and salinity. Fish Physiol. Biochem. 1421–1431. https://doi.org/10.1007/s10695-017-0382-z

Koldewey, H.J., Martin-Smith, K.M., 2010. A global review of seahorse aquaculture. Aquaculture 302, 131–152. https://doi.org/10.1016/j.aquaculture.2009.11.010

Kwan, G.T., Shen, S.G., Drawbridge, M., Checkley, D.M., Tresguerres, M., 2021. Ion-transporting capacity and aerobic respiration of larval white seabass (*Atractoscion nobilis*) may be resilient to ocean acidification conditions. Sci. Total Environ. 791, 148285. https://doi.org/10.1016/j.scitotenv.2021.148285

Lee, C., Kwon, B.-O., Hong, S., Noh, J., Lee, J., Ryu, J., Kang, S.-G., Khim, J.S., 2018. Sub-lethal and lethal toxicities of elevated CO_2 on embryonic, juvenile, and adult stages of marine medaka *Oryzias melastigma*. Environ. Pollut. 241, 586–595. https://doi.org/10.1016/j.envpol.2018.05.091

Luiz, O., Floeter, S., Rocha, L., Ferreira, C., 2013. Perspectives for the lionfish invasion in the South Atlantic: Are Brazilian reefs protected by the currents? Mar. Ecol. Prog. Ser. 485, 1–7. https://doi.org/10.3354/meps10383

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish : a mechanistic approach. Fish Physiol. Biochem. 42, 711–747. https://doi.org/10.1007/s10695-015-0171-5

Martinez-Cardenas, L., Purser, J.G., 2016. Effect of Direct Transfer to Different Salinities on Early Juvenile Pot-bellied Seahorse, *Hippocampus abdominalis* , Survival in Culture Conditions. J. World Aquac. Soc. 47, 201–206. https://doi.org/10.1111/jwas.12268

McCormick, S.D., Regish, A.M., 2018. Effects of ocean acidification on salinity tolerance and seawater growth of Atlantic salmon *Salmo salar* smolts. J. Fish Biol. 93, 560–566. https://doi.org/10.1111/jfb.13656

Miller, G.M., Watson, S.A., Mccormick, M.I., Munday, P.L., 2013. Increased CO_2 stimulates reproduction in a coral reef fish. Glob. Chang. Biol. 19, 3037–3045. https://doi.org/10.1111/gcb.12259

Mota, V.C., Hop, J., Sampaio, L.A., Heinsbroek, L.T.N., Verdegem, M.C.J., Eding, E.H., Verreth, J.A.J., 2018. The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac. Res. 49, 3456–3467. https://doi.org/10.1111/are.13812

Palmtag, M.R., 2017. The Marine Ornamental Species Trade, in: Marine Ornamental Species Aquaculture. Wiley, pp. 3–14. https://doi.org/10.1002/9781119169147.ch1

Planas, M., Burhans, R., Simões, N., 2017. Seahorses and Pipefish, in: Marine Ornamental Species Aquaculture. Wiley, pp. 299–326. https://doi.org/10.1002/9781119169147.ch17

Pouil, S., Tlusty, M.F., Rhyne, A.L., 2019. Aquaculture of marine ornamental fish : overview of the production trends and the role of academia in research progress. Rev. Aquac. 1–14. https://doi.org/10.1111/raq.12381

Ray, P.D., Huang, B.W., Tsuji, Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell. Signal. 24, 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008

Regoli, F., Winston, G.W., Radicals, P., Radi-, H., Phar-, G.W.T.A., 1999. Quantification of Total Oxidant Scavenging Capacity of Antioxidants for Peroxynitrite , Peroxyl Radicals , and Hydroxyl Radicals 105, 96–105.

Rhyne, A.L., Tlusty, M.F., Schofield, P.J., Kaufman, L., Morris, J.A., Bruckner, A.W., 2012. Revealing the Appetite of the Marine Aquarium Fish Trade: The Volume and Biodiversity of Fish Imported into the United States. PLoS One 7, e35808. https://doi.org/10.1371/journal.pone.0035808

Rodrigues, R.V., dos Santos Pedron, J., Romano, L.A., Tesser, M.B., Sampaio, L.A., 2015. Acute responses of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) to acid stress. Aquac. Res. 46, 1241–1247. https://doi.org/10.1111/are.12282

Sanz, A., López-rodríguez, M.J., García-mesa, S., Trenzado, C.E., Ferrer, R.M., Figueroa, J.M.T.D.E., 2017. Are antioxidant capacity and oxidative damage related to biological and autecological characteristics in aquatic insects? J. Limnol. 76, 170–181. https://doi.org/10.4081/jlimnol.2016.1581

Schmidt-Nielsen, K. 2002. Fisiologia Animal: Adaptação e Meio Ambiente. 5. ed. São Paulo: Livraria e Editora Santos. 609 p.

Shartau, R.B., Damsgaard, C., Brauner, C.J., 2019. Limits and patterns of acid-base regulation during elevated environmental CO_2 in fish. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 236, 110524. https://doi.org/10.1016/j.cbpa.2019.110524

Shrivastava, J., Ndugwa, M., Caneos, W., De Boeck, G., 2019. Physiological trade-offs, acid-base balance and ion-osmoregulatory plasticity in European sea bass (*Dicentrarchus labrax*)juveniles under complex scenarios of salinity variation, ocean acidification and high ammonia challenge. Aquat. Toxicol. 212, 54–69. https://doi.org/10.1016/j.aquatox.2019.04.024

Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability , development and biochemical responses of sand smelt larvae. Sci. Total Environ. 563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091

Silveira, R.B., Siccha-ramirez, R., Rodrigo Santos Silva, J., Oliveira, C., 2014. Morphological and molecular evidence for the occurrence of three *Hippocampus* species (Teleostei: Syngnathidae) in Brazil. Zootaxa 3861, 317–332. https://doi.org/10.11646/zootaxa.3861.4.2

Smith, K.F., Behrens, M.D., Max, L.M., Daszak, P., 2008. US drowning in unidentified fishes: Scope, implications, and regulation of live fish import. Conserv. Lett. 1, 103–109. https://doi.org/10.1111/j.1755-263X.2008.00014.x

Thornhill, D.J., 2012. Ecological impacts and practices of the coral reef wildlife trade, Defenders of Wildlife.

Timmons, M.B., Ebeling, J.M., 2010. Recirculating Aquaculture, 2° edition. ed. Northeastern Regional Aquaculture Center, Ithaca, NY.

Tseng, Y.C., Hwang, P.P., 2008. Some insights into energy metabolism for osmoregulation in fish. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 148, 419–429. https://doi.org/10.1016/j.cbpc.2008.04.009

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharmacol. 13, 57–149. https://doi.org/10.1016/S1382-6689(02)00126-6

Wabnitz, C., Taylor, M., Green, E., Razak, T., 2003. From Ocean to Aquarium: The Global Trade in Marine Ornamental Species. UNEP-WCMC, Cambridge, UK.

Wang, S., Jiang, Z., Mao, M., Mao, S., Sun, Y., Sui, Y., 2018. Effects of seawater pH on survival, growth, energy budget and oxidative stress parameters of juvenile turbot *Scophthalmus maximus*. Iran. J. Fish. Sci. 17, 675–689. https://doi.org/10.22092/ijfs.2018.116814

Wang, Y., Branicky, R., Noë, A., Hekimi, S., 2018. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. jcb.201708007. https://doi.org/10.1083/jcb.201708007

Wasielesky, W., Bianchini, A., Santos, M.H.S., Poersch, L.H., 1997. Tolerance of juvenile flatfish Paralichthys *orbignyanus* to acid stress. J. World Aquac. Soc. 28, 202–204. https://doi.org/10.1111/j.1749-7345.1997.tb00857.x

Welch, M.J., Munday, P.L., 2015. Contrasting effects of ocean acidification on reproduction in reef fishes. Coral Reefs 35, 1–9. https://doi.org/10.1007/s00338-015-1385-9

Witttenrich, ML. 2007. The complete illustrated breeder's guide to marine aquarium fishes. T.F.H Publications. Neptune city. 301p.

Yang, S.H., Kang, C.K., Kung, H.N., Lee, T.H., 2014. The lamellae-free-type pseudobranch of the euryhaline milkfish (*Chanos chanos*) is a Na^+, K^+-ATPase-abundant organ involved in hypoosmoregulation. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 170, 15–25. https://doi.org/10.1016/j.cbpa.2013.12.018

2. HIPÓTESE

A exposição ao ambiente ácido é mais onerosa em água marinha (alta salinidade), considerando o conforto isosmótico proporcionado pela menor salinidade em água salobra.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Estudar a produção dos peixes ornamentais marinhos, *Amphiprion percula* e *Hippocampus reidi*, em diferentes pH's em sistemas de recirculação em água marinha (SW) e estuarina (BW).

3.2 OBJETIVOS ESPECÍFICOS

Verificar o dano subletal do pH em juvenis do peixe-palhaço *A. percula* a nível de status oxidativo;

Avaliar alterações no status oxidativo em órgãos específicos de juvenis do peixe-palhaço *A. percula* submetidos a pH ácidos em SW e BW;

Avaliar alterações de estresse, principalmente no status oxidativo, de juvenis do cavalo-marinho *H. reidi* submetidos a pH ácidos em SW e BW

Avaliar o desempenho e o status oxidativo de recém-nascidos e juvenis do *H. reidi* em diferentes pH's e salinidades;

Avaliar o desempenho zootécnico de juvenis de *A. percula* em diferentes pH's e salinidades, considerando o retorno as condições controle;

4. CAPÍTULO 1: Does acidification lead to impairments on oxidative status and survival of orange clownfish *Amphiprion percula* juveniles?

Artigo publicado na revista Fish Physiology and Biochemistry

https://doi.org/10.1007/s10695-021-00942-9

Authors: Mario Davi Dias Carneiro[a,b*], Lucas Campos Maltez[a], Ricardo Vieira Rodrigues[a], Miquel Planas[b], Luís André Sampaio[a].

[a] Laboratório de Piscicultura Estuarina e Marinha, Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil
[b] Department of Ecology and Marine Resources, Instituto de Investigaciones Marinas (CSIC), Vigo, Spain.

[*]Author for correspondence (marioiddc@gmail.com)

Abstract.

The nitrification process in recirculating aquaculture systems can reduce water pH. Fish can also be exposed to water acidification during transport, an important feature in the aquarium industry, as live fish can be kept in a closed environment for more than 24h during overseas aerial transportation. Therefore, it is important to study the responses of fish to acidic environments. We investigated the impacts of acute exposure to decreasing pH levels in orange clownfish *Amphiprion percula* juveniles on their survival and oxidative stress status. Fish were exposed to pH 5, 6, 7, and 8 for 96h. We observed significant reduction in survival (85%) and protein damage as measured by P-SH (protein thiol) for fish maintained at pH 5. Despite no effects on survival or oxidative damage, fish exposed to pH 6 showed an increase in their antioxidant defense systems, demonstrating this pH level could not be suitable for them as well. Furthermore, there were no negative effects for fish kept at pH 7, compared to those maintained at pH 8 during this short-term evaluation.

Keywords: anemonefish, aquarium, RAS, reef fish, stress.

Introduction

Acidification is a threat to aquatic organisms in aquaculture. In fish, Wasielesky et al (1997) observed that flatfish (*Paralichthys orbignyanus)* juveniles survived when exposed to pH 5.2 for 96h, but ventilation rate in juveniles maintained at pH 6.1 was increased in comparison to fish in controls (pH 8.0). Juvenile cobia (*Rachycentron canadum*) survived after an exposure to pH 5.5 for 24h, but damage in gills and skin epithelium were noticed in fish kept at pH 6.0 (Rodrigues et al., 2015). However, it is noteworthy that some teleost fish may not be harmed by certain levels of acidification. The growth of turbot (*Psetta maxima)* juveniles was not harmed when reared in pH 5.7 compared to those reared in pH 7.5 (Mota et al. 2018). Furthermore, turbot (*P. maxima)* growth rate was higher in juveniles raised at pH 6.3-7.3 than those reared at pH 8.3-8.8 (S. Wang et al., 2018). Improvement of fish performance was also observed when exposed to smaller pH acidification. Growth of Atlantic salmon (*Salmo salar)* smolts was improved when they were reared in pH 7.7 compared to those raised at pH 8.1 (McCormick and Regish, 2018). Regarding clownfish, there is scarce information on the effects of acidification. The reproductive output of *Amphiprion melanopus* (Miller et al. 2013) and *Amphiprion percula* (Welch and Munday 2015) was improved when the breeders were maintained in a CO_2 rich environment, which decreased pH to 7.5-7.8 in comparison to pH 8.1.

Water pH reduction can be severe in recirculating aquaculture systems (RAS), because the nitrification process releases H^+. If no action is taken to restore alkalinity reserves, pH can be reduced to 5.7-6.0 in a couple of days (Ebeling et al., 2006; Mota et al., 2018). The intensity of acidification in a RAS will be influenced by the quantity of food introduced in the system, because as more protein is consumed, the larger will be the activity of the biological filter in order to oxidize all excreted ammonia (Timmons and Ebeling, 2010). Additionally, fast pH reduction can occur during fish transport due to increased CO_2 concentration (Cohen et al. 2018), causing gill damage and ion regulation dysfunction (Lim et al., 2003a).

The exposure to water acidification can cause oxidative stress in aquatic organisms, including fish (Han et al., 2018; Mai et al., 2010; Tiedke et al., 2013; Wang et al., 2009). Aerobic organisms rely on oxygen to survive. However, the aerobic metabolism results in the production of ROS (reactive oxygen species), among other reactive products (Lushchak, 2016). In order to counterattack the formation of ROS, they also rely on an antioxidant defense system, which is composed by enzymes and molecules with antioxidant capacity. The imbalance between the production of oxidants molecules and the antioxidant defenses, in favor of oxidizing agents, may increase the levels of oxidative damage in macromolecules such as lipids, proteins and DNA, thus compromising their biological functions (Halliwell & Gutteridge 2015). Tiedke et al. (2013) considered unlikely that acidification itself triggers an overproduction of ROS. Instead, oxidative stress associated with acidification may be the result of an increase in aerobic metabolism involved in physiological adjustments for ionic and acid-base balance maintenance. In addition, acidification may reduce the hemoglobin affinity for oxygen through the Bohr effect, and the resulting hypoxia is a condition known for inducing oxidative stress in fish (Johannsson et al., 2018).

The marine aquarium industry relies heavily on the capture of wild fish (Olivotto et al., 2016; Pouil et al., 2019), but production of some species in captivity has been increasing. This is the case of clown fish *A. percula*, a native species of the Eastern Indian and West Pacific oceans (Thornhill, 2012). Its global market is estimated at around 70,000-100,000 individuals, with 80% originating from countries outside their native range, thus suggesting the importance of aquaculture for the international ornamental fish market (Maison and Graham 2015).

The aim of the present study was to evaluate survival, antioxidant responses and oxidative damage levels in juvenile *A. percula* exposed to short term seawater acidification.

Material and methods

The trials were conducted at the facilities of the Marine Fish Culture Laboratory (LAPEM) of the Institute of Oceanography of Federal University of Rio Grande (FURG), in southern Brazil. All experimental procedures involving fish manipulation were approved by the Ethics Committee on Animal Use of FURG (# 23116.003734/2018-86).

Juvenile *A. percula* were produced at LAPEM following the protocols of Hoff (1996). Briefly, naturally spawned eggs were allowed to hatch in the broodstock tank. Newly hatched larvae were transferred to larviculture tanks, where larvae were fed on live prey (rotifers and *Artemia*) in “green water” (*Nannochloropsis oceanica*) until they were fully weaned into dry diets. Two months old fish (weight 263.5±77.5 mg, length 24.4±2.4 mm) were transferred from the rearing tanks (pH 8) into 12 circular tanks (40 L) distributed in four RAS. Eleven fish were placed in each tank, where they were acutely exposed in triplicate to four pH levels: 5, 6, 7, and 8 for 96h. Each RAS unit was equipped with a pH controller (Tecna Evo 603, Seko, Brazil), which continuously monitored and added an appropriate volume of an acidic solution (HCl 3% - 0,36 M) in order to maintain the desired pH levels. The fish were fasted throughout the trial.

The water quality was monitored every morning and kept as follows: salinity: 33±1 (refractometer ATAGO S/Milli-E, Japan); temperature: 26.4±0.1 °C and oxygen 6.3±0.2 mg O_2 L^{-1} (Oxymeter 550A, YSI, USA); total ammonia nitrogen 0.06±0.01 N-NH_4^+ + NH_3 mg L^{-1} (UNESCO 1983); nitrite 0.0 N-NO_2^- mg L^{-1} (Bendschneider and Robinson 1952). The actual pH levels were 5.01±0.01, 5.97±0.01, 6.99±0.01 and 8.05±0.01 (pH meter EcoSense pH 100A, YSI, USA). Alkalinity at pH 5, 6, 7, and 8 increased with increasing pH (14±1, 19±1, 28±1, 143±1 $CaCO_3$ mg L^{-1}, respectively) (Eaton et al., 2005).

At the end of the experimental period, five fish from each tank were euthanized using a lethal benzocaine hydrochloridrate bath (300 ppm). Whole-body samples were immediately frozen in liquid nitrogen and stored at -80°C until biochemical analyses.

Biochemical analyses

Individual whole-body samples were homogenized (homogenizer Marconi, MA 590/Agata, Brazil) (1:4 – w/v) in an ice-cold homogenization buffer (100 mM Tris–HCl, 0,1 mM EDTA, pH 7.8, and 1% triton X-100 (v/v)) according Castro et al. (2012). The samples were then centrifuged at 10,000 *g* for 30 minutes at 4 °C (SOLAB SL-703, Brazil). Thus, every homogenate, contains 20% of the original sample mass, and this is taken into consideration for normalization of the results per tissue. The supernatants were stored at -80 °C until they were thawed for the biochemical assays described below. All analyses were performed in 96-well plates using a spectrofluorometer (BioTek, Synergy HT, USA).

The total protein content of the supernatant described above was determined by biuret protein assay using bovine serum albumin as standard (40 mg mL^{-1}). A commercial kit was used (Proteínas Totais, Doles, Brazil), results were read at 550 nm.

The Glutathione-S-transferase (GST) activity was measured adding to 15 µL of sample homogenate, 1mM of reduced glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). The final solution was read at 340 nm every minute for 5 min (Habig, 1974).

The determination of the total antioxidant capacity against peroxyl radicals (ACAP) through reactive oxygen species (ROS) followed the method described by

Amado et al. (2009). Homogenates (10 µL previously diluted to 2.0 mg protein mL^{-1}) were added to ABAP (20 µM 2,2-azobis-2-methylpropionamidine dihydrochloride), as peroxyl radicals generator, or distilled water, in conjunction with H2DCF-DA (40 µM of 2′, 7′ dichlorofluorescein diacetate). Fluorescence of the resulting fluorochromes were determined (excitation 485 nm; emission 520 nm) with readings every 5 min for 30 min. For interpretation of the results, a higher relative area means a lower antioxidant capacity. ACAP relative fluorescent area were calculate as follows:

$$ACAP = (ROS_{\text{area with ABAP}} - ROS_{\text{area without ABAP}}) / ROS_{\text{area without ABAP}}.$$

The non-proteic (NP-SH) and proteic thiols (P-SH) were measured based on DTNB (5,5- dithiobis-(2-nitrobenzoic acid; Sigma) reactions. Homogenized samples (50 µL) were deproteinized with trichloroacetic acid (TCA 50% w/v) and distilled water for 15 minutes in an ice bath. After centrifugation (3,000 *g*, at 4°C for 15 min) the supernatants were used to NP-SH reaction. The pellet formed by the precipitated protein was re-suspended with 30 µL of homogenization buffer for determination of P-SH content. Both readings were done at 405 nm absorbance according Sedlak and Lindsay (1968).

Lipid peroxidation (LPO) was evaluated following the protocol of reactive substances to thiobarbituric acid (TBARS) (Oakes & Van Der Kraak, 2003). In order to determine LPO, TBA (2-thiobarbituric acid) was added to 20 µL of the homogenized sample, the resulting fluorescence of the supernatant is read at 515 nm (excitation) and 580 nm (emission). TBARS levels are plotted in function of a calibration curve of 1,1,3,3-tetramethoxypropane (TMP) and expressed as nmol TMP per mg of tissue.

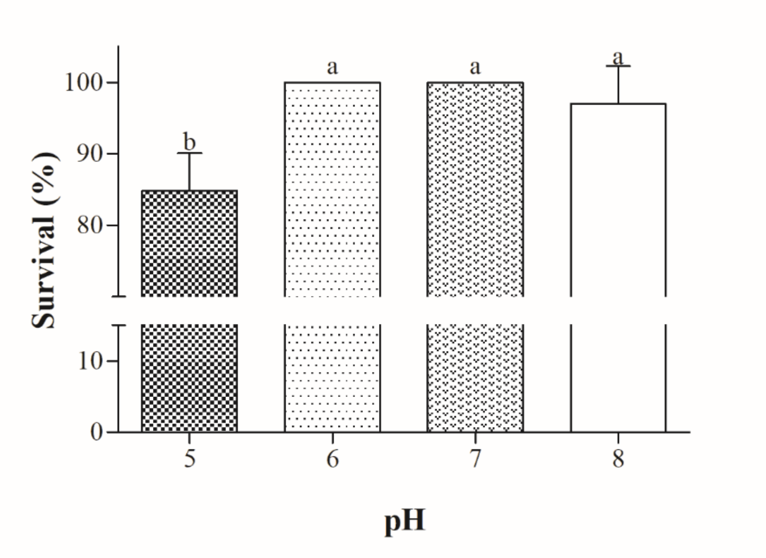
Statistical analyses

The experimental design was totally randomized. Normality and homoscedasticity were verified using Shapiro Wilk's and Levene's test, respectively. If data were not normal and/or homoscedastic, the Rank transformation was applied (Friedman, 1937). One-way ANOVA followed by Newman-Keuls test were used for identifying differences among treatments. The minimum significance level was set at 5% ($P<0.05$) for all cases. All data were expressed as average ± standard deviation.

Results

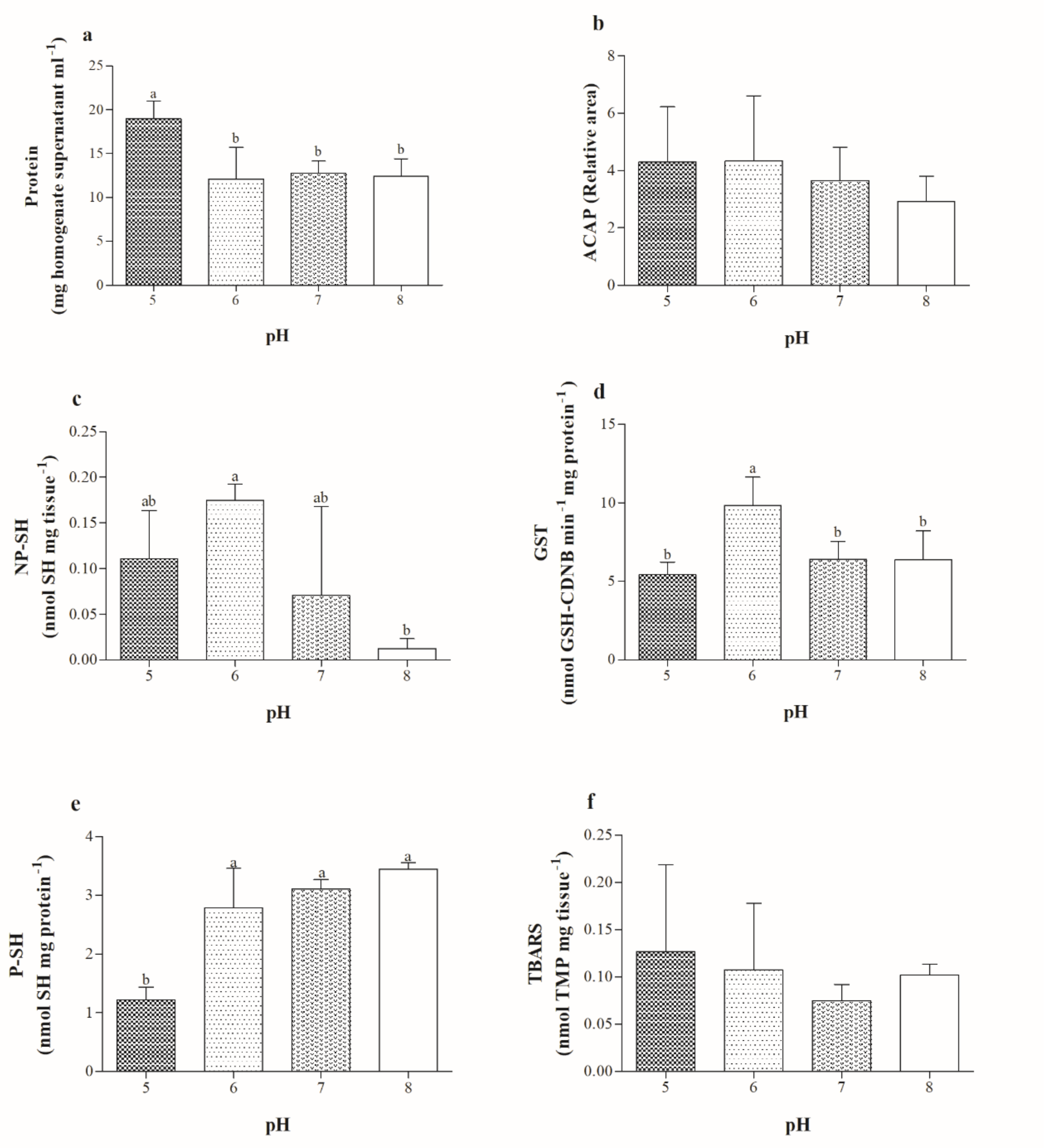
Survival of *A. percula* was significantly influenced by pH (P<0.01). While nearly 100% survival was achieved for fish maintained at pH 6-8, only 85% of fish survived after 96 h exposure to pH 5 (Figure 1).

Figure 1 - Survival of juvenile *Amphiprion percula* after acute exposure to varying pH levels for 96h. Values are means ± SD. Data are expressed as means ± SD (n=3). Different letters indicate significant differences among treatments (Newman-Keuls test, p<0.05).



All biochemical results are show in figure 2. The protein content increased in fish exposed to pH 5 (P<0.05) (figure 2a). Despite not significant (P>0.05), ACAP for fish exposed to acidic environment was 26.6% and 35.9% higher (respectively for pH 5 and 6) than in fish maintained at pH 8 (higher values mean lower antioxidant capacity) (figure 2b). The GST activity (figure 2c) and the content of NP-SH (figure 2d) showed a similar response pattern. Both parameters were significantly higher at pH 6 than at pH 8 (P<0.05), but there were no significant differences regarding NP-SH content for fish kept at pH 5, 6, and 7 (P>0.05). Regarding actual oxidative damage, we observed significative lower P-SH content (P<0.01) for fish kept at pH 5 in comparison with all other treatments (figure 2e). However, *A. percula* juveniles showed no effect of water pH on lipid peroxidation (P>0.05) for TBARS (figure 2f) among all treatments.

Figure 2 - a- Total protein content of the supernatant was determined by biuret protein assay using bovine serum albumin as standard (40 mg mL^{-1}), **b**- total antioxidant capacity against peroxyl radicals (ACAP), **c**- activity of glutathione-S-transferases (GST) determined following the conjugation of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB), **d**- content of non-proteic (NP-SH) and **e**- proteic thiols (P-SH), and **f**- levels of reactive substances to thiobarbituric acid (TBARS) using 1,1,3,3-tetramethoxypropane (TMP) as standard. All analyses were done in whole-body homogenates of juvenile *Amphiprion percula* after acute exposure to different pH values for 96h. Data are expressed as means ± SD (n=15). Different letters indicate significant differences among treatments (Newman-Keuls test, $p<0.05$).



Discussion

In aquaculture systems pH can be reduced due to increased H^+ production during the nitrification process and also by increased CO_2 concentration. Results of studies focusing in acidification cause by increased CO_2 will show stress responses due to the pH reduction itself, but also to the toxicity of CO_2. These studies often attempt to simulate the reality of ocean acidification, which is not the case of the present investigation.

Even though mortality increased at the lowest pH tested (pH=5), it is noteworthy that deaths occurred at the last day of the trial (96h). Similarly, juvenile cobia *R. canadum* did not die when exposed to pH 5.5 for 24h (Rodrigues et al., 2015). That finding supports the high capacity of marine fish to withstand acidification. Although fish are resilient to acidic conditions (McCormick and Regish, 2018; Miller et al., 2013; Mota et al., 2018; Welch and Munday, 2015), impacts of acid or CO_2 acidification may conduct to different survival rates for a given pH level, but higher mortalities occur due to CO_2 toxicity (Kikkawa et al., 2004).

The increased protein content observed at pH 5 would be due to changes in fish metabolism, i.e. selective consumption of carbohydrates/lipids reserves in a stressed condition (Martínez-Álvarez et al., 2002; Tseng and Hwang, 2008) along with increasing abundances of oxidative stress and ionoregulation related proteins (Rodrigues et al., 2015; Tomanek, 2014).

Measurement of antioxidant responses has been conducted in terms of the overall tissue capacity to scavenge ROS. The advantage of such techniques (as TOSC or equivalent methodology) is the capacity to establish an integrated antioxidant response of an organism in response to different ROS (Amado et al., 2009; Regoli et al., 2002). Thus, even though no differences were observed for ACAP in the present study, our results suggest that the fish are expending the antioxidant reserves to combat the acidic stress. The ACAP has rarely been used to assess pH related stress in marine fish, but it is a common biomarker for freshwater fish facing acidic exposition, where a reduction in ACAP content is often demonstrated (Copatti et al., 2019b; Pellegrin et al., 2019).

Reduced glutathione (GSH) is the main component of NP-SH, and its content may increase due to the increased activity of glutamate cysteine ligase, glutathione synthetase and glutathione reductase (GR) that produce or recover GSH, and/or the uptake of substrate amino acids in order to protect the fish from oxidative stress (Srikanth et al. 2013). It works along GST to remove dangerous products from the cells (Blanchette et al., 2007). Enhanced NP-SH and GST activity demonstrated at pH 6 can be interpreted as an antioxidant response triggered to deal with a pro-oxidant condition induced by that level of acidification. Our results showed that the antioxidant system of clownfish juveniles is enhanced at pH 6 compared to any other pH level tested.

The reduction observed in P-SH levels at pH 5 can be interpreted as a protein oxidation evidence. The oxidation of cysteine SH groups can cause intermolecular protein cross-linking and enzyme inactivation, eventually leading to cell death (Colombo et al., 2020; Mitton et al., 2016). It has been pointed out that protein oxidation measured by carbonyl groups increase in Atlantic halibut (*Hippoglossus hippoglossus*) after long-term exposition to pH 7.6 (Carney Almroth et al., 2019a). Furthermore, orange clownfish did not suffer LPO, contrarily to the findings reported after a long-term exposition of flatfish larvae, *Solea senegalensis*, to pH 7.5 (Pimentel et al., 2015) as well as in the brain of a freshwater cyprinid acutely exposed to pH 5.5 (Mukherjee et al., 2019).

The antioxidant mechanisms of orange clown fish succeeded in avoiding lipid peroxidation and protein damage when exposed to a pH level as low as 6. Furthermore, no mortality was observed at that pH. However, at the most severe acidification environment evaluated in the present study (pH 5), orange anemonefish did not perform well GST activity did not follow the same increasing trend observed for fish kept in pH 6, on the contrary, this enzyme seemed to be exhausted, as its activity decreased towards levels observed at normal pH. At this low pH level, it was also observed increased protein damage and higher mortality.

Special care should be taken when rearing this species in a RAS, where pH could fall below 6, and also when transported under high stocking densities (Sampaio et al., 2019; Sampaio and Freire, 2016). As shown in our results, protein damage was already observed even in short term exposition to pH 5, and the antioxidant defense system was under pressure at pH 6, showing that this environment may not be suitable for orange clownfish either. Therefore, considering the antioxidant scenario, *A. percula* should not be acutely exposed to pH 6 or below.

Conclusions

Clownfish juveniles increased their antioxidant defense system as pH is reduced to 6. This is shown by higher content of NP-SH and activity of GST. At that pH level, tissue damage and mortalities were not observed. A further increase in acidification (pH 5) resulted in increased mortality and the failure of the antioxidant defense system to avoid oxidative stress, as shown by protein oxidation.

Acknowledements

The authors are grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for research fellowships provided for L.A. Sampaio (# 307445/2016-0), and to CAPES (Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) for a research grant provided for M.D.D. Carneiro.

References

Amado LL, Garcia ML, Ramos PB, et al (2009) A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci Total Environ 407:2115–2123. https://doi.org/10.1016/j.scitotenv.2008.11.038

Bendschneider, K, Robinson RJ (1952) A new spectrophotometric method for the determination ofnitrite in sea water. J. Mar. Res. 11:87–96

Blanchette B, Feng X, Singh BR (2007) Marine glutathione S-transferases. Mar Biotechnol 9:513–542. https://doi.org/10.1007/s10126-007-9034-0

Carney Almroth B, Bresolin de Souza K, Jönsson E, Sturve J (2019) Oxidative stress and biomarker responses in the Atlantic halibut after long term exposure to elevated CO_2 and a range of temperatures. Comp Biochem Physiol Part - B Biochem Mol Biol 238:1–8. https://doi.org/10.1016/j.cbpb.2019.110321

Castro C, Pérez-Jiménez A, Guerreiro I, et al (2012) Effects of temperature and dietary protein level on hepatic oxidative status of Senegalese sole juveniles (*Solea senegalensis*). Comp Biochem Physiol A Mol Integr Physiol 163:372–378. https://doi.org/10.1016/j.cbpa.2012.07.003

Cohen FPA, Planas M, Valenti WC, et al (2018) Optimizing packing of live seahorses for shipping. Aquaculture 482: 57-64. https://doi.org/10.1016/j.aquaculture.2017.09.024

Colombo GM, Simião S, Schmitz MJ, et al (2020) The role of açaí (*Euterpe oleracea* Mart. 1824) as a chemoprotective agent in the evaluation of antioxidant defence, oxidative damage and histology of juvenile shrimp *Litopenaeus vannamei* (BOONE, 1931) exposed to ammonia. Aquac Res 51: 1551– 1566. https://doi.org/10.1111/are.14503

Copatti CE, Baldisserotto B, Souza C de F, et al (2019) Water pH and hardness alter ATPases and oxidative stress in the gills and kidney of pacu (*Piaractus* *mesopotamicus*). Neotrop Ichthyol 17:1–10. https://doi.org/10.1590/1982-0224-20190032

Eaton AD, Clesceri LS, Rice EW, Greenberg AE (2005) Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington, D.C

Ebeling JM, Timmons MB, Bisogni JJ (2006) Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257:346–358. https://doi.org/10.1016/j.aquaculture.2006.03.019

Friedman M (1937) The Use of Ranks to Avoid the Assumption of Normality Implicit in the Analysis of Variance. J Am Stat Assoc 32:675–701. https://doi.org/10.1080/01621459.1937.10503522

Gattuso J-P, Magnan A, Billé R, et al (2015) Contrasting futures for ocean and society from different anthropogenic CO_2 emissions scenarios. Science 349:aac4722-1–10. https://doi.org/10.1126/science.aac4722

Habig WH, Pabst MJ, Jakoby WB (1974) Glutathione S-transfer- ases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249:7130–7139

Han S, Wang M, Wang B, et al (2018) A comparative study on oxidative stress response in the hepatopancreas and midgut of the white shrimp *Litopenaeus vannamei* under gradual changes to low or high pH environment. Fish Shellfish Immunol 76:27–34. https://doi.org/10.1016/j.fsi.2018.02.001

Johannsson OE, Giacomin M, Sadauskas- H, et al (2018) Does hypoxia or different rates of re-oxygenation after hypoxia induce an oxidative stress response in *Cyphocharax abramoides* (Kner 1858), a Characid fish of the Rio Negro? Comp Biochem Physiol Part A 224:53–67. https://doi.org/10.1016/j.cbpa.2018.05.019

Kikkawa T, Kita J, Ishimatsu A (2004) Comparison of the lethal effect of CO_2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Mar Pollut Bull 48:108–110. https://doi.org/10.1016/S0025-326X(03)00367-9

Lim LC, Dhert P, Sorgeloos P (2003) Recent developments and improvements in ornamental fish packaging systems for air transport. Aquac Res 34:923–935

Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101:13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Lushchak VI (2016) Contaminant-induced oxidative stress in fish : a mechanistic approach. Fish Physiol Biochem 42:711–747. https://doi.org/10.1007/s10695-015-0171-5

Mai W, Yan J, Wang L, et al (2010) Acute acidic exposure induces p53-mediated oxidative stress and DNA damage in tilapia (*Oreochromis niloticus*) blood cells. Aquat Toxicol 100:271–281. https://doi.org/10.1016/j.aquatox.2010.07.025

Maison KA, Graham KS (2015) Status review report: Orange clownfish (*Amphiprion percula*). Report to National Marine Fisheries Service, Office of Protected Resources. NOAA, Silver Spring.

Martínez-Álvarez RM, Hidalgo MC, Domezain A, et al (2002) Physiological changes of sturgeon *Acipenser naccarii* caused by increasing environmental salinity. J Exp Biol 205:3699–3706

McCormick SD, Regish AM (2018) Effects of ocean acidification on salinity tolerance and seawater growth of Atlantic salmon *Salmo salar* smolts. J Fish Biol 93:560–566. https://doi.org/10.1111/jfb.13656

Miller GM, Watson SA, Mccormick MI, Munday PL (2013) Increased CO_2 stimulates reproduction in a coral reef fish. Glob Chang Biol 19:3037–3045. https://doi.org/10.1111/gcb.12259

Mitton FM, Ribas JL, Gonzalez M, et al (2016) Antioxidant responses in soybean and alfalfa plants grown in DDTs contaminated soils : Useful variables for selecting plants for soil phytoremediation ? Pestic Biochem Physiol 130:17–21. https://doi.org/10.1016/j.pestbp.2015.12.005

Mota VC, Hop J, Sampaio LA, et al (2018) The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac Res 49:3456–3467. https://doi.org/10.1111/are.13812

Mukherjee A, Bhowmick AR, Mukherjee J, Moniruzzaman M (2019) Physiological response of fish under variable acidic conditions: a molecular approach through the assessment of an eco-physiological marker in the brain. Environ Sci Pollut Res 26:23442–23452. https://doi.org/10.1007/s11356-019-05602-3

Pellegrin L, Nitz LF, Maltez LC, et al (2019) Alkaline water improves the growth and antioxidant responses of pacu juveniles (*Piaractus mesopotamicus*). Aquaculture 734713. https://doi.org/10.1016/j.aquaculture.2019.734713

Pimentel MS, Faleiro F, Diniz M, et al (2015) Oxidative Stress and Digestive Enzyme Activity of Flatfish Larvae in a Changing Ocean. PLoS One 10:e0134082. https://doi.org/10.1371/journal.pone.0134082

Pouil S, Tlusty MF, Rhyne AL (2019) Aquaculture of marine ornamental fish : overview of the production trends and the role of academia in research progress. Rev Aquac 1–14. https://doi.org/10.1111/raq.12381

Regoli F, Gorbi S, Frenzilli G, et al (2002) Oxidative stress in ecotoxicology: From the analysis of individual antioxidants to a more integrated approach. Mar Environ Res 54:419–423. https://doi.org/10.1016/S0141-1136(02)00146-0

Rodrigues RV, dos Santos Pedron J, Romano LA, et al (2015) Acute responses of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) to acid stress. Aquac Res 46:1241–1247. https://doi.org/10.1111/are.12282

Sampaio FDF, Freire CA (2016) An overview of stress physiology of fish transport: changes in water quality as a function of transport duration. Fish Fish 17:1055–1072. https://doi.org/10.1111/faf.12158

Sampaio FDF, Silva-de-assis HC, Bettim FL, et al (2019) Water acidification causes death of marine ornamental fish (Perciformes : Pomacentridae) during transport : contributing to the conservation of wild populations. Zoologia 36:1–10. https://doi.org/https://doi.org/10.3897/zoologia.36.e25083

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25:192–205. https://doi.org/10.1016/0003-2697(68)90092-4

Srikanth K, Pereira E, Duarte AC, Ahmad I (2013) Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish–a review. Environ Sci Pollut Res 20:2133–2149. https://doi.org/10.1007/s11356-012-1459-y

Thornhill DJ (2012) Ecological impacts and practices of the coral reef wildlife trade. Defenders of Wildlife, Washington, DC

Tiedke J, Cubuk C, Burmester T (2013) Environmental acidification triggers oxidative stress and enhances globin expression in zebrafish gills. Biochem Biophys Res Commun 441:624–629. https://doi.org/10.1016/j.bbrc.2013.10.104

Timmons MB, Ebeling JM (2010) Recirculating Aquaculture, 2° edition. Northeastern Regional Aquaculture Center, Ithaca, NY

Tomanek L (2014) Proteomics to study adaptations in marine organisms to environmental stress. J Proteomics. https://doi.org/10.1016/j.jprot.2014.04.009

Tseng YC, Hwang PP (2008) Some insights into energy metabolism for osmoregulation in fish. Comp Biochem Physiol - C Toxicol Pharmacol 148:419–429. https://doi.org/10.1016/j.cbpc.2008.04.009

UNESCO (1983) Chemical methods for use in marine environmental monitoring. Manual and Guides 12. Intergovernamental Oceanographic Commissiony, Paris, France

Wang S, Jiang Z, Mao M, et al (2018) Effects of seawater pH on survival, growth, energy budget and oxidative stress parameters of juvenile turbot *Scophthalmus maximus*. Iran J Fish Sci 17:675–689. https://doi.org/10.22092/ijfs.2018.116814

Wang W, Zhou J, Wang P, et al (2009) Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress. Comp Biochem Physiol Part C 150:428–435. https://doi.org/10.1016/j.cbpc.2009.06.010

Welch MJ, Munday PL (2015) Contrasting effects of ocean acidification on reproduction in reef fishes. Coral Reefs 35:1–9. https://doi.org/10.1007/s00338-015-1385-9

Halliwell B, Gutteridge JMC (2015) Free Radicals in Biology and Medicine. Oxford University Press

Oakes KD, Van Der Kraak GJ, (2003) Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquatic Toxicology 63 (4):447-463

Olivotto I, Chemello G, Vargas A, et al (2017) Marine ornamental species culture: From the past to "Finding Dory". General and Comparative Endocrinology 245:116-121

Wasielesky W, Bianchini A, Santos MHS, Poersch LH, (1997) Tolerance of Juvenile Flatfish *Paralichthys orbignyanus* to Acid Stress. Journal of the World Aquaculture Society 28 (2):202-204. https://doi.org/10.1111/j.1749-7345.1997.tb00857.x

5. CAPÍTULO 2: Effects of salinity on the oxidative stress responses of juvenile orange clownfish *Amphiprion percula* acutely exposed to water acidification

Capítulo formatado para a revista Chemosphere

Authors: M. D. Dias Carneiro[a,b*], J. F. Fagundes[a], L. C. Maltez [a], R. V. Rodrigues [a], J. M. Monserrat[c,d], M. Planas[b], L. A. Sampaio[a].

[a] Laboratório de Piscicultura Estuarina e Marinha, Instituto de Oceanografia (IO), Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil.

[b] Department of Ecology and Marine Resources, Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain.

[c] Laboratório de Bioquímica Funcional de Organismos Aquáticos (BIFOA), Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil.

[d] Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil

[*]Author for correspondence (marioiddc@gmail.com)

Abstract

The orange clownfish *Amphiprion percula* is an important species for the aquarium industry. There is increasing interest in improving techniques for its production in captivity. For that, it is important to understand the role of different environmental factors on fish welfare and performance, including pH and salinity. Therefore, the objective of this study was to investigate the oxidative status of juvenile orange clownfish (more specifically on gills, liver, digestive tract, and muscle) exposed for 96 h to different pH levels (5, 6, 7, and 8) and two salinities: brackish water (BW, salinity 11 ± 1) and seawater (SW, salinity 33 ± 1). The survival was 100% in all treatments. The most apparent alteration in protein content was observed in the liver, increasing with decreasing pH in BW, while it remained unaltered in SW. Total antioxidant competence (ACAP) was not affected by pH, but it was hampered in BW, especially in liver and muscle. Activity of glutathione-S-transferase (GST) decreased in the liver when the pH was reduced in BW, but there was no influence of environmental pH on GST activity in SW. The fish kept in both salinities showed an increase in protein protection in the muscle when exposed to acidic pH, as indicated by higher contents in NP-SH. Lipid peroxidation (LPO) in the gills of fish kept in SW increased significantly with acidification. Protein damage, measured as protein sulphdryl group (P-SH), was more marked in the muscle of fish kept in SW at higher pH levels. In conclusion, there was no mortality of orange clownfish exposed to acidic environments both in BW and SW. However, the antioxidant competence decreased in fish exposed to the lowest pH levels tested.

Keywords: reef fish; antioxidant defenses; lipid peroxidation; pH; ornamental aquaculture.

Introduction

The clownfish group is represented by 30 species of the Amphiprioninae subfamily. They inhabit the southern Indo-Pacific oceans at depths between 1 and 10 m, where they live in a protocooperation relationship with anemones during all their lifetime (Fautin and Allen, 1992; Thornhill, 2012). They are an important item in the international ornamental fish market, where *Amphiprion percula* and *Amphiprion ocellaris* together occupy the fifth position in the imports to the USA (Rhyne et al., 2012). The annual global market for *A. percula* comprises around 100,000 individuals and 80% of it is produced by aquaculture (Maison and Graham, 2015).

The role of decreasing pH on aquatic organisms is of concern both in the wild and in rearing systems. In nature, ocean acidification results from increasing levels of CO_2 (Heuer and Grosell, 2014; Ishimatsu et al., 2008; McCormick and Regish, 2018). However, in intensive aquaculture, especially in recirculating aquaculture systems (RAS), the fish must face pH reduction by both, CO_2 increases or due to H^+ released during the nitrification process (Aslam et al., 2019; Ebeling et al., 2006; Mota et al., 2018).

Fish are considered the most tolerant taxa to marine acidification (Melzner et al., 2009). Nevertheless, their performance during the initial development may be impaired, even after a small pH reduction (i.e. ΔpH = -0.5) corresponding to the estimated pH disturbance in ocean acidification scenarios (Frommel et al., 2016; Silva et al., 2016). More intense acidification (i.e. ΔpH = -2.0-2.5) may cause impairments to larger fish (Lee et al., 2018; Rodrigues et al., 2015). However, it is also noteworthy that reducing pH values to a certain level does not induce detrimental effects in fish production (Mota et al., 2018; S. Wang et al., 2018). Clark et al. (2020) pointed out that behavior of coral reef fishes might not be hampered by ocean acidification. Furthermore, it has been shown that the reproductive output of *Amphiprion melanopus* (Miller et al. 2013) and *A. percula* (Welch and Munday 2015) were improved when exposed to limited acidified environments (ΔpH = -0.3-0.4), according the authors due to a GABA receptor alteration and reproduction hormone increasing.

The use of some biomarkers may help to explain the effects of acidification in fish, particularly in Amphiprioninae and other species popular to the aquarium industry. Recent studies revealed impairment of the oxidative status for *A. percula* juveniles (two-month-old) acutely exposed to pH 5 (Carneiro et al., 2021b) as well as augmentation of cortisol levels along with lipid peroxidation in 15 days-old seahorses *Hippocampus reidi* in scenarios of acidification under low salinity (Carneiro et al., 2021a).

Reactive oxygen species (ROS) are normally generated during aerobic metabolism, but fish can increase intracellular ROS production when challenged by environmental changes. Oxidative stress results from unbalanced pro-oxidant and antioxidant conditions, where the higher pro-oxidant levels can induce harmful effects (Halliwell and Gutteridge, 2015). ROS can oxidize macromolecules such as lipids, proteins, and DNA, or change pathways and signalization of cell processes. To avoid these alterations, all aerobic organisms have an antioxidant system, composed of enzymatic and non-enzymatic processes, involved in the maintenance of homeostasis of their oxidative status (Lushchak, 2016). Therefore, the measurements of antioxidant defenses and oxidative damage levels are useful biochemical markers to estimate the stress caused in the organisms by acid-base and osmotic challenges (Martínez-Álvarez et al., 2002; Monserrat et al., 2007).

Alteration in the antioxidant content, enzyme activities, and oxidative damages as consequence of pH reduction have been demonstrated in freshwater (Copatti et al., 2019; Mukherjee et al., 2019), estuarine and marine fishes (Carney Almroth et al., 2019; Maulvault et al., 2018; Sampaio et al., 2018). Regarding salinity, both hypoosmotic and hyperosmotic changes may promote changes in activity and expression levels of antioxidant enzymes (Kim et al., 2017; Zeng et al., 2017). Increased expression and activity of oxidative enzymes were observed for cinnamon clownfish *A. melanopus* after acute exposure to hypoosmotic environments, along with increasing lipid peroxidation (LPO) (Park et al., 2011a). Concerning acidification, *A. percula* showed reduced survival and suffered increased protein damage when exposed to pH 5. Despite no damage to tissue was evident at pH 6, the alterations observed in the oxidative system suggest that this pH level may not be suitable for this species (Carneiro et al. 2021b).

The acid-base regulation in fish is coupled with ionic regulation, because acid-base compensation relies primarily on the direct transfer of H^+ and HCO_3^- across the gills in exchange for Na^+ and Cl^-, respectively. This flux is, in turn, the keystone to maintain the ionic and osmotic balance (Gilmour and Perry, 2009). The plasma osmolality of stenohaline marine teleost varies between 370 and 480 mOsm/kg H_2O (Sampaio & Bianchini, 2002), which corresponds to around 1/3 of seawater salinity. Reef fish, that hardly experience salinity challenges in nature, can face this situation in aquaculture (Dhaneesh et al., 2012; Evangelista et al., 2020). Thus, the capacity of fish to deal with acidification may change with salinity.

The present study aimed to evaluate survival and to assess the oxidative status in different organs of *A. percula* juveniles exposed to acute acidification both in brackish and seawater.

Material and methods

The study was conducted at the Marine Fish Culture Laboratory (LAPEM) from Federal University of Rio Grande (FURG). The fishes utilized were produced and reared in LAPEM according to adapted methodologies described by Hoff (1996). All experimental procedures involving fish manipulation were approved by FURG CEUA (Ethics Committee on Animal Use, #23116.003734/2018-86).

Breeding and rearing conditions

Adult clownfish *Amphiprion percula* were maintained in glass aquaria (40 L), placed in a RAS composed of biological filter, bag filter (5 µm), skimmer, UV treatment, and sump. The photoperiod was set at 14 h light:10 h dark. Broodstock were fed *ad libitum* three times per day with a commercial diet for ornamental marine fishes (Marine Granules, Tetra, Germany), supplemented with a moist diet based on shellfish prepared on site as suggested by Montalvo (2017). A tile was placed in each aquarium to serve as the substrate for spawning. Tiles with eggs remained in the aquarium throughout the incubation period (7-9 days), and they were transferred to larviculture tanks one day before hatching.

The larvae were reared in a 20 L circular tank, with black walls and white bottom, in a semi-static system (20-80% water renewal per day). The photoperiod was fixed in 18 h light: 6 h dark. Rotifers *Brachionus plicatilis* (10 ind. mL^{-1}) were offered from 1 to 5 days after hatching (DAH); followed by *Artemia sp.* nauplii (3-5 ind. mL^{-1}) from 3 to 10 DAH; and enriched *Artemia sp.* metanauplii (2-5 ind. mL^{-1}) from 7 to 15 DAH. Red Pepper (INVE – USA) was used for HUFA enrichment, following the manufacturer instructions. Live microalgae was added to the tanks (*Nannochloropsis oceanica* - 50,000 cells mL^{-1}) during the first 10 days and thereafter the larvae were reared in clear water. The weaning began at 10 DAH. For that, the fish were fed three times a day on a commercial diet (Orange Grow, 300-600 INVE, USA) until apparent satiety. The fish were fully weaned into the commercial diet at 15 DAH.

Weaned juveniles were then reared for seven months in rectangular tanks (50 L) placed in a RAS similar to those used for broodstock maintenance. The photoperiod was fixed in 12 h light:12 h dark. They were fed three times daily until apparent satiety on a commercial diet (Orange Nurse, INVE, USA) with adequate size for their mouth gape.

Water quality was monitored permanently as follows: salinity was measured with a refractometer (ATAGO S/Milli-E, Japan), temperature and oxygen were measured with an oximeter (550A, YSI, USA), and pH with a hand pH meter (EcoSense pH 100A, YSI, USA). Total ammonia (UNESCO, 1983), nitrite (Bendschneider and Robinson (1952) and nitrate were measured using a spectrophotometer. Alkalinity was measured by titration following Eaton et al. (2005). Parameters were kept at: temperature 26 ± 1 °C; salinity range 30-33; pH 8.1 ± 0.2; alkalinity range 120-150 $CaCO_3$ mg L^{-1}; total ammonia nitrogen (TAN): <0.5 N-NH_4^+ + NH_3 mg L^{-1}; nitrite < 0.8 N-NO_2^- mg L^{-1}; and nitrate < 30 N-NO_3^- mg L^{-1}.

Experimental design

Juvenile clownfish (8 months old, weight 2.5±0.3 g, total length 5.0±0.1 cm) were exposed for 96 h, in triplicate, to four different pH levels (5, 6, 7, and 8) in seawater (SW, salinity 33 ± 1) and brackish water (BW, salinity 11 ± 1). Each salinity level was tested in independent trials, in which all fish were siblings but from different spawning events, prior mixed and randomly distributed. The fish were distributed into 12 circular tanks (40 L; 16 fish $tank^{-1}$). Each experimental system included three tanks (40 L) attached to a RAS including sump, bag filter, water pump, and a pH controller. Considering that the fish were not fed during the trial and a low stocking density biofilter was not used in order to avoid potential interferences with the pH balance in each system.

When needed, salinity was reduced by adding dechlorinated local tap water, while pH was adjusted by adding diluted HCl (3% - 0.36 M) to the water using an automated pH controller (Tecna Evo 603, Seko, Brazil). Experimental salinities and pHs were adjusted in each RAS before the fish were transferred from the rearing system.

Water quality parameters and survival were measured every morning (Table 1). A fish was considered dead if it did not respond after being stimulated by the tip of a glass pipette.

Table 1: Water chemistry of seawater (SW – salinity 33) and brackish water (BW – salinity 11) conditions. ‡Data not submitted to statistics. ‡‡There were no significant differences (P>0.05) among water quality parameters, therefore for clarity, only average and standard deviation for each salinity is informed.

Experiment	**SW**				**BW**				
	pH 5	pH 6	pH 7	pH 8	pH 5	pH 6	pH 7	pH 8	
Salinity (‰)	33±1				11±1				‡
Temperature (°C)	26.4±0.10				26.0±0.15				
Oxygen (mg O_2 L^{-1})	6.4±0.29				7.17±0.08				‡‡
ammonia (mg N- NH_4^++NH_3 L^{-1})	0.05±0.03				0.45±0.08				
Nitrite (mg N-NO_2 L^{-1})	0.0±0.0				0.02±0.01				
pH	5.1±0.1	6.0±0.1	7.0±0.1	8.0±0.1	5.0±0.0	6.0±0.0	7.0±0.0	8.0±0.0	‡
Alkalinity (mg $CaCO_3$ L^{-1})	14±1[f]	17±1[e]	29±2[c]	135±3[a]	14±1[f]	16±1[ef]	22 ±1[d]	89±2[b]	

Biochemical analyses

In order to evaluate the effect of salinity and pH on the oxidative status of orange clownfish, 12 fish from each tank were killed in a benzocaine bath (300 ppm) at the end of the experimental period. Their liver, digestive tract, gills, and muscle tissue were excised. Due to the small size of fish, samples from three individuals were pooled together (n=4 samples per tank) and immediately frozen in liquid nitrogen. Samples were immediately stored at -80°C until biochemical assays were completed.

Tissue pools were homogenized (1:5 – w/v) using a sonicator (QSonica, Q55, 50W and 20 kHz, USA) in an adapted ice-cold homogenization buffer (100 mM Tris–HCl, 0,1 mM EDTA, pH 7,8) (Castro et al., 2012). The time and amplitude of sonication for each organ were: 30 s and 40 % of total potency (liver), 60 s and 50% (gills), 60 s and 75 % (muscle), and 30 s and 50 % (digestive tract). Homogenates were centrifuged at 10,000 x *g* for 30 minutes at 4 °C (SOLAB SL-703, Brazil). The supernatants were removed and stored at -80 °C.

All biochemical analyses were performed in a microplate reader (BioTek, Synergy HT, USA). The total protein content of all samples was determined following the biuret assay using a commercial kit (Proteínas Totais, Doles, Brazil) at 550 nm.

Glutathione-S-transferase (GST) activity was measured using 5 µL of the liver homogenates and 10 µL for the homogenates of the other organs (Habig, 1974). Then, supernatant volumes were reacted with 1 mM of reduced glutathione (GSH) and 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB). The formed conjugated was read at 340 nm 1 min intervals for 5 min.

The total antioxidant capacity against peroxyl radicals (ACAP) was determined according to the method described by Amado et al. (2009). All samples were previously diluted with the homogenization buffer to 2.0 mg protein mL^{-1}. The reaction of 10 µL of diluted samples in 127.5 µl of reaction buffer (pH 7.2, 30 mM HEPES, 200 mM KCl, and 1 mM $MgCl_2$) and 7.5 µl of 20 µM 2,2-azobis-2-methylpropionamidine dihydrochloride solution (ABAP) at three of the six wells within 10 µl 2′, 7′ dichlorofluorescein diacetate (H_2DCF-DA) at 40 µM. At 37 °C, the thermolysis of ABAP generated the peroxyl radicals that react with H_2DCF-DA, leading the generation of the fluorescent product DCF, which was read (excitation 485 nm; emission 520 nm) at every 5 min for 30 min. For interpreting the results, a higher relative area means a lower antioxidant capacity (Amado et al., 2009).

Non-proteic (NP-SH) and proteic thiols (P-SH) were measured according to Sedlak and Lindsay (1968), using DTNB (5,5- dithiobis-(2-nitrobenzoic acid; Sigma) reactions. First, 50 µL samples were added to 40 µL of distilled water and deproteinized with 10 µL trichloroacetic acid (TCA 50% w/v). After adding TCA, samples were incubated on ice (15 min) and then centrifuged (3,000 x *g*, at 4°C for 15 min). After adding DTNB, the supernatant absorbance was measured at 405 nm for estimation of NP-SH content. For P-SH, the protein pellet was re-suspended with 50 µL of homogenization buffer and 40 µL of 0.2 M Tris-Base at pH 8.2 were added. Next, 30 µL were removed from this solution and incubated at 50 °C for 30 min; after, addition 10.8 µL of phosphate buffer (0.15 M, pH 7.5) and 4.5 µL of ethanol; Then, 30 µL of this extract were added to 150 µL of 0.2 M Tris-Base at pH 8.2 and 50 µL of DTNB. Finally, it was incubated in ice for 15 min and so centrifuged (3,000 *g*, at room temperature for 15 min) to then read at 405 nm absorbance.

Lipid peroxidation (LPO) was measured as the concentration of substances reactive to thiobarbituric acid (TBARS) (Oakes & Van Der Kraak, 2003). First, 20 µl of butylated hydroxytoluene solution (BHT, 67 µM), 150 µL of 20% acetic acid solution, 150 µL of TBA solution (0.8%), 50 µl of distilled water, and 20 µL of sodium dodecyl sulfate (SDS, 8.1%) were added to the samples (20 µL). Then, the samples were heated at 95 °C for 30 min. Afterwards, 100 µL of distilled water and 500 µL of n-butanol were added to the solution, which was centrifuged (3,000 g for 10 min at 15°C). A volume of 150 µL of the supernatant was used for reading at 515 nm (excitation) and 580 nm (emission). As standard for the construction of a calibration curve, 1,1,3,3-tetramethoxypropane (TMP) was employed.

Statistical analyses

Normality and homoscedasticity were verified using Shapiro Wilk's and Levene's tests, respectively. Two-way ANOVA was used for comparison among results of fish exposed to different pH in BW or SW. Significant ANOVA were followed by the Student-Newman-Keuls multiple comparison test. If data were not normal or not homoscedastic, the Rank transformation test was applied (Friedman, 1937). The significance level was set at 5% for all tests. The data were expressed as average ± standard deviation.

Principal component analyses (PCA) were performed to summarize and visualize globally all the information of the dataset. The PCA were performed in R v.3.6.1 (R Core Team 2019). For that, we used factoMineR v2.3 (Husson et al., 2020), factoextra v1.0.7

(Kassambara, 2020), and corrplot v0.8.4 (Wei et al., 2017) packages. Hierarchical clustering of variables and pH/salinity/tissue was performed with Ward's method (provided the highest agglomerative coefficients) using factoextra v1.0.7 (Kassambara, 2020) and ComplexHeatmap v3.11 (Gu et al., 2016) packages in R. The data values were standardized (mean = 0; sd = 1) for clustering and PCA.

Results

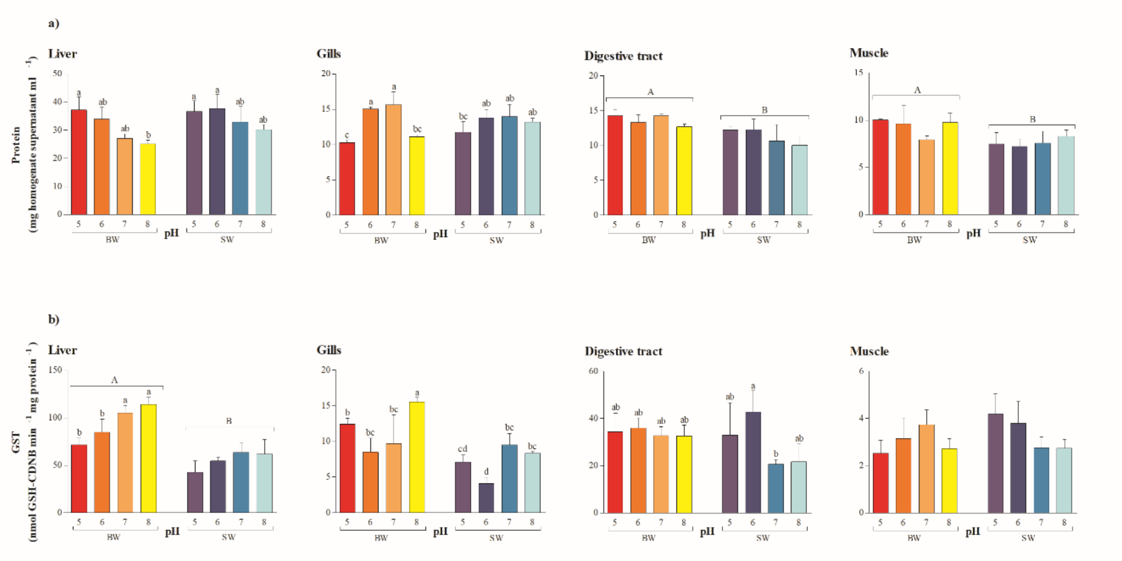
Routine observations did not indicate evident differences in fish activity among treatments. All fishes survived after being exposed to salinity or pH challenges.

Biochemical responses

Protein concentration in the liver showed significant differences between pH 5 and at pH 8 in BW, with former value one-third higher than latter. Differences across pH levels for SW were not significant (P_{pH}= 0.002; $P_{salinity}$= 0.041; $P_{pH*salinity}$= 0.502). In the digestive tract, the highest values were found in fishes reared in BW (P_{pH}= 0.087; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.345). The higher gills protein content were achieved in fish kept at pH 6 and 7 in BW (P_{pH} <0.001; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.025). In the muscle, higher protein levels in fish maintained in BW were 18% higher than those in SW, irrespective of pH (P_{pH}= 0.228; $P_{salinity}$= 0.001; $P_{pH*salinity}$= 0.292) (Figure 1a).

The lower GST activity in the liver was obtained at pH 5-6 in BW, being 30% low than pH 7-8. In addition, the lowest GST values were found in SW when compared with BW (P_{pH} <0.001; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.192). In the digestive tract, significant differences for GST activity were found in SW, where activity at pH 6 was two-fold higher than in pH 7. On the other side, there were no significant differences for GST activity among pH for fish maintained in BW (P_{pH}= 0.028; $P_{salinity}$= 0.162; $P_{pH*salinity}$= 0.164). The highest gill GST activity was at pH 8 in BW, while the lowest activity was observed for fish exposed to pH 6 in SW (P_{pH}= 0.002; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.061). No statistical differences were found for activity of GST in the muscle, even though there was significant interaction, the Newman-Keuls post-hoc did not reveal any differences (P_{pH}= 0.281; $P_{salinity}$= 0.236; $P_{pH*salinity}$= 0.025) (Figure 1b).

Figure 1. (a) Protein and (b) glutathione-S-transferase (GST) of tissue of anemonefish *Amphiprion percula* exposed to different pHs in seawater (SW – S33) and brackishwater (BW – S11) for 96 h. Data are expressed as means ± SD (n=3). Different letters indicate significant differences among treatments (Newman-Keuls test, p<0.05).

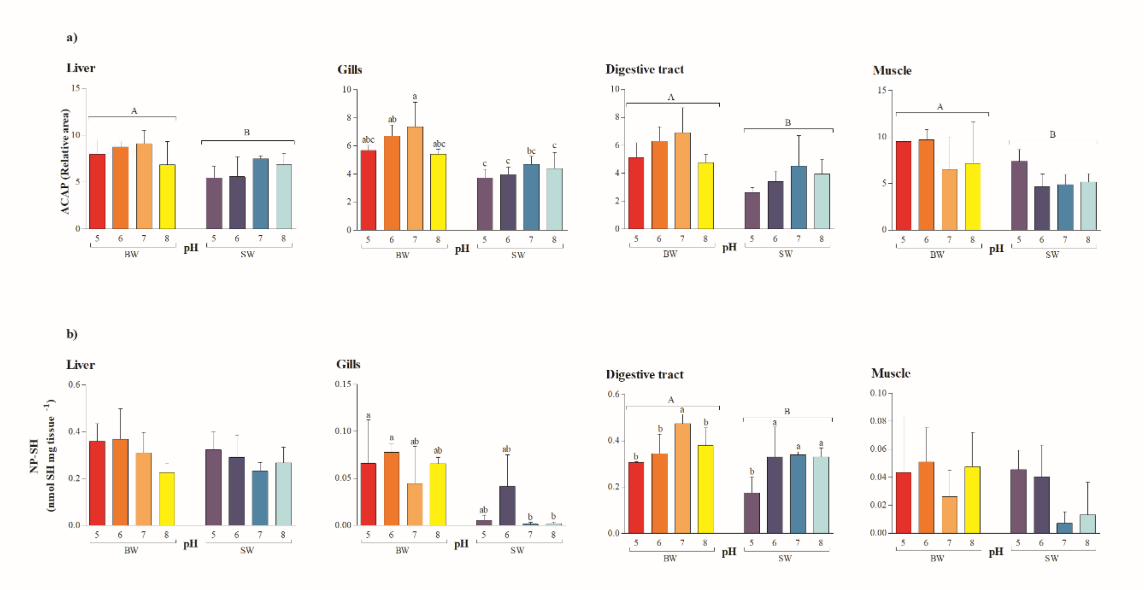


In the liver, independently of pH, fish exposed to BW presented lower ACAP (22% higher relative area) in comparison with fish kept in SW (P_{pH}= 0.287; $P_{salinity}$= 0.009; $P_{pH*salinity}$= 0.325). Similarly, in the digestive tract, no significant differences among pH in each salinity were observed. Even though there was no significant interaction between pH and salinity, ACAP in the digestive tract of fish kept in SW reached up to 2.5-fold higher levels (lower relative area) than those in fish maintained in BW (P_{pH}= 0.107; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.509). The same finding was observed for ACAP in the gills, in which there were not significant differences across the tested pHs for both salinities- Despite no significant interaction between both factors, it was observed higher ACAP (lower relative area) in pH 5, 6 and 8 in SW, when compared with pH 6 and 7 in BW (P_{pH}= 0.088; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.322). The antioxidant capacity in the muscle was 32% higher (lower relative area) in SW than in BW, irrespective of pH (P_{pH}= 0.178; $P_{salinity}$= 0.008; $P_{pH*salinity}$= 0.527) (Figure 2a).

NP-SH levels in the liver did not differ significantly across salinity nor pH levels (*P* of *pH*= 0.169; *P* of *salinity*= 0.266; *P* of $_{pH*salinity}$= 0.541). In the digestive tract, NP-SH level was significantly higher in fish maintained at pH 7 in BW, as when compared to those exposed in SW the pH 5 show a half NP-SH values in comparison with the other

SW pH (P_{pH}= 0.008; $P_{salinity}$= 0.044; $P_{pH*salinity}$= 0.674). In gills, NP-SH values in SW at the two higher pH (7-8) were 98% lower than at pH 5-6 BW (P of $_{pH}$= 0.051; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.855). In the muscle, no significant effects of salinity and pH were found for NP-SH (P_{pH}= 0.151; $P_{salinity}$= 0.127; P of $_{pH*salinity}$= 0.609) (Figure 2b).

Figure 2. (a) Total antioxidant capacity against peroxyl radicals (ACAP) and (b) non-proteic thiols (NP-SH) of tissue of anemone fish *Amphiprion percula* exposed to different pHs for 96 h at different salinities: seawater (SW – S33) and brackishwater (BW – S11) for 96 h. Data are expressed as means ± SD (n=3). Different letters indicate significant differences among treatments (Newman-Keuls test, p<0.05).

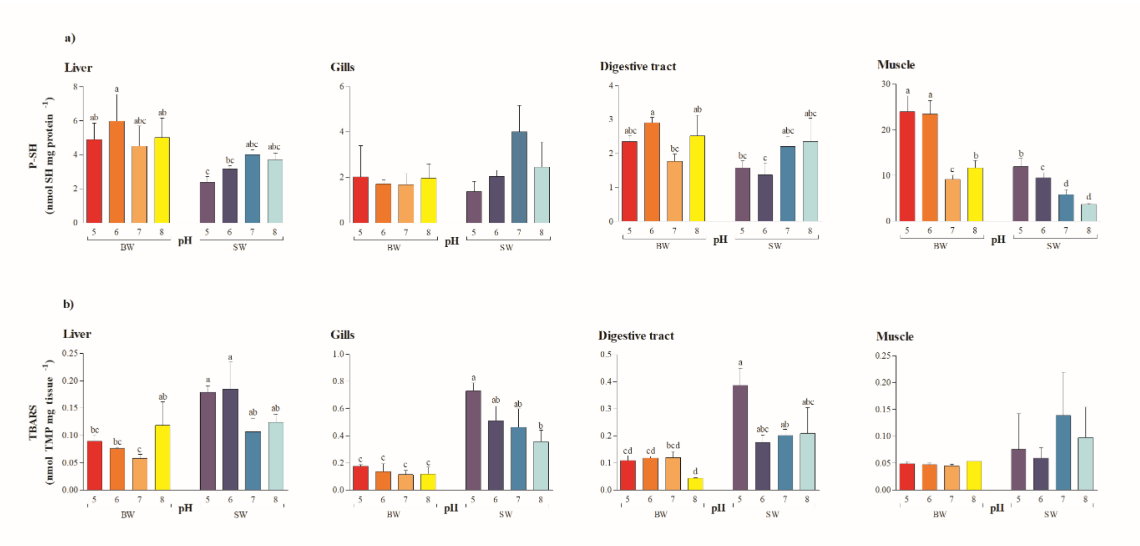


The lowest liver P-SH levels were observed at pH 5 in SW and the highest was registered at pH 6 in BW, being 60 % higher in the latter (*P pH*= 0.356; *P salinity* <0.001; $P_{pH*salinity}$= 0.141). For the digestive tract, the level of P-SH at pH 6 in BW was significantly higher than digestive tract of fish exposed to pH 5-6 in SW (P_{pH}= 0.175; $P_{salinity}$= 0.004; $P_{pH*salinity}$= 0.002). There were no significant differences for P-SH in gills (P_{pH}= 0.244; $P_{salinity}$= 0.234; P of $_{pH*salinity}$= 0.278). The highest values of P-SH in the muscle were observed at pH 5-6 for fish in BW, which was five-fold higher than those exposed to pH 7-8 in SW (P_{pH} <0.001; $P_{salinity}$ <0.001; $P_{pH*salinity}$= 0.011) (Figure 3a).

There was no significant effect of pH on the concentration of TBARS in the liver of fish kept in SW. However, lipid peroxidation for fish kept at pH 5-6 in SW was higher than those fish kept in the same pH levels in BW (P_{pH}= 0.007; $P_{salinity}$ <0.001; $P_{pH*salinity}$=

0.039). Similarly, the LPO level in the digestive tract of clownfish exposed to pH 5 in SW was high compared to all pH levels in BW (P of pH= 0.041; $P_{salinity}$ <0.001; P of $_{pH*salinity}$= 0.116). In the gills, there were no significant effect of pH for TBARS among fish kept in BW. Meanwhile, in SW, two-fold higher TBARS values were observed for clownfish exposed to pH 5 when compared to those maintained in pH 8 (P_{pH}= 0.006; $P_{salinity}$ <0.001; P of $_{pH*salinity}$= 0.735). Regarding muscle, there were no statistical differences for TBARS among pH and salinities (P_{pH}= 0.901; P of $salinity$= 0.401; P of $_{pH*salinity}$= 0.460) (Figure 3b).

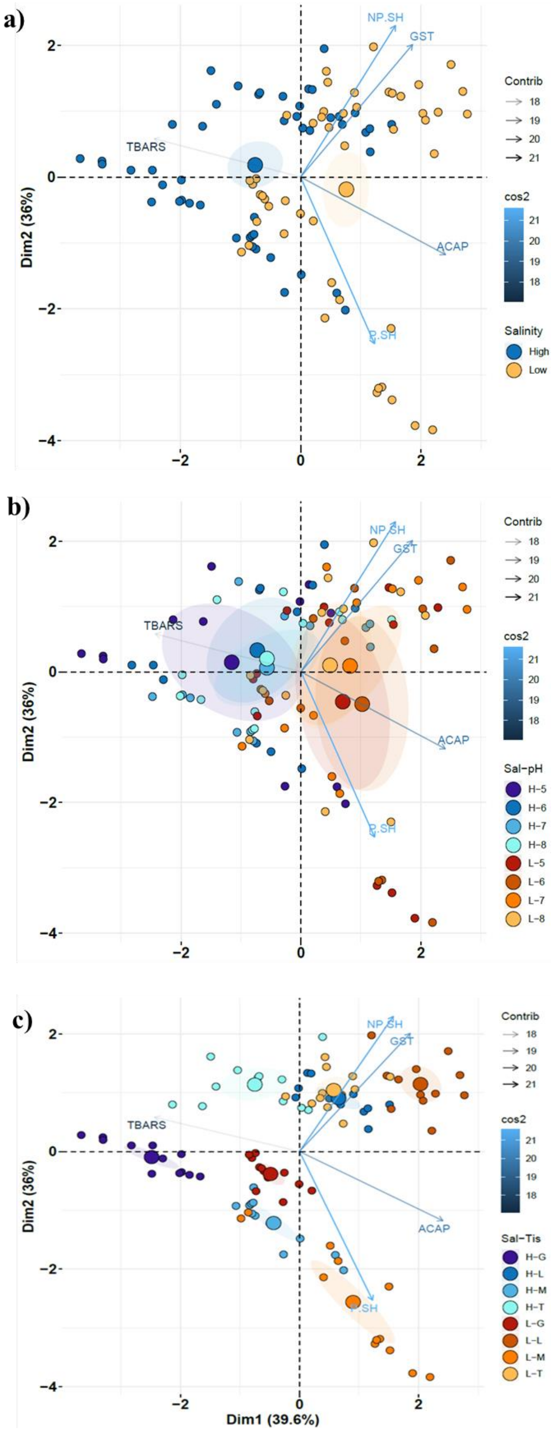
Figure 3. (a) Proteic thiols (P-SH) and (b) reactive substances to thiobarbituric acid (TBARS), respectively as protein damage and lipid peroxidation (LPO), of tissue of anemonefish *Amphiprion percula* exposed to different pHs in seawater (SW – S33) and brackishwater (BW – S11) for 96 h. Data are expressed as means ± SD (n=3). Different letters indicate significant differences among treatments (Newman-Keuls test, p<0.05).



Principal Component Analysis (PCA)

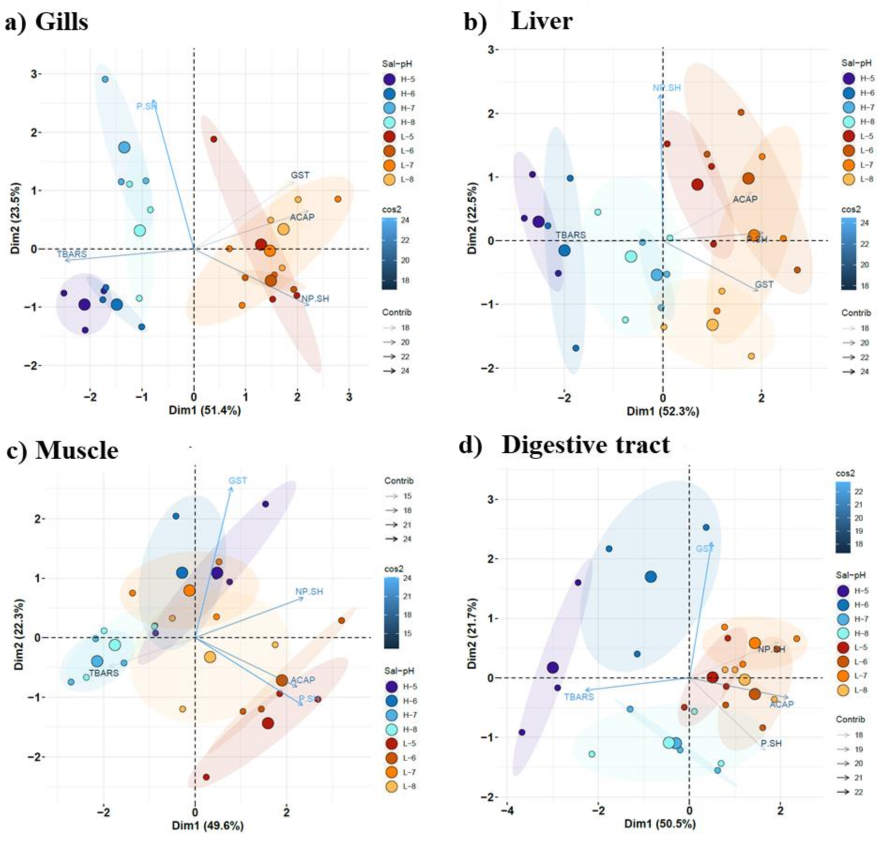
The PCA performed on the biomarkers assessed to characterize globally the oxidative status in clownfish kept under different salinity and pH levels. The first two factors explained 75.6 % of the total variability. The global analysis for salinities showed increased TBARS values in SW and higher P-SH (lower protein damage) and ACAP (low antioxidant capacity) values in BW (Figure 4a). A detailed analysis of the plot for salinity-pH comparisons showed that all centroids were close each other for high salinity conditions (Figure 4b). However, the centroids for fish maintained in brackish water under more acidic conditions (pH 5-6) were not so close to those under pH 7-8 (Figure 4b), indicating higher NP-SH and GST values in the former. The response of tissues to changing environments revealed differences between gills–muscle and liver-digestive tract (Figure 4c). NP-SH and GST levels in gills and muscle were lower. Furthermore, TBARS and P-SH in gills-digestive tract were higher and lower, respectively than in muscle-liver.

Figure 4. Global Principal Component Analyses (PCA) results for *A. percula* exposed to seawater (SW – salinity 33) or brackishwater (BW– salinity 11) regarding: a) global results (H –high salinity = SW; L – low salinity = BW), b) results across salinity and pH levels (5-8), and c) results across salinity and tissues (G- gills; L – liver; M – muscle; T - digestive tract). Analyses performed on dataset including enzymatic activities and contents related to oxidative stress. Only the variables with the highest contributions (cos^2 > 0.5) are indicated. Ellipses correspond to centroid value ± 1 s.d. (shaded areas).



The PCA for specific organs explained 74.8% of total variability. The most influencing biomarkers were TBARS and ACAP. The plots revealed major differences in gills and digestive tract at high salinities and low pH (Figures 5a, d), and in liver and muscle at low salinities at high and low pH, respectively (Figures 5b, c). In most cases, TBARS values were negatively related with ACAP, GST, P-SH and NP-SH, depending on the tissue. Under SW maintenance, TBARs increased in most tissues and acidic conditions, except in muscle of juveniles maintained at low pH levels. Changes in pH levels at low salinity resulted in similar changes of biomarkers in both gills and digestive tract (Figures 5a, d). Under those conditions, biomarkers (e.g., especially ACAP, GST and P-SH) in liver and muscle were markedly affected by changes in environmental conditions (Figures 5b, c). Interestingly, the cellular damage in muscle was higher under SW and normal pH conditions (Figure 5c).

Figure 5. Specific Principal Component Analyses (PCA) results for *A. percula* exposed to seawater (SW – salinity 33) or brackishwater (BW– salinity 11) and pH levels regarding tissues (a) Liver, (b) Digestive tract, (c) Gills, and (d) Muscle. (H –high salinity = SW; L – low salinity = BW),



Discussion

In the present study, we investigated the oxidative status on different organs of juvenile orange clownfish *A. percula*, and we assessed changes in biomarkers potentially implicated in acute exposures of fish to low pH, both in BW or SW. The overall response of juvenile clownfish to acidification suggests that the organs investigated (liver, gills, muscle, and digestive tract) were not severely compromised when exposed to pH as low as 6, but a further decrease to pH 5 resulted in significant oxidative stress responses, especially in BW. Those responses brought knowledge on the welfare of fish being reared in potentially stressful acidic conditions (Ashley, 2007). The assessment of the oxidative status in different organs, as shown by PCA analyses, can provide information on specific responses imposed by a stressful environment like acidic pH (Lushchak, 2016). For example, catalase and non-proteic sulfhydryl in the gills act as antioxidants in *Chasmagnathus granulata*, while other antioxidant enzymes and non-enzymatic components could be involved with hepatopancreas (Maciel et al., 2004)

We observed changes in the content of homogenate soluble protein. Protein content increased in the liver of orange clownfish exposed to pH 5 in BW. The content also increased in the muscle of fish kept in BW, independently of pH level. This finding might have been caused by changes in fish metabolism under stressful conditions (i.e., selective consumption of carbohydrates/lipids reserves) (Martínez-Álvarez et al., 2002; Tseng and Hwang, 2008). For example, an hiperglicemia has been reported in *A. melanopus* after 3 days of starvation (Choi et al., 2012). Hence, the augmented protein content in the liver could be explained by the mobilization of glycogen and carbohydrates in order to deal with the stressful condition, and/or the period of fasting during the trial. Increases in CAT, SOD, and GPx levels were reported in the liver of *A. melanopus* under stressful conditions (Choi et al., 2012). Regarding gills, increases in the abundance of oxidative stress-related proteins may be induced by changes in both salinity and pH (e.g., pH 6-7 in BW), since this occurred in fish exposed to stressful environments (Tomanek, 2014). Acidification may reduce the hemoglobin affinity for oxygen through the Bohr effect, and the resulting oxidative stress is a known disorder in fish submitted to hypoxia (Johannsson et al., 2018). However, an increase in respiration proteins (globins-protein) of zebrafish *Danio rerio* was observed after exposure to an acidic environment (pH 4). That augmentation was triggered by the role of globins in the aerobic energy metabolism or likely by a specific function in the antioxidant defense (Tiedke et al, 2013).

GST activity was lower in the gills and liver of fish exposed to acidic conditions in BW. This finding agrees with the downregulation of Mu-GST in the gills of shrimp *Litopenaeus vannamei* at pH 5.6 after 24 h (Zhou et al., 2009). Considering that the gill is an important organ in the ion regulation and acid-base process (Burggren and Bautista, 2019), the decreased GST activity of fish exposed to an acidic environment in low salinity water suggests a negative effect of pH on that enzyme, since pH has a pivotal effect on enzymes, proteins, lipids, carbohydrates and nucleic acids (Burggren and Bautista, 2019; Shartau et al., 2019).

ACAP levels in juvenile orange clownfish were not affected by acidic exposure in any salinity. However, we observed ACAP changes associated with salinity in all tissues investigated, especially in liver, gills, and muscle. A general trend for higher antioxidant capacity against peroxyl radicals (low relative area) was observed in SW, while the use of these reserves was more evident in BW. A higher metabolism to deal with osmoregulation was expected in SW, resulting in the production of more ROS and thus, triggering stronger antioxidant responses (Strobel et al., 2013). However, the lower antioxidant capacity observed in BW could have been triggered by the isosmotic comfort, and consequently the lower production of ROS associated to osmoregulatory activity. However, contrary to the present study, lower ACAP was observed in *Poecilia vivipara* maintained in salinity 25 (Leitemperger et al., 2019).

It is well known that ROS can virtually interact with all cellular components, (e.g., lipids, carbohydrates, proteins, nucleic acids, etc). When oxidized by ROS, these molecules can be degraded or transformed into toxic end products (Lushchak, 2016). The thiol redox state is characterized by the levels of non-protein (NP-SH) and protein thiols (P-SH), as well as by various protein and non-protein disulfide (Chen et al., 2008). The NP-SH is an indirect estimator of GSH (Maciel et al., 2004). In *Poecilia reticulata* exposed to acidic environment along with salinity challenges, no changes in GSH content were observed (Moniruzzaman et al., 2018). These authors explained that molecular mechanisms involved in oxidative status and the associated transduction pathways (i.e., which regulate intermediary metabolism during stress-related anoxia/hypoxia) might be involved in the activation and maintenance of antioxidant enzymes and GSH levels in response to pH and salinity stress. On the other hand, PCA plots show that higher NP-SH values were more associated with the digestive tract (TGI) and liver compared to gills and muscle, whereas GST values were more related with gills and liver. GSH is produced by the liver and transported to other tissues, where it will be used for antioxidant protection (Lushchak, 2012). However, PCA results revealed that the relationship between NP-SH and GSH was associated to the consumption of NP-SH like GSH in the liver. Similarly, low GSH content in the brains of *Labeo rohita* and *Cirrhinus cirrhosus* maintained at pH 5.5 has been reported (Mukherjee et al., 2019).

Oxidation of cysteine SH groups can cause intermolecular protein cross-linking and enzyme inactivation, leading eventually to cell death (Mitton et al., 2016). Then, reductions in P-SH levels can be used as a reversible protein oxidation index. Nonetheless, the muscle in fish kept in both salinities showed an increase in P-SH levels at acidic pH, suggesting the activation of an antioxidant response to cope with a more pro-oxidant condition, turning the intracellular redox state more reductive. PCA plots showed higher GST activities and higher levels of P-SH mainly in the digestive tract and liver. This finding supports the role of the digestive tract as osmoregulatory organ, by intestinal HCO_3^- secretion, water and salt absorption, and the ensuing effects on acid–base balance (Genz et al., 2008) and the GST function in the protection of this organ enzymatic activities.

S-glutathionylation targets proteins in response to fluctuations in the redox state of cellular glutathione pools, in low GSH/GSSG ratios, promoting cell signaling for adaptation to environmental changes (Mailloux et al., 2020). Afterwards, seahorse juveniles showed increased GSSG and oxidative stress condition (OSI) as a result of salinity changes (Carneiro et al, 2021a). However, protein damage, indicated by low P-SH levels, increased in smaller *A. percula* exposed to SW and pH 5 (Carneiro et al, 2021b). In the present study, muscle P-SH increase is also supported by the expenditure of muscle ACAP in BW mentioned above. Another explanation for the high levels of muscle P-SH is a potential reduction in fish metabolism under acidic environments, which would cause a smaller ROS production and consequently lower oxidative damage in that organ. Nonetheless, since routine activities of fish not seems affected by pH this muscle responses were not expected, and more investigation is needed to ascertain how this tissue reacts to salinity and pH changes.

Intense alterations in [H^+] and other ions can lead to mitochondrial hyperpolarization and cell death (Matsuyama and Reed, 2000). Excess LPO in biological membranes increases the permeability to H^+ and other ions, changing their membrane potential, which can lead to membrane rupture and loss of cell or organelle content (Gutteridge, 1995). However, an acidic environment can also promote beneficial effects.

It has been reported that stressed fishes can activate their anaerobic energy production pathways (Sokolova, 2013). A high pCO_2 condition (Δ pH = -0.4) increased electron transport system (ETS) and lactate dehydrogenase (LDH) activity in sand smelt larvae (*Atherina presbyter*). This finding was associated with oxidative enzyme activation, coupled to lower LPO and growth enhancement (Silva et al., 2016). In turbot (*Scophthalmus maximus*), acidification increased the energy allocated for growth, triggering higher growth in fish reared at pH 6.3-7.3 compared to those at pH 8.3-8.8, even if LPO was higher at acidic conditions (S. Wang et al., 2018).

Fish maintained in SW at pH 5 showed higher LPO in the gills. This specific response could be associated with the ion regulation function of this organ, which is in direct contact with acidic water. Furthermore, TBARS values were also higher in fish kept in SW for all tissues. The low gills TBARS levels at BW was paralleled by a high antioxidant effort, since the expenditure of ACAP (i.e, high relative area), was noticed in BW in ion regulatory related organs, as gills (Tseng and Hwang, 2008).

The anaerobic metabolism in seabream larvae was intensified when exposed to an acidic environment, as a short-term metabolic strategy that may help in homeostasis maintenance (Pimentel et al., 2020). Also, (i) the aerobic and non-aerobic energy metabolism, (ii) the mitochondrial proton leak, and (iii) interactions with metabolites of non-mitochondrial energy metabolism relies upon H^+ (Kuno et al., 2016; Robergs, 2017). H^+ can interact with pro-oxidant molecules (e.g. $H^+ + OH^- \rightleftharpoons H_2O$), helping to reduce oxidative stress (Sampaio et al., 2018). However, higher concentrations of H^+ and other ions can trigger mitochondrial hyperpolarization and cell death (Matsuyama and Reed, 2000).

Our results suggest that more than one metabolic pathway, (i) as acid-base, (ii) ion regulation, and (iii) mobilization of substrates in aerobic or non-aerobic strategies are influenced by the exposure to acidic pH changes, especially when maintained under different salinity environments.

Conclusion

Salinity level affected the oxidative status in juvenile orange clownfish *Amphiprion percula* and revealed differential organ-specific responses to deal with environmental acidification. Acidic pH levels influenced the oxidative status of fish, protein increase, reduced GST activity and ACAP expenditure at pH 5 in BW in liver as gills GST inactivation in this environment condition, it was also observed higher LPO in gills of fish exposed to pH 5 in SW. Digestive tract and muscle showed high protein levels and ACAP expenditure in BW. P-SH values increased in pH 5-6, revealing a protection by S-glutathionylation process, as a positive relation in P-SH and GST were found in digestive tract, showing the role of GST in this process in that organ. Thus, liver and gills are important organs to investigate the effect of acidification in different salinities, since they were the most affected organs by the environmental changes applied. However, orange clownfish exposed to a moderate acidic environment (up to pH 6) did not show alterations in their oxidative status, as evidenced mostly by the stable LPO levels in most organs.

Acknowledgments

The Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil) provided access to the Portal de Periódicos and financial support through Programa de Excelência Acadêmica-PROEX (Brazil). L.A. Sampaio and J.M Monserrat are recipient of research fellowship provided by Brazilian CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and M.D.D. Carneiro was granted by CAPES. We acknowledge the co-tutelage agreement between FURG (Brazil) and Universidade de Vigo (Spain) for M.D.D. Carneiro, which is within the scope of the Capes PrInt Program (public notice 041/2017). We also are thankful to Ane Medeiros, Diego Alba and João Timm for help in biochemical analyses.

References

Abdalla, R.P., Kida, B.M.S., Pinheiro, J.P.S., Oliveira, L.F., Martinez, C.B.F., Moreira, R.G., 2019. Exposure to aluminum, aluminum + manganese and acid pH triggers different antioxidant responses in gills and liver of *Astyanax altiparanae* (Teleostei: Characiformes: Characidae) males. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 215, 33–40. https://doi.org/10.1016/j.cbpc.2018.09.004

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123. https://doi.org/10.1016/j.scitotenv.2008.11.038

Aminot, A.; Chaussepied, M. Manuel des analyses chimiques en milieu marin. Brest, E: C.N.E.X.O., 1983. 395 p.

Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59, 95–104. https://doi.org/10.1016/S0006-2952(99)00301-9

Ashley, P.J., 2007. Fish welfare: Current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199–235. https://doi.org/10.1016/j.applanim.2006.09.001

Aslam, S.N., Navada, S., Bye, G.R., Mota, V.C., Terjesen, B.F., Mikkelsen, Ø., 2019. Effect of CO_2 on elemental concentrations in recirculating aquaculture system tanks. Aquaculture 511, 734254. https://doi.org/10.1016/j.aquaculture.2019.734254

Bendschneider K, Robinson RJ (1952) A new spectrophotometric method for the determination of nitrite in sea water. J Mar Res 11:87–96

Burggren, W., Bautista, N., 2019. Invited review: Development of acid-base regulation in vertebrates. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 236, 110518. https://doi.org/10.1016/j.cbpa.2019.06.018

Carneiro, M.D.D., García-Mesa, S., Sampaio, L.A., Planas, M., 2021a. Primary, secondary, and tertiary stress responses of juvenile seahorse *Hippocampus reidi* exposed to acute acid stress in brackish and seawater. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 255, 110592. https://doi.org/10.1016/j.cbpb.2021.110592

Carneiro, M.D.D., Maltez, L.C., Rodrigues, R.V., Planas, M., Sampaio, L.A., 2021b. Does acidification lead to impairments on oxidative status and survival of orange clownfish *Amphiprion percula* juveniles? Fish Physiol. Biochem. 47, 841–848. https://doi.org/10.1007/s10695-021-00942-9

Carney Almroth, B., Bresolin de Souza, K., Jönsson, E., Sturve, J., 2019. Oxidative stress and biomarker responses in the Atlantic halibut after long term exposure to elevated CO_2 and a range of temperatures. Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 238, 110321. https://doi.org/10.1016/j.cbpb.2019.110321

Castro, C., Pérez-Jiménez, A., Guerreiro, I., Peres, H., Castro-Cunha, M., Oliva-Teles, A., 2012. Effects of temperature and dietary protein level on hepatic oxidative status of Senegalese sole juveniles (*Solea senegalensis*). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 163, 372–378. https://doi.org/10.1016/j.cbpa.2012.07.003

Chen, W., Zhao, Y., Seefeldt, T., Guan, X., 2008. Determination of thiols and disulfides via HPLC quantification of 5-thio-2-nitrobenzoic acid. J. Pharm. Biomed. Anal. 48, 1375–1380. https://doi.org/10.1016/j.jpba.2008.08.033

Choi, C.Y., Shin, H.S., Choi, Y.J., Kim, N.N., Lee, J., Kil, G.S., 2012. Effect of LED light spectra on starvation-induced oxidative stress in the cinnamon clownfish *Amphiprion melanopus*. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 163, 357–363. https://doi.org/10.1016/j.cbpa.2012.07.005

Clark, T.D., Raby, G.D., Roche, D.G., Binning, S.A., Speers-Roesch, B., Jutfelt, F., Sundin, J., 2020. Ocean acidification does not impair the behaviour of coral reef fishes. Nature 577, 370–375. https://doi.org/10.1038/s41586-019-1903-y

Copatti, C.E., Baldisserotto, B., Souza, C. de F., Monserrat, J.M., Garcia, L., 2019. Water pH and hardness alter ATPases and oxidative stress in the gills and kidney of pacu (*Piaractus mesopotamicus*). Neotrop. Ichthyol. 17, 1–10. https://doi.org/10.1590/1982-0224-20190032

Dhaneesh, K. V., Ajith Kumar, T.T., Swagat, G., Balasubramanian, T., 2012. Breeding and mass scale rearing of clownfish *Amphiprion percula*: feeding and rearing in brackishwater. Chinese J. Oceanol. Limnol. 30, 528–534. https://doi.org/10.1007/s00343-012-1184-x

Díaz, M.E., Furné, M., Trenzado, C.E., García-Gallego, M., Domezain, A., Sanz, A., 2010. Antioxidant defences in the first life phases of the sturgeon *Acipenser naccarii*. Aquaculture 307, 123–129. https://doi.org/10.1016/j.aquaculture.2010.06.026

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg AE., 2005. Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington, D.C.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358. https://doi.org/10.1016/j.aquaculture.2006.03.019

Esbaugh, A.J., Ern, R., Nordi, W.M., Johnson, A.S., 2016. Respiratory plasticity is insufficient to alleviate blood acid–base disturbances after acclimation to ocean acidification in the estuarine red drum, *Sciaenops ocellatus*. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 186, 97–109. https://doi.org/10.1007/s00360-015-0940-6

Evangelista, I.R., dos Santos, L.N., dos Santos, A.F.G.N., 2020. Influence of salinity, temperature and photoperiod on eye asymmetry of *Amphirion ocellaris* larvae. Aquaculture 521, 734976. https://doi.org/10.1016/j.aquaculture.2020.734976

Fautin D., Allen, G.R., 1992. Anemonefishes and their host sea anemones. Western Australian Museum, Perth, 160 p.

Friedman, M., 1937. The Use of Ranks to Avoid the Assumption of Normality Implicit in the Analysis of Variance. J. Am. Stat. Assoc. 32, 675–701. https://doi.org/10.1080/01621459.1937.10503522

Frommel, A.Y., Margulies, D., Wexler, J.B., Stein, M.S., Scholey, V.P., Williamson, J.E., Bromhead, D., Nicol, S., Havenhand, J., 2016. Ocean acidification has lethal and sub-lethal effects on larval development of yellowfin tuna , *Thunnus albacares*. J. Exp. Mar. Bio. Ecol. 482, 18–24. https://doi.org/10.1016/j.jembe.2016.04.008

Genz, J., Taylor, J.R., Grosell, M., 2008. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta.* J. Exp. Biol. 211, 2327–2335. https://doi.org/10.1242/jeb.016832

Gilmour, K.M., Perry, S.F., 2009. Carbonic anhydrase and acid-base regulation in fish. J. Exp. Biol. 212, 1647–1661. https://doi.org/10.1242/jeb.029181

Grosell, M., 2006. Intestinal anion exchange in marine fish osmoregulation. J. Exp. Biol. 209, 2813–2827. https://doi.org/10.1242/jeb.02345

Gutteridge, J.M.C., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin. Chem. 41, 1819–1828.

Habig, W.H., Pabs,t M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Halliwell, B., Gutteridge, J.M., 2015. Free radicals in biology and medicine. Oxford University Press, USA

Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. AJP Regul. Integr. Comp. Physiol. 307, R1061–R1084. https://doi.org/10.1152/ajpregu.00064.2014

Hoff, F.H., 1996. Conditioning, spawning and rearing of fish with emphasis on marine clownfish. Aquaculture Consultants, Incorporated.

Hwang, P., Lee, T., Lin, L., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am. J. Physiol. Integr. Comp. Physiol. 301, R28–R47. https://doi.org/10.1152/ajpregu.00047.2011

Hwang, P.-P., Lee, T.-H., 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 148, 479–497. https://doi.org/10.1016/j.cbpa.2007.06.416

Ishimatsu, A., Hayashi, M., Kikkawa, T., 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. 373, 295–302. https://doi.org/10.3354/meps07823

Johannsson, O.E., Giacomin, M., Sadauskas-Henrique, H., Campos, D., Braz-Mota, S., Heinrichs-Caldas, W., Baptista, R., Wood, C.M., Almeida-Val, V.M.F., Val, A.L., 2018. Does hypoxia or different rates of re-oxygenation after hypoxia induce an oxidative stress response in Cyphocharax abramoides (Kner 1858), a Characid fish of the Rio Negro? Comp. Biochem. Physiol. Part A 224, 53–67. https://doi.org/10.1016/j.cbpa.2018.05.019

Kang, C.-K., Yang, W.-K., Lin, S.-T., Liu, C.-C., Lin, H.-M., Chen, H.-H., Cheng, C.-W., Lee, T.-H., Hwang, P.-P., 2013. The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 181–91. https://doi.org/10.1016/j.cbpa.2012.08.010

Kim, J., Park, H., Kim, K., 2017. Growth performance, oxidative stress, and non-specific immune responses in juvenile sablefish, *Anoplopoma fimbria*, by changes of water temperature and salinity. Fish Physiol. Biochem. 1421–1431. https://doi.org/10.1007/s10695-017-0382-z

Kuno, M., Li, G., Moriura, Y., Hino, Y., Kawawaki, J., Sakai, H., 2016. Acid-inducible proton influx currents in the plasma membrane of murine osteoclast-like cells.

Pflugers Arch. Eur. J. Physiol. 468, 837–847. https://doi.org/10.1007/s00424-016-1796-7

Laiz-Carrión, R., Sangio-Alvarellos, S., Guzmán, J.M., Río, M.P.M., Míguez, J.M., Soengas, J.L., Mancera, J.M., 2004. Energy Metabolism in Fish Tissues Related to Osmoregulation and Cortisol Action : Fish Growth and Metabolism. Environmental, Nutritional and Energy metabolism in fish tissues related to osmoregulation and cortisol 179–188. https://doi.org/10.1023/B

Lee, C., Kwon, B.O., Hong, S., Noh, J., Lee, J., Ryu, J., Kang, S.G., Khim, J.S., 2018. Sub-lethal and lethal toxicities of elevated CO2 on embryonic, juvenile, and adult stages of marine medaka *Oryzias melastigma*. Environ. Pollut. 241, 586–595. https://doi.org/10.1016/j.envpol.2018.05.091

Leitemperger, J., Müller, T.E., Cerezer, C., Marins, A.T., de Moura, L.K., Loro, V.L., 2019. Behavioural and biochemical parameters in guppy (*Poecilia vivipara*) following exposure to waterborne zinc in salt or hard water. Mol. Biol. Rep. 46, 3399–3409. https://doi.org/10.1007/s11033-019-04802-x

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Lushchak, V.I., 2012. Glutathione Homeostasis and Functions: Potential Targets for Medical Interventions. J. Amino Acids 2012, 1–26. https://doi.org/10.1155/2012/736837

Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish: a mechanistic approach. Fish Physiol. Biochem. 42, 711–747. https://doi.org/10.1007/s10695-015-0171-5

Maciel, F.E., Rosa, C.E., Santos, E.A., Monserrat, J.M., Nery, L.E.M., 2004. Daily variations in oxygen consumption, antioxidant defenses, and lipid peroxidation in the gills and hepatopancreas of an estuarine crab. Can. J. Zool. 82, 1871-1877. https://doi.org/10.1139/Z04-182

Mailloux, R.J., Gill, R., Young, A., 2020. Protein S-glutathionylation and the regulation of cellular functions. In: Oxidative Stress. Elsevier, pp. 217–247. https://doi.org/10.1016/B978-0-12-818606-0.00013-4

Maison, K.A., Graham, K.S., 2015. Status review report: Orange clownfish (*Amphiprion percula*). Report to National Marine Fisheries Service, Office of Protected Resources. July, 2015. 67 p.

Martínez-Álvarez, R.M., Hidalgo, M.C., Domezain, A., Morales, A.E., García-Gallego, M., Sanz, A., 2002. Physiological changes of sturgeon Acipenser naccarii caused by increasing environmental salinity. J. Exp. Biol. 205, 3699–3706.

Maulvault, A.L., Barbosa, V., Alves, R., Anacleto, P., Camacho, C., Cunha, S., Fernandes, J.O., Ferreira, P.P., Rosa, R., Marques, A., Diniz, M., 2018. Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. Aquat. Toxicol. 202, 65–79. https://doi.org/10.1016/j.aquatox.2018.06.016

McCormick, S.D., Regish, A.M., 2018. Effects of ocean acidification on salinity tolerance and seawater growth of Atlantic salmon *Salmo salar* smolts. J. Fish Biol. 93, 560–566. https://doi.org/10.1111/jfb.13656

Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.O., 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: Pre-adaptation through lifestyle and ontogeny? Biogeosciences 6, 2313–2331. https://doi.org/10.5194/bg-6-2313-2009

Miller, G.M., Watson, S.A., Mccormick, M.I., Munday, P.L., 2013. Increased CO_2 stimulates reproduction in a coral reef fish. Glob. Chang. Biol. 19, 3037–3045. https://doi.org/10.1111/gcb.12259

Mitton, F.M., Ribas, J.L., Gonzalez, M., Miglioranza, K.S.B., Monserrat, J.M., 2016. Antioxidant responses in soybean and alfalfa plants grown in DDTs contaminated soils : Useful variables for selecting plants for soil phytoremediation ? Pestic. Biochem. Physiol. 130, 17–21. https://doi.org/10.1016/j.pestbp.2015.12.005

Moniruzzaman, M., Mukherjee, J., Jacquin, L., Mukherjee, D., Mitra, P., Ray, S., Chakraborty, S.B., 2018. Physiological and behavioural responses to acid and osmotic stress and effects of Mucuna extract in Guppies. Ecotoxicol. Environ. Saf. https://doi.org/10.1016/j.ecoenv.2018.07.053

Monserrat, J.M., Martínez, P.E., Geracitano, L.A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., Bianchini, A., 2007. Pollution biomarkers in estuarine animals: Critical

review and new perspectives. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 146, 221–234. https://doi.org/10.1016/j.cbpc.2006.08.012
Montalvo, A.J., 2017. Considerations for Developing a Marine Ornamental Hatchery, in: Marine Ornamental Species Aquaculture. Wiley, pp. 159–173. https://doi.org/10.1002/9781119169147.ch11
Mota, V.C., Hop, J., Sampaio, L.A., Heinsbroek, L.T.N., Verdegem, M.C.J., Eding, E.H., Verreth, J.A.J., 2018. The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac. Res. 49, 3456–3467. https://doi.org/10.1111/are.13812
Mukherjee, A., Bhowmick, A.R., Mukherjee, J., Moniruzzaman, M., 2019. Physiological response of fish under variable acidic conditions: a molecular approach through the assessment of an eco-physiological marker in the brain. Environ. Sci. Pollut. Res. 26, 23442–23452. https://doi.org/10.1007/s11356-019-05602-3
Park, M.S., Shin, H.S., Choi, C.Y., Na Kim, N., Park, D.-W., Kil, G.-S., Lee, J., 2011. Effect of hypoosmotic and thermal stress on gene expression and the activity of antioxidant enzymes in the cinnamon clownfish, Amphiprion melanopus. Animal Cells Syst. (Seoul). 15, 219–225. https://doi.org/10.1080/19768354.2011.604941
Pimentel, M.S., Faleiro, F., Machado, J., Pousão-Ferreira, P., Rosa, R., 2020. Seabream larval physiology under ocean warming and acidification. Fishes 5, 1–13. https://doi.org/10.3390/fishes5010001
Rhyne, A.L., Tlusty, M.F., Schofield, P.J., Kaufman, L., Morris, J.A., Bruckner, A.W., 2012. Revealing the Appetite of the Marine Aquarium Fish Trade: The Volume and Biodiversity of Fish Imported into the United States. PLoS One 7, e35808. https://doi.org/10.1371/journal.pone.0035808
Robergs, R.A., 2017. Competitive cation binding computations of proton balance for reactions of the phosphagen and glycolytic energy systems within skeletal muscle. PLoS One 1–24. https://doi.org/https://doi.org/10.1371/journal.pone.0189822
Rodrigues, R.V., dos Santos Pedron, J., Romano, L.A., Tesser, M.B., Sampaio, L.A., 2015. Acute responses of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) to acid stress. Aquac. Res. 46, 1241–1247. https://doi.org/10.1111/are.12282
Sampaio, E., Lopes, A.R., Francisco, S., Paula, J.R., Pimentel, M., Maulvault, A.L., Repolho, T., Grilo, T.F., Pousão-Ferreira, P., Marques, A., Rosa, R., 2018. Ocean acidification dampens physiological stress response to warming and contamination

in a commercially-important fish (*Argyrosomus regius*). Sci. Total Environ. 618, 388–398. https://doi.org/10.1016/j.scitotenv.2017.11.059

Sampaio, L.A., Freitas, L.S., Okamoto, M.H., Louzada, L.R., Rodrigues, R. V., Robaldo, R.B., 2007. Effects of salinity on Brazilian flounder *Paralichthys orbignyanus* from fertilization to juvenile settlement. Aquaculture 262, 340–346. https://doi.org/10.1016/j.aquaculture.2006.09.046

Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 25:192–205. https://doi.org/10.1016 /0003-2697(68)90092-4

Shrivastava, J., Ndugwa, M., Caneos, W., De Boeck, G., 2019. Physiological trade-offs, acid-base balance and ion-osmoregulatory plasticity in European sea bass (*Dicentrarchus labrax*) juveniles under complex scenarios of salinity variation, ocean acidification and high ammonia challenge. Aquat. Toxicol. 212, 54–69. https://doi.org/10.1016/j.aquatox.2019.04.024

Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability , development and biochemical responses of sand smelt larvae. Sci. Total Environ. 563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091

Sinha, A.K., AbdElgawad, H., Zinta, G., Dasan, A.F., Rasoloniriana, R., Asard, H., Blust, R., De Boeck, G., 2015. Nutritional status as the key modulator of antioxidant responses induced by high environmental ammonia and salinity stress in European sea bass (*Dicentrarchus labrax*). PLoS One 10, 1–29. https://doi.org/10.1371/journal.pone.0135091

Sokolova, I.M., 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr. Comp. Biol. 53, 597–608. https://doi.org/10.1093/icb/ict028

Strobel, A., Graeve, M., Poertner, H.O., Mark, F.C., 2013. Mitochondrial acclimation capacities to ocean warming and acidification are limited in the Antarctic Nototheniid fish, *Notothenia rossii* and *Lepidonotothen squamifrons*. PLoS One 8. https://doi.org/10.1371/journal.pone.0068865

Tiedke, J., Cubuk, C., Burmester, T., 2013. Environmental acidification triggers oxidative stress and enhances globin expression in zebrafish gills. Biochem. Biophys. Res. Commun. 441, 624–629. https://doi.org/10.1016/j.bbrc.2013.10.104

Tomanek, L., 2014. Proteomics to study adaptations in marine organisms to environmental stress. J. Proteomics. https://doi.org/10.1016/j.jprot.2014.04.009

Tresguerres, M., Hamilton, T.J., 2017. Acid-base physiology, neurobiology and behaviour in relation to CO_2-induced ocean acidification. J. Exp. Biol. 220, 2136–2148. https://doi.org/10.1242/jeb.144113

Tseng, Y.C., Hwang, P.P., 2008. Some insights into energy metabolism for osmoregulation in fish. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 148, 419–429. https://doi.org/10.1016/j.cbpc.2008.04.009

UNESCO (1983) Chemical methods for use in marine environmen- tal monitoring. Manual and Guides 12. Intergovernamental Oceanographic Commissiony, Paris, France

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ. Toxicol. Pharmacol. 13, 57–149. https://doi.org/10.1016/S1382-6689(02)00126-6

Welch, M.J., Munday, P.L., 2015. Contrasting effects of ocean acidification on reproduction in reef fishes. Coral Reefs 35, 1–9. https://doi.org/10.1007/s00338-015-1385-9

Wang, S., Jiang, Z., Mao, M., Mao, S., Sun, Y., Sui, Y., 2018. Effects of seawater pH on survival, growth, energy budget and oxidative stress parameters of juvenile turbot *Scophthalmus maximus*. Iran. J. Fish. Sci. 17, 675–689. https://doi.org/10.22092/ijfs.2018.116814

Zeng, L., Ai, C.X., Wang, Y.H., Zhang, J.S., Wu, C.W., 2017. Abrupt salinity stress induces oxidative stress via the Nrf2-Keap1 signaling pathway in large yellow croaker *Pseudosciaena crocea*. Fish Physiol. Biochem. 43, 955–964. https://doi.org/10.1007/s10695-016-0334-z

Zhou, J., Wang, W., Wang, A., He, W., Zhou, Q., Liu, Y., Xu, J., 2009. Glutathione S-transferase in the white shrimp *Litopenaeus vannamei*: Characterization and regulation under pH stress. Comp. Biochem. Physiol. Part C 150, 224–230. https://doi.org/10.1016/j.cbpc.2009.04.012

6. CAPÍTULO 3: Primary, secondary, and tertiary stress responses of juvenile seahorse *Hippocampus reidi* exposed to acute acid stress in brackish and seawater

Artigo publicado na revista Comparative Biochemistry and Physiology, Part B
https://doi.org/10.1016/j.cbpb.2021.110592

Authors: M.D. Dias Carneiro[a,b*], S. García-Mesa [c], L.A. Sampaio [b], M. Planas[a]

[a] Department of Ecology and Marine Resources, Instituto de Investigaciones Marinas (CSIC), Vigo, Spain.

[b] Laboratório de Piscicultura Estuarina e Marinha, Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil.

[c] Department of Zoology, University of Granada, Campus Universitario de Fuentenueva, Granada, Spain.

[*]Author for correspondence (marioiddc@gmail.com)

Abstract

Seahorse *Hippocampus reidi* is a vulnerable species, inhabiting estuarine and coastal waters. The safety of acidic environments for fish has been considered in terms of ocean acidification in nature and decreasing pH in intensive aquaculture systems. This study aimed to investigate the effects of acute exposition (96 h) of juvenile seahorses to different pH (5, 6, 7, and 8) in brackish (BW - salinity 11) or seawater (SW - salinity 33). For that, we studied the responses of cortisol, oxidative stress, and survival, thus covering primary, secondary, and tertiary stress responses. In SW, cortisol levels were not altered for fish maintained at pH 5 and 8. However, in BW, cortisol was higher for fish kept at pH 5. Regarding secondary stress responses, only GST activity increased with acidification in SW. However, acidification in BW caused biochemical alterations at enzymatic level (SOD, GST, GPx) and glutathione metabolism, accompanied by reduction of antioxidant capacity (TEAC) and increased lipid peroxidation (TBARS). Survival was always above 90% and it did not differ significantly among pH levels. Our

results suggest that *H. reidi* juveniles are more vulnerable to acidic exposure in BW than in SW.

Keywords: Syngnathidae; Biochemistry; Oxidative stress; ROS.

Introduction

Seahorses are members of the Syngnathidae family and *Hippocampus* genus. These vulnerable species of fishes are widespread in tropical and subtropical regions of all oceans, occupying several distinct habitats, including mangroves, seagrasses, and reef zones (Foster and Vincent, 2004). The most important seahorse species for the aquarium trade is *Hippocampus reidi* (Cohen et al., 2017). It is an euryhaline species, inhabiting estuarine areas along the Western Atlantic coast (Hora et al., 2016; Rosa et al., 2002). Thus, it is expected that this species would also tolerate pH fluctuations along with changes in salinity, as usually riverine waters have lower pH than coastal waters. Juvenile *H. reidi* grows better at intermediate salinity (10-20) than at higher salinities, probably because those salinities are closer to their isosmotic point, which was estimated to be equivalent to salinity 11.7 (Hora et al., 2016). Additionally, excretion of juvenile *H. reidi* transported in high salinity water (25-35) was found to reduce water pH further than those transported at intermediate salinity (15) (Cohen et al., 2018).

Rearing techniques are available for a reduced number of seahorse species (Koldewey and Martin-Smith, 2010; Planas et al., 2017a), and many aspects of the rearing conditions still need to be fully addressed (Cohen et al., 2016). Most species are reared under intensive conditions (Olivotto et al., 2011), and the use of recirculating aquaculture systems (RAS) is increasing for economic purposes, furthermore, it allows more flexibility to select the location of the rearing facility (Calado, 2017). However, fishes maintained in RAS might be exposed to extreme pH values, which can be stressful or lethal for aquatic species. Fortunately, Teleostei is the most-tolerant taxa to acidification among marine animals (Melzner et al., 2009). This is an important feature for aquaculture, since fish can face increasing levels of hazardous acidification when maintained under intensive captive conditions. Regarding RAS, the CO_2 released by fishes as well the action of nitrifying bacteria can reduce pH. In theory, pH can fall below 6.0 within a few days if alkalinity is not adjusted (Aslam et al., 2019; Ebeling et al., 2006; Mota et al., 2018), but commercial RAS companies routinely correct pH by adding sodium bicarbonate. Despite their tolerance to decreasing pH, damages to fish exposed to an acidic environment were observed, especially during early developmental stages. Yellowfin tuna (*Thunnus albacares*) larvae reduced growth and survival when submitted to a pH reduction, from pH 8.1 to 6.9 (Frommel et al., 2016). An acute exposition of juvenile cobia (*Rachycentron canadum*) to pH 5.5 resulted in skin and gills damages, including an increase in chloride cell count (Rodrigues et al., 2015). Conversely, the growth of turbot (*Psetta maxima*) was not impaired, even at a pH as low as 5.7 (Mota et al., 2018). Larvae of some species, such as the sand smelt (*Atherina presbyter*), show contradictory stress responses when facing a CO_2 rich environment. While the activity of some enzymes to prevent oxidative stress was reduced when maintained at pH 7.6, their growth parameters were improved (Silva et al., 2016).

Stress in fish can be divided into three stages (Wendelaar Bonga, 1997). The primary stress includes endocrine changes in catecholamines and corticosteroids. Cortisol is one of the primary response hormones, involved among other functions in acid-base and ion regulation. For example, *Amphiprion melanopus* submitted to a hypoosmotic shock, from salinity 35 to 17.5, showed an increase in plasma cortisol levels (Park et al., 2011b). Several biomarkers encompass the secondary stress responses, among them, the oxidative status of aquatic organisms (Lushchak, 2011). Oxidative stress can be defined by the situation, acute or chronic, when the concentration of reactive oxygen species (ROS) increases, leading to oxidative modification of cellular constituents and resulting in disturbance of cellular metabolism and regulatory pathways (Lushchak, 2016). There are several biomarkers related to oxidative stress and it is increasingly recognized that oxidative stress physiology is key for understanding the proximate mechanisms basic to the evolution of strategies and responses to environmental changes (Costantini et al., 2011). Finally, the tertiary stress responses encompass changes in growth, reproduction, and survival (Barton, 2002).

Considering the ecological importance and the potential of *H. reidi* for aquaculture, this study aimed to assess primary, secondary, and tertiary stress responses of juvenile seahorses after acute exposure to environmental acidification under two salinity scenarios: seawater (SW) and brackish water (BW), with emphasis on the oxidative stress-related responses. We are considering the hypotheses that juvenile seahorse, an estuarine species, would present a better response to acidic stress when kept in BW than in SW.

Material and methods

Breeding and newborn production

Groups of *H. reidi* breeders were maintained in a RAS (recirculation aquaculture system), specifically designed for seahorse reproduction (Planas et al., 2008), at Instituto de Investigaciones Marinas (IIM-CSIC) in Vigo (Spain). Seawater was pumped from the adjacent coast, filtered (5 µm) and UV treated before being used. Daily average exchange rates averaged 10-15%. The fish were maintained under a constant temperature (26±1 °C), 33±1 salinity, and pH 8.1± 0.1. Adult seahorses were fed twice daily on a diet consisting of enriched adult *Artemia sp.* (EG, AF, MC450; Iberfrost®) (Planas et al., 2017b) and frozen Mysidacea (Ocean Nutrition, Spain).

The freshly released offspring were siphoned out, counted, and stocked (3 fish L^{-1}) in 30 L rectangular aquaria. The aquaria were maintained in a RAS (Randazzo et al., 2018) under the same water conditions as adults. Newborn seahorses were fed during the first 5 days with copepods (*Acartia tonsa*) retained in a 125 µm mesh; afterward, the juveniles were fed on copepods (180 µm mesh) and *Artemia* nauplii (*Artemia sp.*), maintaining a density of 1-2 prey mL^{-1}. Wastes and uneaten food were removed twice daily by siphoning the aquaria.

Experimental design

Seahorse juveniles (11 days after male's pouch release - DAR) were exposed to seawater (SW – 33 salinity) and brackish water (BW – 11 salinity) at four pH levels: 5, 6, 7, and 8. The juveniles were transferred from the holding aquaria to 16 plastic beakers (2 L each), comprising four replicas for each pH level. The stocking density was equal to 10 seahorses per beaker. Since a unique brood could not provide the necessary juveniles for the whole study, the experiment was carried out with two batches: the first batch (18.3±0.5 mm and 11.9±1.4 mg) was exposed to SW, and the second batch (18.2±1.0 mm and 15.1±2.1 mg) to BW. Both batches were released by the same broodstock group.

The experimental beakers were placed in a water bath system equipped with a temperature controller. Aeration was provided in order to supply oxygen for the fish. The juveniles were fed 12 hours prior to each experimental run and fasted during 96h exposure to experimental conditions. The pH levels were adjusted in each beaker before fish were added to the experimental units. Appropriate doses of an acidic solution (HCl 3% - 0,36 M) were used to reach the desired pH. The pH of each beaker was measured daily (Crison, Micro pH 2001, Spain) and, if necessary, corrected by adding a small volume of diluted HCl solution. Oxygen concentration and temperature were also checked daily (Hach, HQ40d, Spain). At the end of the experiment, the following parameters were measured: salinity (refractometer Atago S/Milli-E, Japan), total ammonia nitrogen (TAN) by spectrophotometry (Cesil, CE 3040, England) according to Solorzano (1969), nitrite and nitrate (segmented flow analyzer AMS Alliance, Futura, Italy) (Hansen and Grasshoff, 1983), alkalinity (Titrino Metrohm, 716 DMS, Brazil), and *p*CO2 was calculated using CO2SYS according Lee et al. (2016). Prior to stocking the fish, water was aerated attempting to remove excess CO_2, thus assuring the results of the experiment reflect the desired pH and not excess CO_2. However, as estimates of CO_2 content do not consider how much of it was stripped, especially at low alkalinities (Summerfelt et al., 2015), it is possible the results overestimated the actual CO_2 concentration seahorse faced in the present experiment. Water quality parameters are presented in table 1.

Table – 1: Water chemistry of seawater (SW – salinity 33) and brackish water (BW – salinity 11) conditions. ‡‡There were no significant differences (P>0.05) among water quality parameters, therefore for clarity, only average and standard deviation for each salinity are informed. ‡Data not submitted to statistics.

Experiment	**SW**				**BW**				
	pH 5	pH 6	pH 7	pH 8	pH 5	pH 6	pH 7	pH 8	
Salinity (‰)	33±2				11±2				‡
Temperature (°C)	25.9±0.15				26.0±0.16				
Oxygen (mg O_2 L^{-1})	6.7±0.03				6.6±0.03				‡‡
TAN (mg N- NH_4^++NH_3 L^{-1})	0.26±0.05				0.28±0.04				
Nitrite (mg N-NO_2 L^{-1})	0.04±0.01				0.02±0.01				
pH	5.2±0.1	6.2±0.1	7.2±0.1	8.2±0.1	5.1±0.0	6.0±0.0	7.0±0.0	7.9±0.0	‡
Alkalinity (µM kg^{-1})	0.046±0.004	0.081±0.011	0.354±0.007	2.45±0.021	0.060±0.003	0.088±0.004	0.242±0.007	0.932±0.014	‡
pCO_2 (µatm)	12,799.9±945.3	1,754.2±42.3	776.7±42.2	440±11.9	23,309.2±2,398.2	3,633.4±95.1	1,044.8±47.2	552.5±24.1	‡

Mortality was checked daily, and dead individuals were removed and counted. At the end of the experiment, all survivors were counted and euthanized using an overdose of MS-222 (100 mg L^{-1}), flash-frozen in liquid nitrogen, and stored (-80°C) until further biochemical analyses.

Animal maintenance, handling, and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (Real Decreto 1201/2005, 10th October 2005) and the Regional Government Xunta de Galicia (REGA ES360570202001/16 /EDU-FOR07/MP001).

Stress analyses

Samples preparation

Samples were homogenized (1:9-w:v) (Poly Tron, PT 2100), as follows. Pools of five to seven seahorses (whole-body) from each beaker were homogenized in ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v:v) Triton X-100, pH 7.8. After centrifugation (30,000 *g* for 30 min at 4 °C) (SIGMA, 3K30), the resulting supernatants were stored at -80 °C for determination of cortisol, protein, enzymes, TBARS, and TEAC. Pools of three seahorses from each beaker were homogenized in 10 mM HCl and 1.3 % SSA buffer (1:9-w:v), centrifuged at 20,000 *g* for 10 min at 4 °C, and the supernatants were stored at -80 °C for analyses of glutathione (GSSG and GSH).

Analytical Assays

All analyses were performed in duplicate at 25 ± 0.5 °C in 96-well microplates (UVStar®, Greiner Bio-One, Frickenhausen, Germany) using a microplate reader (Bio-Tek PowerWave X, USA). The optimal substrate concentration to measure the maximal specific activity was previously established by preliminary assays for each enzyme and GSH/GSSG determination. The enzymatic reaction was initiated by the addition of the homogenate.

Primary stress response

Cortisol level was measured using a commercial kit (Cortisol ELISA kit, Cayman, USA) following the protocol informed by the manufacturer. This assay is based on the competition between cortisol and cortisol- acetylcholinesterase (AChE) conjugate (cortisol tracer) for a limited number of cortisol-specific mouse monoclonal antibody binding sites. This antibody-cortisol (either free or tracer) complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm.

Secondary stress responses

Superoxide dismutase (SOD) was assessed by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals (Mccord and Fridovich, 1969). One activity unit was defined as the amount of enzyme necessary to bring about a 50% inhibition of the ferricytochrome c reduction rate measured at 550 nm, using a reaction mixture comprised of 50 mM potassium phosphate buffer (pH of 7.8), 0.1 mM EDTA 0.1 mM xanthine, 0.013 mM cytochrome c, and 0.024 IU mL^{-1} xanthine oxidase.

DT-diaphorase (DTD) activity was measured in the reaction mixture contained 50 mM Tris–HCl (pH 7.3), 50μM DCPIP (2,6-dichlorophenol indophenol) and 0.5 mM NADH. The control reaction contained distilled water instead of the sample extract. The DTD activity was assessed as the difference between sample activity and control reading at 600 nm (Sturve et al., 2005).

Catalase (CAT) activity was analyzed by measuring the decrease of the H_2O_2 concentration in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 10.6 mM H_2O_2 (freshly prepared) at 240 nm (Aebi, 1984).

Glucose 6-P dehydrogenase (G6PDH) was analyzed according to Lupiañez et al. (1987) with some modifications (Barroso et al., 1994). The change in absorbance of NADPH at 340 nm was monitored to determine the enzymatic activity, containing 50 mM imidazole-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM NADP, and 1 mM glucose-6-phosphate.

Glutathione peroxidase (GPx) was indirectly measured according to Flohé and Günzler (1984). A freshly prepared GR solution (2.4 U ml^{-1} in 0.1 M potassium phosphate buffer, pH 7.0) was added to 50 mM potassium phosphate buffer pH 7.0, 0.5 mM EDTA, 1 mM sodium azide, 0.15 mM NADPH, 0.15 mM cumene hydroperoxide and 1mM GSH (glutathione reduced). NADPH consumption was monitored spectrophotometrically at 340 nm.

Glutathione reductase (GR) was assayed as described by Carlberg and Mannervik (1975), by measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH, and 0.15 mM glutathione oxidized (GSSG).

Glutathione-S transferase (GST) activity was monitored spectrophotometrically at 340 nm by the formation of glutathione-CDNB-conjugate according to Habig (1974). The reaction mixture comprised 0.1 M phosphate buffer (pH 6.5), 1.2 mM glutathione reduced (GSH), and 1.23 mM solution of 1-chloro-2,4- dinitrobenzene (CDNB) in ethanol, which was prepared just before the assay.

For every enzyme activity, except for SOD, one unit or milliunit is defined as the amount of enzyme required to transform 1 µmol or nmol of substrate/min under the conditions defined for each assay. The soluble protein of all samples was determined following Bradford (1976) using standard bovine serum albumin. Enzyme activity was referred to protein content.

Glutathione (GSH) was measured according to Anderson (1985), partially modified by Baker et al. (1990), and adapted to microtiter plate by Vandeputte et al. (1994). Both tGSH (total glutathione) and GSSG (oxidized glutathione) were measured in the same sample. Samples for GSSG determination had GSH derivatized by adding TEA and 2-vinylpyrimidine. This process blocks the interactions with GSH. The final enzymatic reagent was freshly prepared (0.71 mM DTNB, 0.24 mM NADPH, 0.08% SSA, and 1.2 IU mL^{-1} GR (glutathione reductase) was used to determine both tGSH and GSSG levels. The DTNB oxidized GSH to GSSG, which was restored to GSH by GR. The reaction was initiated by quickly adding GR to each well. The increase of absorbance was monitored at 415 nm at 25 ± 0.5 °C. GSH levels were calculated by subtracting GSSG values from tGSH. OSI (oxidative stress index) relates the amount of GSSG concerning tGSH, according to the following equation: OSI = (2GSSG/tGSH)×100.

Trolox equivalent antioxidant capacity (TEAC) was measured according to Erel (2004). ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) in an acidic medium (10 mmol of ABTS in acetate buffer 30 mmol L^{-1}, pH 3.6) that is oxidized by hydrogen peroxide, turning into an emerald-green color. The reduction of this compound in the presence of antioxidants comes from samples in reactive medium, acetate buffer 0.4 mol L^{-1} (pH 5.8), results in a loss of color, measured at 595 nm, proportional to the total antioxidant capacity of the extract. Antioxidant activity refers to the equivalent of a water-soluble analog of vitamin E (Trolox) used as a standard. The results are expressed in terms of µmol of Trolox-equivalent antioxidant capacity per liter of tissue extract (µM).

Thiobarbituric acid reactive substances (TBARS) were assessed by the Buege and Aust (1978) protocol. It assumes the samples contain malondialdehyde (MDA) resulting from LPO, which reacts with 2-thiobarbituric acid (TBA). The samples were incubated with a final reagent concentration of 7% TCA (Trichloroacetic acid), 0.21% TBA, and 1 mM BHA (butylhydroxyanisole) for 20 min at 100°C. After samples were cooled in an ice-water bath and centrifuged at 10,000 g for 3 min, the absorbance of the supernatant was read at 535 nm, using MDA as standard. Results were expressed as nmol equivalent MDA mg^{-1} of protein.

Tertiary stress response

Fish laying on the bottom of the beaker were considered dead if they did not move even after being touched by the tip of a glass pipette. Immobile fish that responded to the mechanical stimulus was considered moribund. Although the behavior and activity of seahorses along the experiment were not strictly explored, differences among treatments for some aspects of their general behavior (i.e. positioning, activity) were visually observed and annotated. Percent survival was calculated as:

Survival (%): (*Final number of fishes /Initial number of fishes) × 100*

Data analyses

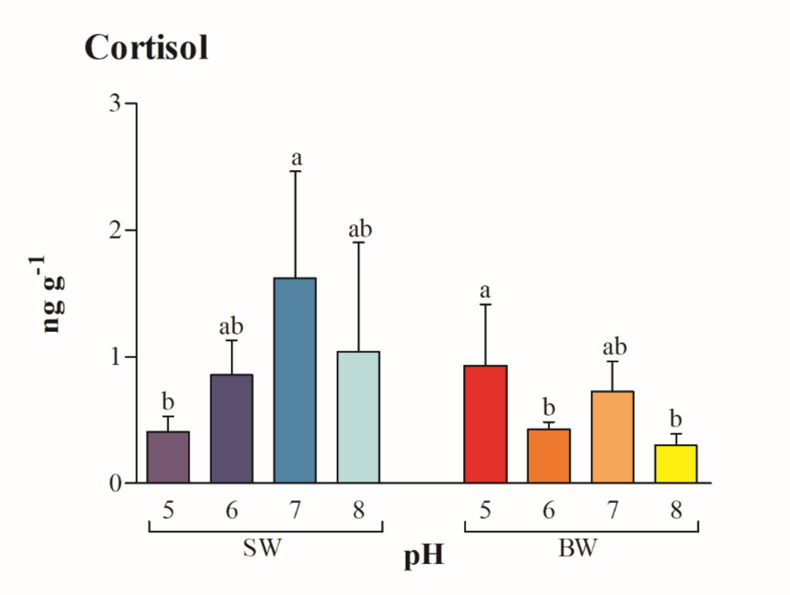
The experimental design was randomized. Data normality and homoscedasticity were analyzed with Shapiro Wilk's and Levene's test, respectively. The rank transformation was applied to data which were not normal or homoscedastic (Friedman, 1937). One-way ANOVA followed by the Tukey HSD *post hoc* test was applied to compare differences in data for fish exposed to decreasing pH levels in SW or BW. The significance level was set at 5% ($P<0.05$). Values are expressed as mean ± standard deviation. All data were analyzed using Statistica 7.0 software package.

Results

Primary stress response

The cortisol response in seahorses exposed to the four experimental pH levels differed depending on the salinity level tested. There was no significant difference on cortisol level among those exposed to pH 5, 6, and 8 in SW (Figure 1), but there was a significant difference for seahorse maintained in pH 5 and 7 ($P= 0.022$). However, in BW, we observed a significant three-fold increase of cortisol concentration for seahorse exposed to pH 5 compared to pH 8 (P= 0.009) (Figure 1).

Figure 1: Cortisol level (Mean±S.D.) in seahorses *Hippocampus reidi* juveniles exposed to different pHs for 96 h in seawater (SW – salinity 33) and brackish water (BW – salinity 11). Different letters indicate differences among pH to each salinity (P<0.05) by Tukey test.

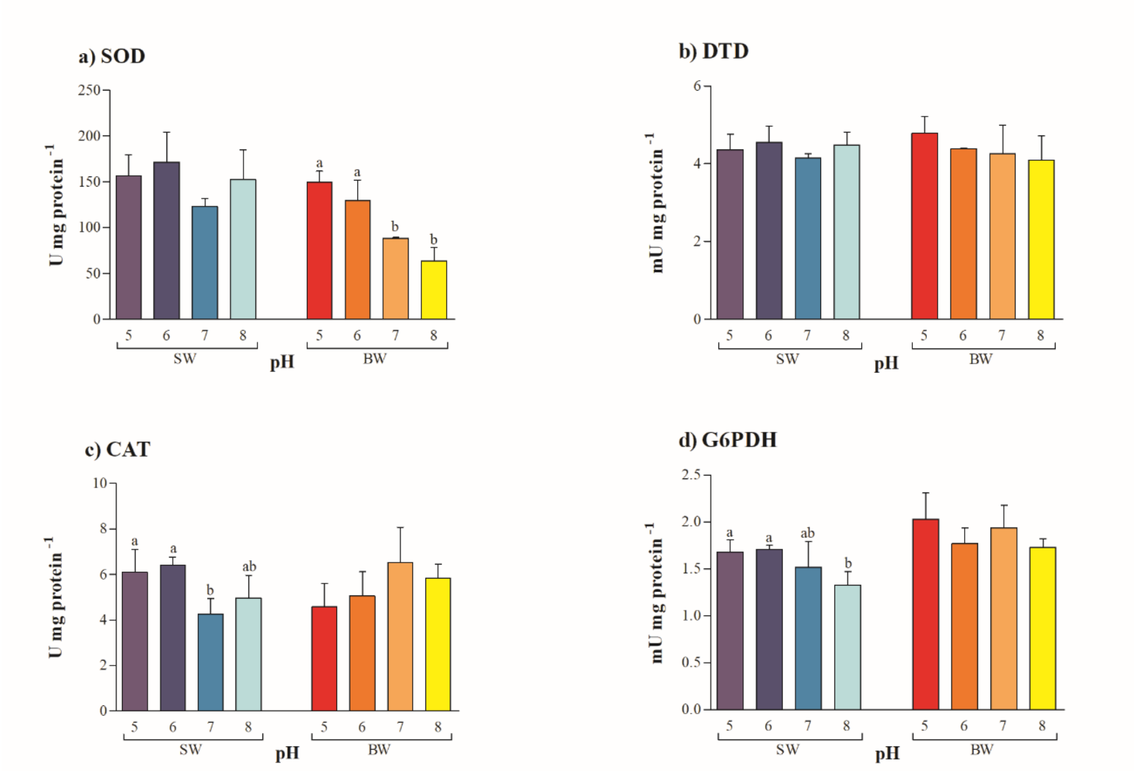


Secondary stress responses

There were no significant differences in SOD activity across pH values in SW (P= 0.120) (Figure 2a). However, SOD activity in BW juveniles was inversely related to pH levels (P< 0.001) being more than twofold high at pH 5 (Figure 2a). DTD activity was not influenced by the pH level in both salinities (P= 0.848 in SW and P= 0.345 in BW) (Figure 2b). The CAT activity of fish maintained in SW was significantly lower (43%) at pH 7 compared to other pH levels (P= 0.009), but it did not differ significantly across pH treatments when seahorses were kept in BW (P= 0.118), even being 30% lower at pH 5 compared with pH 8 (Figure 2c).

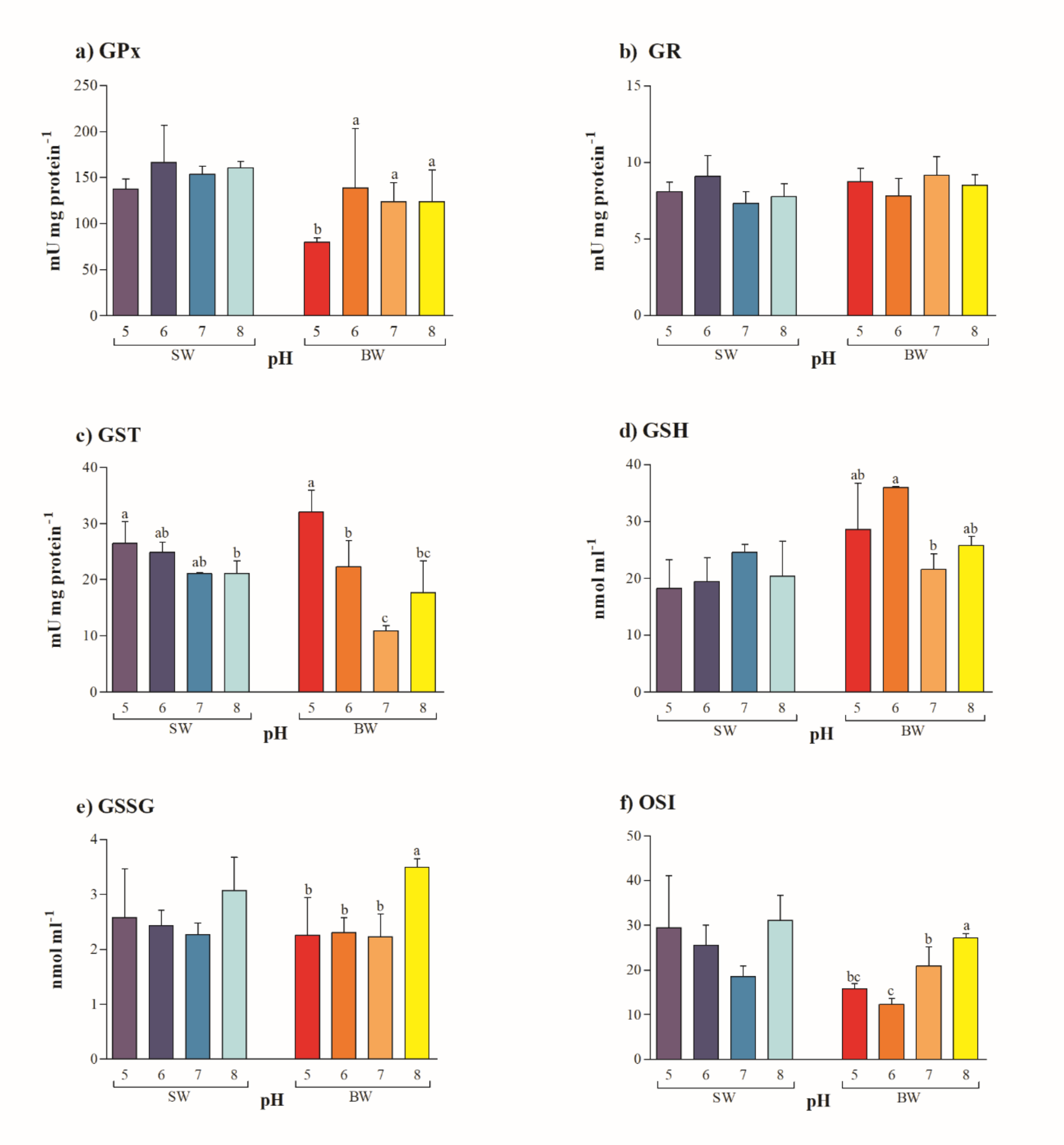
The G6PDH activity was affected by pH in SW (P= 0.032). At lower pHs (5 and 6), the activity increased significantly (around 30%) when compared to pH 8. However, there were no significant differences among fish maintained at different pHs in BW, even though we observed a 20% increase in G6PDH for fish maintained in pH 5 compared to pH 8 (P= 0.077) (Figure 2d).

Figure 2: SOD, DTD, CAT, G6PDH index (Mean±S.D.) of seahorses *Hippocampus reidi* exposed to different pHs for 96 h in seawater (SW – salinity 33) and brackish water (BW – salinity 11). Different letters indicate differences among pH to each salinity (P<0.05) after Tukey test.



Lowering pH levels in SW did not affect glutathione metabolism, as shown by activities of GPx (P= 0.131) and GR (P= 0.091), along with GSH (P= 0.270) and GSSG (P= 0.152) content (Figure 3a, b, d, e). However, GST increased significantly (P= 0.027) at pH 5 (Figure 3c). Conversely, except for GR activity (P= 0.326), glutathione metabolism was significantly affected by pH in BW (Figure 3). GPx activity at pH 5 was 54 – 73% lower than at higher pHs (P= 0.014), whereas GST activity was significantly higher at pH 5 (P< 0.001), there was about two-fold increase in comparison to fish maintained in pHs 7 and 8 (Figure 3a, c). GSH (P= 0.017) was significantly higher at pH 6 compared to pH 7, but there was no significant difference for fish maintained in pH 5 and 8 (Figure 3d). Finally, GSSG (P= 0.010) was 55% higher at pH 8 compared to lower pHs (Figure 3e).

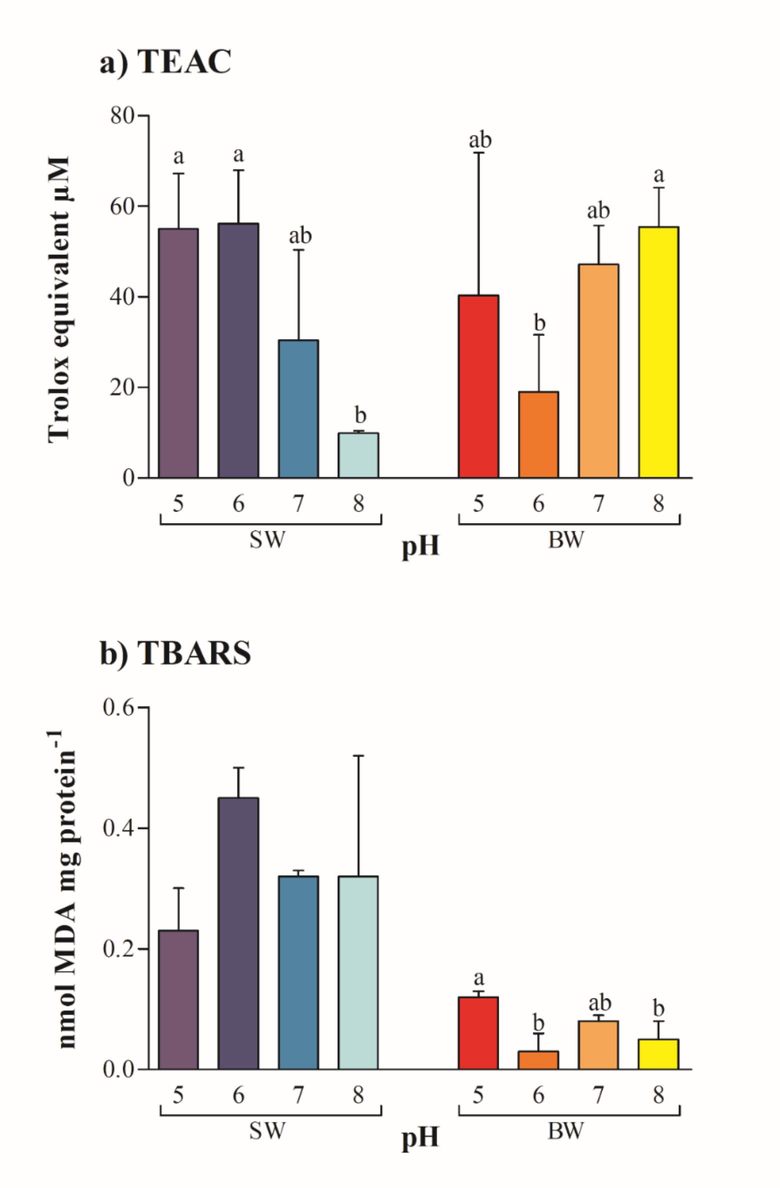
Figure 3: GPx, GR, GST, GSH, GSSG, and OSI index (Mean±S.D.) of seahorses *Hippocampus reidi* exposed to different pHs for 96 h in seawater (SW – salinity 33) and brackish water (BW – salinity 11). Different letters indicate differences among pH to each salinity (P<0.05) by Tukey test.



There were no significant differences for OSI across pH levels in SW (P= 0.100). However, there was a significant increase in OSI with increasing pH in BW, which was almost two-fold higher for juvenile seahorse maintained in pH 8 (P< 0.001) (Figure 3f). We observed depletion of TEAC content in SW seahorses, it decreased significantly with increasing pH (P= 0.015) being fivefold lower at pH 8. On the contrary, TEAC was not altered among pHs, except for a significant drop in its content for seahorses maintained in pH 6 (P= 0.032), which is 35% lower than that of fish kept in pH 8 (Figure 4a).

TBARS was not significantly affected by pH levels in SW (P= 0.094). However, a significant effect on LPO was noticed in juveniles maintained in BW, and lipid damage increased by 140% with decreasing pH. The highest LPO was observed for fish exposed to pH 5 (P= 0.007), and there were no significant differences among other pH levels (P> 0.05) (Figure 4b).

Figure 4: TEAC and TBARS index (Mean±S.D.) of seahorses *Hippocampus reidi* exposed to different pHs for 96 h in seawater (SW – salinity 33) and brackish water (BW – salinity 11). Different letters indicate differences among pH to each salinity (P<0.05) by Tukey test.

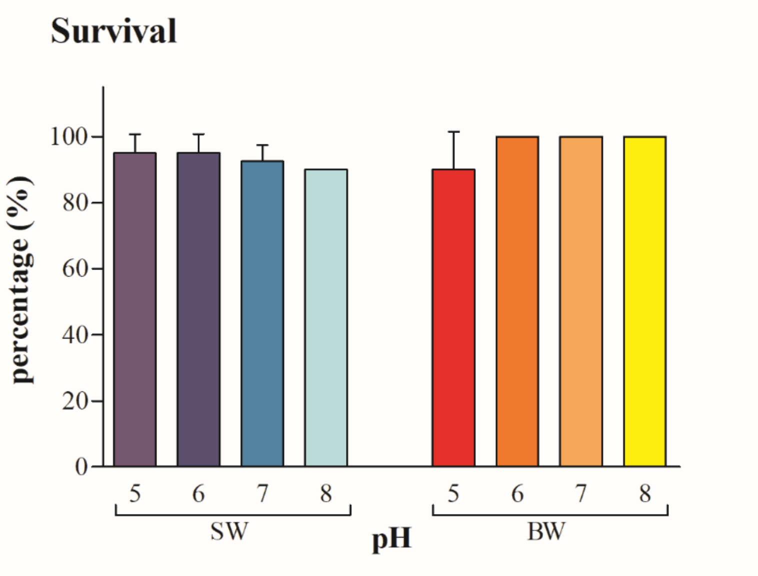


Tertiary stress response

Fish exposed to pH 5 in BW, remained most of the time on the bottom of the beakers. Fish maintained in higher pHs swam evenly along the water column. However, it is noteworthy, that swimming activity in pH 6 was slower than in pH 7 and 8. After 48h, we observed 2 to 3 fish laying immobile on the bottom of the beakers with pH 5 in BW. These fish, however, once stimulated by the glass pipette, started moving again, and are called moribund.

Dead fish were observed only at 96h. Seahorse survival was high, and it was not significantly affected by pH levels at both salinities tested. Survival ranged from 90 to 95% (P = 0.644) for juveniles kept in SW and from 90 to 100% (P = 0.073) for those exposed to BW (Figure 5).

Figure 5: Survival (Mean±S.D.) of seahorses *Hippocampus* juveniles exposed to different pHs for 96 h in salt water (SW – salinity 33) and brackish water (BW – salinity 11). Different letters indicate differences among pH to each salinity (P<0.05) by Tukey test.



Discussion

Seahorses are batch spawners providing several broods along the breeding season. The average brood size for *H. reidi* is about 300-350 newborns (Randazzo et al., 2018) and bearing in mind the mortality rate during the first days of life, using one single batch to perform the whole study would not ensure the number of seahorses needed for the experiments. Therefore, the present study was split in two independent trials, one in SW and another in BW, using for this purpose two consecutive broods originated from the same broodstock. Our experience shows seahorse from different broods perform similarly, including growth rate and survival (Randazzo et al., 2018). Thus, it is not likely to obtain different stress responses due to the use of different broods. Even tough, we chose not to compare the results obtained in each environment with statistical analysis, in order to avoid ascertaining an incorrect conclusion.

Recirculating aquaculture systems are now widely used to produce estuarine and marine fish (Tomoda et al. 2005). It is well known that increasing stocking density in a RAS may lead to acidification (Aslam at al., 2019), and as such it is important to understand the role of low pH in fish. Therefore, in this study we looked after several biomarkers to understand the role of acute exposure of juvenile seahorse to low pH in SW and BW, a plethora of responses that may add to understand the welfare of fish being reared in potentially stressful conditions (Ashley, 2007). The overall response of juvenile seahorse to acidification leads us to understand they were not severely compromised when exposed to pH 6, but further acidification showed a poorer response, especially in BW, as detailed below. It is noteworthy, that the stress responses observed for seahorse exposed to acidification, can also be due to elevated pCO_2 concentration, which reached 12,800 and 23,300 μatm in SW and BW respectively (for pH 5.0). Although high, our CO_2 levels are below the median lethal CO_2 concentration for medaka (*Oryzias melastigma*) estimated by Lee et al. (2016) at pCO_2 = 95,268 μatm (pH 5.91). Additionally, using HCl or CO_2 for acidification may lead to different results, mortality of larvae of *Pagrus major* exposed to pH 6.2 was equal to 61.2% when pH reduction was induced by increasing CO_2 (pCO_2 = 48,852.7 μatm), and only 1.6% when HCl was used to reduce pH, thus confirming CO_2 toxicity (Kikkawa et al., 2004). Anyway, the possible confounding effect of low pH associated with elevated CO_2 should be further investigated.

Primary stress response

Evidence that SW is a less stressful environment than BW for juvenile seahorse acutely exposed to pH 5 begins with the cortisol response. We only observed sustained elevated cortisol for fish exposed to pH 5 in BW, not in SW. Cortisol is a typical primary stress response, including thermal and chemical stressors (Zarantoniello et al. 2021), and salinity as well (Park et al 2011). Mota et al. (2017) reported increased cortisol level for rainbow trout reared in low pH (5.8) for 70 days, but primary stress response has also been observed for fish after acute exposure to acidic environment (Goss and Wood, 1988).

Summarizing, at the primary stress response level, seahorses are not affected after acute exposure to acidic environment in SW, on the opposite, they respond with increasing cortisol level in BW as the environment is acidified to pH 5.

Secondary stress responses

SODs are the first-line enzymes acting against oxygen free radicals and the unique enzymes interacting specifically with superoxide and, thus capable to control the levels of ROS and RNS (reactive nitrogen species) (Y. Wang et al., 2018). We observed different effects due to environmental acidification depending on water salinity. For example, SOD activity increased in an acidified environment in BW, but not in SW. Such an increase implies a better antioxidant stress response to low pH, which is in agreement with previous studies in marine fish (i.e. *Argyrosomus regius*, *Dicentrarchus labrax,* and *Hippoglossus hippoglossus*) exposed to an acid environment (Carney Almroth et al., 2019b; Maulvault et al., 2018a; Sampaio et al., 2018). However, we observed that increased SOD activity did not prevent ROS damages observed in BW.

The product of SOD is peroxide (H_2O_2), which is further reduced to water and oxygen by CAT and/or GPx. SOD activity increased for seahorses exposed to the acidic environment in BW, but the same was not observed for CAT activity, which remained unaltered at different pHs. GPx activity was even reduced at pH 5, thus peroxide removal may have been hampered in BW. On the other side, the activity of those enzymes was not influenced by pH in SW, suggesting juvenile seahorse would be eliminating ROS adequately, which may be proven correct, as there was no LPO in SW. Halliwell and Gutteridge (2015) already mentioned that an imbalance in SOD and CAT activities may result in an insufficient elimination of ROS, thus leading to oxidative stress. Activation of this system was observed in the brain of two Cyprinidae species (Mukherjee et al., 2019), in which SOD and CAT were high at pH 8.0 but both dropped drastically at pH 5.5. Nevertheless, a smaller acidification (ΔpH = - 0.4 units) did not disturb CAT activity neither in *Dicentharchus labrax* (Maulvault et al., 2018a) nor in *Argyrosomus regius (*ΔpH = - 0.5 units*)* (Sampaio et al., 2018).

The enzyme G6PDH produces NADPH, which is involved with enzymatic antioxidant defenses (Lushchak, 2011), but it is not specific for this function, as it is also involved in other metabolic pathways (Stanton, 2012). Anyway, we observed increased

G6PDH activity for seahorses maintained in SW at low pHs (5-6), supporting the idea of a larger energy demand to reduce the oxidative stress pressure in acidic pHs. However, in BW, seahorses were not able to increase G6PDH activity when exposed to low pHs, thus making it more difficult to cope with the acidic stress.

GST has a protective role against harmful compounds. It catalyzes the actions of GSH for detoxification of ROS or enhances their elimination from the organism (Blanchette et al., 2007). GST activity in juvenile seahorses increased at lower pHs in both salinities, aiming for the elimination of secondary products of peroxidation (Fridovich, 1998). However, GST activity was unable to avoid, along with the other detoxification machinery, LPO in juveniles maintained in BW. Similar results were reported in Atlantic halibut and European sea bass kept at low pH (ΔpH= - 0.4 units) (Carney Almroth et al., 2019b; Maulvault et al., 2018a).

Glutathione is a tripeptide used directly, or by enzymes as a co-factor. In an organism, glutathione can be present in its reduced form (GSH) or as an oxidized molecule (GSSG). The regeneration of GSH is possible by the action of glutathione reductase (GR), an enzyme which also depends on the NADPH content (Halliwell and Gutteridge, 2015). The glutathione metabolism in seahorse juveniles exposed to SW was not affected by pH, except for GST activity. On the other hand, glutathione metabolism was affected, as shown by the levels in GSH, GSSG, GST, and GPx for BW seahorses. Mukherjee et al. (2019) reported reduced levels of GSH in the brain of *Labeo rohita* and *Cirrhinus cirrhosis* exposed to pH 5.5, indicating its role in the elimination of free radicals. Furthermore, these authors detected an increase in brain GR activity in an acidic environment. However, GR activities in seahorses were not affected by the pH level independently of salinity.

Thus H^+ interaction could be the reason why GSSG pools were similar among experimental pHs in SW, once GR did not suffer modification in any treatments. Since H^+ is involved in aerobic and non-aerobic energy metabolism as mitochondrial proton leak or interaction with metabolites of non-mitochondrial energy metabolism (Kuno et al., 2016; Robergs, 2017). Furthermore, ionic steady-state implies consequences in the redox cycle (Halliwell and Gutteridge, 2015).

It is important to remember that ROS are dynamic (Lushchak, 2011), being continuously generated and eliminated. The imbalance between antioxidant and pro-oxidant (ROS) factors in favor of the pro-oxidant, leads ultimately to oxidative stress with damage in macromolecules (Lushchak, 2016). TEAC is a generalist anti-oxidant parameter, composed of - SH groups of proteins (53.0%), uric acid (33.1%), vitamin C (4.7%), bilirubin (2.4%), vitamin E (1.7%) and others (5.2% - glutathione, etc.) (Erel, 2004). Therefore, it can be considered indicative of the antioxidant capacity of a tissue (Sanz et al., 2017). In the present study, the compensatory mechanisms to combat free radicals failed in BW. Consequently, seahorse juveniles exposed to acidic environments showed a higher degree of LPO (increased TBARS). High LPO results in loss of membrane permeability and fall in their potential, sometimes leading to its rupture, along with loss of cell or organelle content (Gutteridge, 1995). The highest levels of LPO in the brain of two Cyprinidae (*C. cirrhosis* and *L. rohita*) occurred at pH 5.5 (Mukherjee et al., 2019). Similarly, TBARS also increased in flatfish (*Solea senegalensis*) larvae exposed to pH reduction (Pimentel et al., 2015).

The TEAC content in juveniles kept in BW was reduced with decreasing pH within the range of 6 to 8. Simultaneously, TBARS remained low at this pH range, demonstrating mobilization of TEAC to combat LPO. However, further reduction of pH resulted in increased TBARS, despite TEAC level was similar to those in fish kept at pH 7 and 8. This shows a failure of the anti-oxidant system when seahorse juveniles were exposed to the most severe acidic condition, which could not be compensated even in the presence of TEAC, which for some reason was not mobilized in this situation.

Summarizing, seahorses maintained in SW and exposed to reduced pH did not suffer LPO even when exposed to pH 5. Furthermore, most enzymes and macromolecules involved in the antioxidant process were not influenced by pH. However, seahorse juveniles exposed to BW were less tolerant to lower pH values, showing disruptions on the activity of several enzymes and on the content of antioxidant biomolecules, resulting in increased LPO at pH 5.

Tertiary stress responses

Fish, among other aquatic organisms, are the most tolerant taxa to acidic stress (Melzner et al., 2009). Regarding seahorses, Faleiro et al. (2015) observed behavioral changes (i.e. reduced activity levels) combined with a reduction of feeding and ventilation rates for adult *Hippocampus guttulatus* exposed to hypercapnia (ΔpH = -0.5 units). Other fish species show tolerance to even lower pH levels. The Brazilian flounder *Paralichthys orbignyanus* exhibits full survival of individuals after exposure to pH 5.2 for 96h (Wasielesky et al, 1997). Another Pleuronectiformes, the turbot *Psetta maxima*, also performs well in low pH, its growth was not hampered when reared at pH 5.7 (Mota et al., 2018). In the present study, the survival of juvenile seahorse was always above 90% and it does not seem to have been influenced by pH, even at the lowest pH tested. However, the juveniles seemed to be physiologically affected on a different manner depending on the treatment. For example, moribund fish were observed laying on the bottom of the experimental units following 48h exposure to pH 5 in BW. It is important to mention that moribund fish were not observed in SW, further suggesting better tolerance to acidic stress in SW than in BW. Moribund seahorses were not observed under SW conditions at pH 6, but direct observations showed that the swimming of juveniles was slower than those kept in pH 7 and 8.

Conclusions

Fish can be highly tolerant to acidic stress and seahorses are not an exception. We observed high survival, even when seahorse juveniles were exposed to pH values as low as 5. Considering the overall primary, secondary, and tertiary stress levels here studied, our results seem to indicate lower levels of stress for seahorses maintained in SW compared to those exposed to acidic stress in BW, thus we reject our initial hypothesis that as an estuarine species, *H. reidi* would be more tolerant to acidification in BW.

Acknowledgments

We are thankful to Alexandro Chamorro, Lourdes Nieto, Jesús Muras, José Padín, Susana Bastero and Carmen Castro by their support in fish maintenance and water quality analyses. The authors are grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for research fellowships provided for L.A. Sampaio (# 307445/2016-0), and to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) for a research grant provided for M.D.D. Carneiro. Also, we are very grateful to A. Pérez-Jiménez, principal of the research group "Nutrición y alimentación en peces" (RNM-156) (University of Granada) for granting the necessary permissions to perform the analyses at Campus de Excelencia Internacional del Mar (CeiMAR) and for financial support.

References

Aebi, H., 1984. Catalase din vitroT. Methods Enzymol, 105, 121-127.

Ashley, P.J., 2007. Fish welfare: Current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199–235. https://doi.org/10.1016/j.applanim.2006.09.001

Aslam, S.N., Navada, S., Bye, G.R., Mota, V.C., Terjesen, B.F., Mikkelsen, Ø., 2019. Effect of CO_2 on elemental concentrations in recirculating aquaculture system tanks. Aquaculture 511, 734254. https://doi.org/10.1016/j.aquaculture.2019.734254.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem. 190, 360–365. https://doi.org/10.1016/0003-2697(90)90208-Q.

Barroso, J.B., García-Salguero, L., Peragón, J., de la Higuera, M., Lupiáñez, J.A., 1994. The influence of dietary protein on the kinetics of NADPH production systems in various tissues of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 124, 47–59. https://doi.org/10.1016/0044-8486(94)90353-0.

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 42, 517–525. https://doi.org/10.1093/icb/42.3.517.

Bignami, S., Sponaugle, S., Cowen, R.K., 2013. Response to ocean acidification in larvae of a large tropical marine fish, *Rachycentron canadum*. Glob. Chang. Biol. 19, 996–1006. https://doi.org/10.1111/gcb.12133.

Bignami, S., Sponaugle, S., Cowen, R.K., 2014. Effects of ocean acidification on the larvae of a high-value pelagic fisheries species , mahi-mahi *Coryphaena hippurus* 21, 249–260. https://doi.org/10.3354/ab00598.

Blanchette, B., Feng, X., Singh, B.R., 2007. Marine glutathione S-transferases. Mar. Biotechnol. 9, 513–542. https://doi.org/10.1007/s10126-007-9034-0.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Meth. Enzymol. 52, 302–310.

Calado, R., 2017. Location, in: Marine Ornamental Species Aquaculture. Wiley, pp. 75–79. https://doi.org/10.1002/9781119169147.ch5.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Carney Almroth, B., Bresolin de Souza, K., Jönsson, E., Sturve, J., 2019. Oxidative stress and biomarker responses in the Atlantic halibut after long term exposure to elevated CO_2 and a range of temperatures. Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 238, 110321. https://doi.org/10.1016/j.cbpb.2019.110321.

Cohen, F.P.A., Planas, M., Valenti, W.C., Lillebø, A., Calado, R., 2018. Optimizing packing of live seahorses for shipping. Aquaculture 482, 57–64. https://doi.org/10.1016/J.AQUACULTURE.2017.09.024.

Cohen, F.P.A., Valenti, W.C., Planas, M., Calado, R., 2016. Seahorse Aquaculture, Biology and Conservation: Knowledge Gaps and Research Opportunities. Rev. Fish. Sci. Aquac. 25, 100–111. https://doi.org/10.1080/23308249.2016.1237469.

Cominassi, L., Moyano, M., Claireaux, G., Howald, S., Mark, F.C., Zambonino-Infante, J.-L., Bayon, N. Le, Peck, M.A., 2019. Combined effects of ocean acidification and temperature on larval and juvenile growth, development and swimming performance of European sea bass (*Dicentrarchus labrax*). PLoS One 14, 1–22. https://doi.org/10.1371/journal.pone.0221283.

Costantini, D., Marasco, V., Møller, A.P., 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 2011, 447–456. https://doi.org/10.1007/s00360-011-0566-2.

Crespel, A., Anttila, K., Lelièvre, P., Quazuguel, P., Le Bayon, N., Zambonino-Infante, J.-L., Chabot, D., Claireaux, G., 2019. Long-term effects of ocean acidification upon energetics and oxygen transport in the European sea bass (*Dicentrarchus labrax*, Linnaeus). Mar. Biol. 166, 116. https://doi.org/10.1007/s00227-019-3562-9.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358. https://doi.org/10.1016/j.aquaculture.2006.03.019.

Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem. 37, 277–285. https://doi.org/10.1016/j.clinbiochem.2003.11.015.

Faleiro, F., Baptista, M., Santos, C., Aurélio, M.L., Pimentel, M., Pegado, M.R., Paula, J.R., Calado, R., Repolho, T., Rosa, R., S, S., D, Q., M, Manning, Z, C., M, Marquis, KB, A., M, T., HL, M., 2015. Seahorses under a changing ocean: the impact of warming and acidification on the behaviour and physiology of a poor-swimming bony-armoured fish. Conserv. Physiol. 3, 1–7. https://doi.org/10.1093/conphys/cov009.

Flohé, L., Günzler, W.A., 1984. Assays of Glutathione Peroxidase. Methods Enzymol. 105, 114–120.

Foster, S.J., Vincent A.C.J., 2004. Life history and ecology of seahorses: implications for conservation and management. J. Fish Bio. 65, 1-61. https://doi.org/10.1111/j.0022-1112.2004.00429.x.

Fridovich, I., 1998. Oxygen toxicity : a radical explanation. J. Exp. Biol. 1209, 1203–1209.

Friedman, M., 1937. The Use of Ranks to Avoid the Assumption of Normality Implicit in the Analysis of Variance. J. Am. Stat. Assoc. 32, 675–701. https://doi.org/10.1080/01621459.1937.10503522.

Frommel, A.Y., Margulies, D., Wexler, J.B., Stein, M.S., Scholey, V.P., Williamson, J.E., Bromhead, D., Nicol, S., Havenhand, J., 2016. Ocean acidification has lethal and sub-lethal effects on larval development of yellowfin tuna , *Thunnus albacares*. J. Exp. Mar. Bio. Ecol. 482, 18–24. https://doi.org/10.1016/j.jembe.2016.04.008.

Goss, G.G., Wood, C.M., 1988. The effects of acid and acid/aluminum exposure on circulating plasma cortisol levels and other blood parameters in the rainbow trout, Salmo gairdneri. J. Fish Biol. 32, 63–76. https://doi.org/10.1111/j.1095-8649.1988.tb05335.x.

Gutteridge, J.M.C., 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin. Chem. 41, 1819–1828.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130–7139.

Halliwell B, Gutteridge JM (2015) Free radicals in biology and medicine. Oxford University Press, USA.

Hansen, H.P., Grasshoff, K., 1983. Automated chemical analysis. In: Grasshoff, K., Ehrdhardt, M., Kremling, K. (Eds.), Methods of Seawater Analysis, second ed. Verlag Chemie, Weinheim, pp. 347–395.

Hora, M. dos S.C. da, Joyeux, J.-C., Rodrigues, R.V., Sousa-Santos, L.P. de, Gomes, L.C., Tsuzuki, M.Y., 2016. Tolerance and growth of the longsnout seahorse *Hippocampus reidi* at different salinities. Aquaculture 463, 1–6. https://doi.org/10.1016/j.aquaculture.2016.05.003.

Huang, J., Qin, G., Zhang, B., Tan, S., Sun, J., Lin, Q., 2020. Effects of food, salinity, and ammonia-nitrogen on the physiology of juvenile seahorse (*Hippocampus erectus*) in two typical culture models in China. Aquaculture 520, 734965.

https://doi.org/10.1016/j.aquaculture.2020.734965.

Ishimatsu, A., Hayashi, M., Kikkawa, T., 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. 373, 295–302. https://doi.org/10.3354/meps07823.

Kikkawa, T., Kita, J., Ishimatsu, A., 2004. Comparison of the lethal effect of CO_2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Mar. Pollut. Bull. 48, 108–110. https://doi.org/10.1016/S0025-326X(03)00367-9.

Koldewey, H.J., Martin-Smith, K.M., 2010. A global review of seahorse aquaculture. Aquaculture 302, 131–152. https://doi.org/10.1016/j.aquaculture.2009.11.010

Kuno, M., Li, G., Moriura, Y., Hino, Y., Kawawaki, J., Sakai, H., 2016. Acid-inducible proton influx currents in the plasma membrane of murine osteoclast-like cells. Pflugers Arch. Eur. J. Physiol. 468, 837–847. https://doi.org/10.1007/s00424-016-1796-7.

Lee, C., Hong, S., Kwon, B.O., Lee, J.H., Ryu, J., Park, Y.G., Kang, S.G., Khim, J.S., 2016. Lethal and sub-lethal effects of elevated CO_2 concentrations on marine benthic invertebrates and fish. Environ. Sci. Pollut. Res. 23, 14945–14956. https://doi.org/10.1007/s11356-016-6622-4.

Lupiañez, J.A., Adroher, F.J., Vargas, A.M., Osuna, A., 1987. Differential behaviour of glucose 6-phosphate dehydrogenase in two morphological forms of Trypanosoma cruzi. Int. J. Biochem. 19, 1085–1089. https://doi.org/10.1016/0020-711X(87)90310-7.

Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish : a mechanistic approach. Fish Physiol. Biochem. 42, 711–747. https://doi.org/10.1007/s10695-015-0171-5.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006.

Maulvault, A.L., Barbosa, V., Alves, R., Anacleto, P., Camacho, C., Cunha, S., Fernandes, J.O., Ferreira, P.P., Rosa, R., Marques, A., Diniz, M., 2018. Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. Aquat. Toxicol. 202, 65–79. https://doi.org/10.1016/j.aquatox.2018.06.016.

Mccord, J., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.O., 2009. Physiological basis for high CO_2 tolerance in marine ectothermic animals: Pre-adaptation through lifestyle and ontogeny? Biogeosciences 6, 2313–2331. https://doi.org/10.5194/bg-6-2313-2009.

Miller, G.M., Watson, S.A., Donelson, J.M., McCormick, M.I., Munday, P.L., 2012. Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nat. Clim. Chang. 2, 858–861. https://doi.org/10.1038/nclimate1599.

Mota, V.C., Hop, J., Sampaio, L.A., Heinsbroek, L.T.N., Verdegem, M.C.J., Eding, E.H., Verreth, J.A.J., 2018. The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac. Res. 49, 3456–3467. https://doi.org/10.1111/are.13812.

Mota, V.C., Martins, C.I.M., Eding, E.H., Canário, A.V.M., Verreth, J.A.J., 2017. Cortisol and testosterone accumulation in a low pH recirculating aquaculture system for rainbow trout (*Oncorhynchus mykiss*). Aquac. Res. 48, 3579–3588. https://doi.org/10.1111/are.13184.

Mukherjee, A., Bhowmick, A.R., Mukherjee, J., Moniruzzaman, M., 2019. Physiological response of fish under variable acidic conditions: a molecular approach through the assessment of an eco-physiological marker in the brain. Environ. Sci. Pollut. Res. 26, 23442–23452. https://doi.org/10.1007/s11356-019-05602-3.

Olivotto, I., Planas, M., Simões, N., Holt, G.J., Avella, M.A., Calado, R., 2011. Advances in breeding and rearing marine ornamentals. J. World Aquacult. Soc. 42, 135–166. http://doi.org/10.1111/j.1749-7345.2011.00453.x.

Paglia, D. E., Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med, 70, 158–169.

Park, M.S., Shin, H.S., Kil, G.S., Lee, J., Choi, C.Y., 2011. Monitoring of Na^+/K^+-ATPase mRNA expression in the cinnamon clownfish, *Amphiprion melanopus*, exposed to an osmotic stress environment: Profiles on the effects of exogenous hormone. Ichthyol. Res. 58, 195–201. https://doi.org/10.1007/s10228-011-0207-5.

Pimentel, M.S., Faleiro, F., Diniz, M., Machado, J., Pousão-Ferreira, P., Peck, M.A., Pörtner, H.O., Rosa, R., 2015. Oxidative Stress and Digestive Enzyme Activity of Flatfish Larvae in a Changing Ocean. PLoS One 10, e0134082. https://doi.org/10.1371/journal.pone.0134082.

Planas, M., Chamorro, A., Quintas, P., Vilar, A., 2008. Establishment and maintenance of threatened long-snouted seahorse, *Hippocampus guttulatus*, broodstock in

captivity. Aquaculture 283, 19–28. https://doi.org/10.1016/j.aquaculture.2008.06.023.
Planas, M., Burhans, R, Simões, N., 2017a. Seahorses and pipefish.: in: Marine Ornamental Species Aquaculture. Wiley, pp. 299-326. https://doi.org/10.1002/9781119169147.ch17.
Planas, M., Silva, C., Quintas, P., Chamorro, A., Piñero, S., 2017b. Ongrowing and enhancement of n-3 HUFA profile in adult *Artemia*: short- vs long-time enrichment. J. Appl. Phycol. 29, 1409–1420. https://doi.org/10.1007/s10811-016-1016-z.
Randazzo, B., Rolla, L., Ofelio, C., Planas, M., Gioacchini, G., Vargas Abúndez. J. A., Giorgini, E., Olivotto, I., 2018. The influence of diet on the early development of two seahorse species (*H. guttulatus* and *H. reidi*): Traditional and innovative approaches. Aquaculture 490, 75-90. https://doi.org/ 10.1016/j.aquaculture.2018.02.029.
Robergs, R.A., 2017. Competitive cation binding computations of proton balance for reactions of the phosphagen and glycolytic energy systems within skeletal muscle. PLoS One 1–24. https://doi.org/https://doi.org/10.1371/journal.pone.0189822.
Rodrigues, R.V., dos Santos Pedron, J., Romano, L.A., Tesser, M.B., Sampaio, L.A., 2015. Acute responses of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) to acid stress. Aquac. Res. 46, 1241–1247. https://doi.org/10.1111/are.12282.
Rosa, I.L., Dias, T.L., Baum, J.K., 2002. Threatened fishes of the world: *Hippocampus reidi* Ginsburg , 1933 (Syngnathidae). Environ. Biol. of Fishes 64, 378. https://doi.org/10.1023/A:1016152528847
Sampaio, E., Lopes, A.R., Francisco, S., Paula, J.R., Pimentel, M., Maulvault, A.L., Repolho, T., Grilo, T.F., Pousão-Ferreira, P., Marques, A., Rosa, R., 2018. Ocean acidification dampens physiological stress response to warming and contamination in a commercially-important fish (*Argyrosomus regius*). Sci. Total Environ. 618, 388–398. https://doi.org/10.1016/j.scitotenv.2017.11.059.
Sanz, A., López-rodríguez, M.J., García-mesa, S., Trenzado, C.E., Ferrer, R.M., Figueroa, J.M.T.D.E., 2017. Are antioxidant capacity and oxidative damage related to biological and autecological characteristics in aquatic insects? J. Limnol. 76, 170–181. https://doi.org/10.4081/jlimnol.2016.1581.
Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability , development and biochemical responses of sand smelt larvae. Sci. Total Environ.

563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091.

Stanton, R.C., 2012. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. IUBMB Life 64, 362–369. https://doi.org/10.1002/iub.1017.

Sturve, J., Stephensen, E., Förlin, L., 2005. Effects of redox cycling on DT diaphorase activity in the liver of rainbow trout (*Oncorhynchus mykiss*). Comp. Hepatol. 8, 1–8. https://doi.org/10.1186/1476-5926-4-4.

Summerfelt, S.T., Zühlke, A., Kolarevic, J., Reiten, B.K.M., Selset, R., Gutierrez, X., Terjesen, B.F., 2015. Effects of alkalinity on ammonia removal, carbon dioxide stripping, and system pH in semi-commercial scale water recirculating aquaculture systems operated with moving bed bioreactors. Aquac. Eng. 65, 46–54. https://doi.org/10.1016/j.aquaeng.2014.11.002.

Tomoda, T., Fushimi, H., Kurokura, H., (2005). Performance of a closed recirculation system for larviculture of red sea bream, *Pagrus major*. Fish. Sci. 71, 1179‑1181. https://doi.org/10.1111/j.1444-2906.2005.01079.x.

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biol. Toxicol. 10, 415–421. https://doi.org/10.1007/BF00755791.

Wang, X., Wang, S., Li, C., Chen, K., Qin, J.G., Chen, L., Li, E., 2015. Molecular Pathway and Gene Responses of the Pacific White Shrimp *Litopenaeus vannamei* to Acute Low Salinity Stress. J. Shellfish Res. 34, 1037–1048. https://doi.org/10.2983/035.034.0330.

Wang, Y., Branicky, R., Noë, A., Hekimi, S., 2018. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. jcb.201708007. https://doi.org/10.1083/jcb.201708007.

Wasielesky, W., Bianchini, A., Santos, M.H.S., Poersch, L.H., 1997. Tolerance of juvenile flatfish *Paralichthys orbignyanus* to acid stress. J. World Aquac. Soc. 28, 202–204. https://doi.org/10.1111/j.1749-7345.1997.tb00857.x.

Welch, M.J., Munday, P.L., 2015. Contrasting effects of ocean acidification on reproduction in reef fishes. Coral Reefs 35, 1–9. https://doi.org/10.1007/s00338-015-1385-9.

Wendelaar Bonga, S.E., 1997. The Stress Response in Fish. Physiol. Rev. 77, 591–625.

Yan, J.J., Hwang, P.P., 2019. Novel discoveries in acid-base regulation and osmoregulation: A review of selected hormonal actions in zebrafish and medaka.

Gen. Comp. Endocrinol. 277, 20–29. https://doi.org/10.1016/j.ygcen.2019.03.007.
Zarantoniello, M., Bortoletti, M., Olivotto, I., Ratti, S., Poltronieri, C., Negrato, E., Caberlotto, S., Radaelli, G., Bertotto, D., 2021. Salinity, Temperature and Ammonia Acute Stress Response in Seabream (*Sparus aurata*) Juveniles: A Multidisciplinary Study. Animals 11, 97. https://doi.org/10.3390/ani11010097.

7. CAPÍTULO 4: Implications of salinity and acidic environments on fitness and oxidative stress parameters in early developing seahorses Hippocampus reidi

Capítulo formatado para a revista Science of the Total Environment.

M.D. Dias Carneiro[a,b*], S. Garcia-Mesa[c], L.A. Sampaio[b], M. Planas[a*]

[a] Departamento de Ecología y Recursos Marinos, Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain

[b] Laboratório de Piscicultura Estuarina e Marinha, Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil

[c] Department of Zoology, University of Granada, Campus Universitario de Fuentenueva, 18071 Granada, Spain

[*]Authors for correspondence: M.D. Dias Carneiro (marioiddc@gmail.com) and M. Planas (mplanas@iim.csic.es)

Highlights

- Survival and growth were impaired in seahorse juveniles exposed to chronic acidic environment in brackish water.
- Glutathione (GSH) roles are a key factor under salinity and pH challenges.
- Seahorse newborns raised in brackish water showed low tolerance to acidification and a decrease in antioxidant capacity.
- Growth of seahorse juveniles resulted enhanced in acidic seawater.

Abstract

Water acidification has important effects on aquatic species, affecting natural environmental conditions and *ex-situ* rearing production systems. The impact of acid pH are unknown to seahorses *Hippocampus reidi* (Ginsburg, 1933). Thereby, this work investigated the implications of pH upon growth and oxidative stress of seahorses, Newborns (0-21 days after the male's pouch release -DAR) and juveniles (21-50 DAR) reared at acid (6.5) and control

(8.0) pH in brackish (BW - salinity 11) or seawater (SW - salinity 33). There was no effect of pH on the growth of newborn seahorse reared in SW. However, the growth of those reared in BW was harmed in the pH 6.5. Accordingly, survival increase in fish kept at pH 6.5 SW, regardless the age-dependent increases in oxidative status. The superoxide dismutase, DT-diaphorase, and oxidative stress index increased in BW, but survival and growth just lowering at pH 6.5-BW. In a second trial, juveniles kept at pH 6.5 grew better than at pH 8.0. In conclusion, rearing seahorses at acid pH (6.5) and seawater (33) can improve survival, growth, and oxidative status condition. As well as acidic pH (6.5) and low salinity (11) harmed fish fitness and oxidative stress, and should be avoided.

Introduction

Seahorses (Genus *Hippocampus*; Family Syngnathidae) are fishes whose natural populations threatened due to Traditional Chinese Medicine (dried specimens) and aquarium trade (Koldewey and Martin-Smith, 2010). The average annual volume of seahorse trade reported in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) from 2004 to 2011was estimated in 5,7 million dried specimens and 116,000 live individuals (Foster et al., 2016). However, according Kuo and Vincent (2018) 37 millions of dried seahorse coming from bycatch are trade anually.In the light of these facts, all seahorse species are considered vulnerable by IWT (Illegal Wildlife Trade) and included in Appendix II of CITES since 2002 (Foster et al., 2019).

The production of endangered species in captivity is an alternative activity to reduce exploitation, ensuring traceability of the product in the market (Cohen et al., 2013). Only a few seahorses species are commercially traded for ornamental aquaria and the tropical species *Hippocampus reidi* is one of the most important species for ornamental uses (Cohen et al., 2017; Planas et al., 2017a). As well, most currently seahorses traded as ornamental specimens come from captive breeding (Foster et al., 2016). Seahorses can be raised in recirculating aquaculture facilities (Hora and Joyeux, 2009; Olivotto et al., 2008), as using multi-trophic rearing systems, along with shrimps and oysters (Fonseca et al., 2015) what might imply pH and salinity alterations.

Much of marine ornamentals are produced far from coastal areas in order to avoid high land costs and to improve biosecurity and commercialization (Calado, 2017; Tlusty, 2002). Hence, the use of recirculating aquaculture system (RAS) would result potentially advantageous. RAS use is increasing on the last years, disseminating aquaculture facilities anywhere (Timmons and Ebeling, 2010). However, water quality in RAS may achieved undesirable conditions trough rearing period. Salinity and pH are important factors for facilities, since cause implications in the physiological condition of fishes (Copatti et al., 2019; Kim et al., 2017; Lemos et al., 2018; Park et al., 2011; Pellegrin et al., 2019). The water can be acidified in RAS due to alkalinity reduction and H^+ released by the nitrification process (Ebeling et al., 2006). Besides, an increase in water salinity may affect negatively nitrification in bio filters that are at least 60% increased in a freshwater bio filters compared to seawater systems (Timmons and Ebeling, 2010).

The isosmotic point of *H. reidi* is reached at 11.67 salinity, as well grow better at brackish (slinity10 to 20) than seahorses reared in seawater. (Hora et al., 2016). Growth and survival enhancement at brackish has also been reported in other seahorse species. For instance, the survival improved in early juveniles of *H. abdominalis* kept at 10-15 salinity (Martinez-Cardenas and Purser, 2016). However, other species do not respond similarly, as reported in *H. erectus* that displayed higher sensibility to ammonia and downregulation in oxidative enzymes when exposed to low salinities (Huang et al., 2020a).

In temperate seahorses, *H. guttulatus*, an increase in temperature resulted in increased oxygen consumption at both pH 8.0 and 7.5 (normocapnia *vs* hypercapnia conditions) (Faleiro et al., 2015). This finding suggests an increased metabolic oxygen demand in mitochondrial respiration (electron transport system – ETS), caused by temperature at different pH scenarios,hen which is directly linked with the mobilization or use of energy sources for fish fitness, (Simčič et al., 2015; Sokolova, 2013; Sokolova et al., 2012). Thereby, changes in salinity and pH may alter energy metabolism and the generation of reactive oxygen species (ROS) (Lushchak, 2016; Sampaio et al., 2018; Shrivastava et al., 2019). Seahorses have high antioxidant activities; however, the effects of varying culture conditions on their antioxidant state are poorly know, deserving special assessment (Tseng et al., 2020).

Changes in biochemical status of fishes might be consequence of environmental alterations, which promote an imbalance between prooxidants and antioxidants molecules, named oxidative stress, leading to damages in cellular macromolecules (e.g., lipids, proteins, and DNA), or pathway changes (Lushchak, 2011, 2016; Stoliar and Lushchak, 2012). Regarding *Hippocampus reidi,* the oxidative status (total phenolic content, metal chelating activity, DHHP radical scavenging activity and ferric reducing antioxidant power) were significantly higher at salinity 15 and 20 (Tseng et al., 2020). Nevertheless, the acute exposure to acidic pH under brackish water (salinity 11) lead to stress increases on cortisol, biochemical and ethology level (Carneiro et al., 2021). However, chronic responses and possible adaptive responses for acidic pH and salinity alterations are misunderstanding.

The present study aimed to assess the biological and physiological age-dependent responses of *Hippocampus reidi* juveniles exposed to acidic environments under two salinity conditions: brackish and seawater. Understanding the impact of those conditions on the oxidative status in developing juveniles will contribute to improve the rearing conditions for the species.

Material and Methods

Ethics

Fish rearing, handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (R.D. 1201/2005, 10th October 2005) and the Regional Government Xunta de Galicia (Reference REGA ES360570202001/ 16/EDU-FOR07/MP001).

Live preys culture

Copepods and *Artemia* nauplii, enriched metanauplii, and enriched adults used for feeding seahorses were produced as follows: copepods (*Acartia tonsa*) were cultivated in 700 L tanks at 26–27 °C and 35-38 salinity, at initial densities of 1 copepod mL^{-1}. Copepods were fed every two days on the microalgae *Rhodomonas lens* (10^3 cells mL^{-1}). Siphoning of the culture tanks and water renewals (10% of the total volume) were carried out three times per week.

Artemia cysts (EG, AF, MC450; Iberfrost, Spain) were hatched at 28 °C for 20 h in 20 L units, and the hatched nauplii collected on a 125 µm mesh gently rinsed with tap-water and transferred to 20 L units for metanauplii production (100 *Artemia* mL^{-1}). Metanauplii of several ages (1-4 days) and sizes were enriched twice daily on a mixture consisting of live microalgae *Phaeodactylum tricornutum* (10^7 cells mL^{-1}), Red Pepper (0.015 g L^{-1}, Bernaqua, Belgium), and dried Spirulina (0.03 g L^{-1}, Iberfrost, Spain) (Planas et al., 2017b; Randazzo et al., 2018). Adult enriched *Artemia* was grown in 100 L units at 26–28 °C with aeration and constant lightning. A long-time enrichment (3–6 days) was carried out in adult *Artemia* from day 16 onwards on a mixture consisting of live microalgae *P. tricornutum* and *Isochrysis galbana* (10^7 cells mL^{-1}), Red Pepper (0.015 g L^{-1}, Bernaqua, Belgium), and dried Spirulina (0.03 g L^{-1}, Iberfrost, Spain) (Planas et al., 2017b).

Seahorse breeding

Adult seahorses *Hippocampus reidi* Ginsburg, 1933 were maintained in *ad hoc* aquaria (Planas et al., 2008) at Instituto de Investigaciones Marinas (IIM-CSIC) in Vigo (Spain). Three aquaria sub-units of 160 L each (85 height × 75 length × 50 wide cm) working as RAS were used as husbandry and breeding aquaria. The aquaria were supplied with seawater filtered (5 µm), UV treated, and daily exchanged of 10-15%. The breeders were maintained at 33±1 salinity, pH 8.1±0.1, constant temperature (26±1ºC) and 14L:10D photoperiod (Olivotto et al., 2008). The seahorses were fed twice daily on live long-time enriched adult *Artemia* spp. (EG, AF, MC450; Iberfrost, Spain) and frozen mysidaceans *Neomysis* sp. (Ocean Nutrition, Spain).

Trial 1: Effect of pH and salinity on newborn seahorses

Two salinities were assayed in newly released newborns of *H. reidi*: brackish water (BW; 11 salinity) and seawater (SW; 33 salinity). Since a unique brood could not provide the necessary juveniles for the whole study, the trial was carried out uning two broods (one brood per salinity level). For each salinity, newborn seahorses were transferred directly from the breeding aquarium to six 30 L pseudo-Kreisel experimental aquaria (120 fishes per aquarium). The newborn were maintained under a 12L:12D photoperiod regime and the water conditions during the experiment are provided in table 1. Three aquaria were maintained at pH 8.0, whereas the other three were adjusted and subsequently maintained at pH 6.5. For the maintenance of pH 6.5, water was prepared at pH 6.5 and strongly aerated for 24 hours before water renewal. The whole RAS systems were filled with water adjusted at the desired pH level for at least 24h before the onset of the experiment. This procedure guaranteed the elimination of CO_2 in excess, thus assuring that the results of the experiment reflected the desired pH instead of CO_2 in excess (Kikkawa et al., 2004). Each group of three aquaria worked as a RAS system including a sump, UV filtration, and biological filters (perforated plastic bio-balls) (Blanco et al., 2014; Randazzo et al., 2018).

The levels of pH were regularly monitored and adjusted by adding pre-tested volumes of hydrochloric acid solutions (3% HCl, 0.36 M) to the water used for daily water renewal. Water conditions were monitored twice daily, including pH (Crison, Micro pHmeter 2001, Spain), salinity (Atago S/Milli-E, Japan), dissolved oxygen, and temperature (Hach, HQ40d, Spain), and alkalinity (Eaton et al., 2005). Total ammoniacal nitrogen (TAN = NH_4^+ + NH_3^-) was measured by spectrophotometry (Cecil, spectrophotometer CE 3040, England) according to Solorzano (1969), while nitrite (NO_2^-) and nitrate (NO_3^-) were analyzed by segmented flow analyzer (AMS Alliance, Futura, Italy) following Hansen and Grasshoff (1983). All nitrogen compounds were checked twice a week (Table 1).

Table 1: Trial 1 - Water conditions (mean ± standard deviation) for the maintenance of *Hippocampus reidi* newborns reared in seawater (SW – salinity 33) or brackish water (BW – salinity 11) at pH 6.5 or 8.0 for 21 days (0-21 days after the male's pouch release – DAR).

	SW		**BW**	
	pH 6.5	**pH 8**	**pH 6.5**	**pH 8**
Salinity (‰)	33±1		11±1	
pH	6.6±0.01	8.1±0.0	6.5±0.01	7.8±0.01
Alkalinity (mg $CaCO_3$ L^{-1})	22±5 [b]	138±5 [a]	20±5 [b]	62±5 [a]
Temperature (°C)	26.1±0.1		26.1±0.2	
Oxygen (mg O_2 L^{-1})	6.53±0.03		6.56±0.01	
TAN (mg N- NH_4^++NH_3 L^{-1})	0.21±0.04		0.13±0.02	
Nitrite (mg N-NO_2 L^{-1})	0.02±0.0		0.05±0.05	
Nitrate (mg N-NO_3 L^{-1})	0.13±0.03		0.11±0.01	

The seahorses were reared until 21 DAR (days after male's pouch release), the age at which the newborns undergo important morphological and histological developing changes (Novelli et al., 2015). The newborns were fed twice daily (1-3 prey mL^{-1}) on copepods *Acartia tonsa* retained by 125 µm mesh size (1-5 DAR), a mixture of copepods (*A. tonsa*), filtered by 180 µm mesh and *Artemia* nauplii (6-10 DAR), or *Artemia* nauplii and 24h-enriched metanauplii (1:1) (11-21 DAR). At days 0, 2, 7, 14, and 21 DAR, seahorse newborns were sampled for analytical and biochemical procedures as described below.

Dead seahorses were daily removed (8:00 am and 3:00 pm) from the aquaria and counted. Wastes and uneaten food were removed by siphoning (around 30% total volume) the bottom of the aquaria.

Trial 2: Effect of acidification on seahorse juveniles reared in SW at pH 8.0

This trial was carried out using 21 DAR juveniles produced as performed in trial 1 (SW and pH 8.0). A total of 204 juveniles were transferred to 6 pseudokreisel aquaria (34 juveniles $aquarium^{-1}$; 1.1 juveniles L^{-1}) filled with SW at 6.5 (acidic environment) or 8.0 pH (control) (3 aquaria per pH level). The fishes were reared for 4 weeks and fed on *Artemia* metanuplii enriched for 48, 72 or 96h, depending on the size of seahorses. The feeding schedule was as follows: *Artemia* metanauplii 48-72h for 21-30 DAR, 72-96h metanauplii for 31-45 DAR, and 96 h metanauplii for >45 DAR. The water conditions were monitored and adjusted as described for trial 1, and was showed in table 2. The juveniles were measured and weighed at the start (21 DAR), middle (two weeks; 35 DAR), and end (four weeks; 49 DAR) of the trial.

Table 2: Trial 2 - Water conditions (mean ± standard deviation)for the maintenence of *Hippocampus reidi* juveniles reared in seawater (SW – salinity 33) at pH 6.5 or 8.0 for four weeks (21-49 days after the male's pouch release –DAR).

	pH 6.5	**pH 8**
Salinity (‰)	33±1	
pH	6.6±0.01	8.1±0.0
Alkalinity (mg $CaCO_3$ L^{-1})	21±4[b]	142±7[a]
Temperature (°C)	26.0±0.5	
Oxygen (mg O_2 L^{-1})	6.5±0.5	
TAN (mg N- NH_4^++NH_3 L^{-1})	0.3±0.1	
Nitrite (mg N-NO_2 L^{-1})	0.1±0.05	
Nitrate (mg N-NO_3 L^{-1})	0.2±0.05	

Analytical procedures

Six seahorse juveniles were euthanized by lethal MS-222 immersion (100 mg L^{-1}), weighted (Sartorius, MC210P, Germany), and photographed for length measurements (Lourie et al., 2004) using NIS Elements software (Nikon). The following indices were calculated at 21, 35 and 49 DAR:

- Survival (S, %): (*Final number of fishes /Initial number of fishes) × 100*
- Specific growth rate (SGR, % day^{-1}): $Ln\ w_f - Ln\ w_i /t\ x100$, where w_f and w_i are the final and initial mean weight, and *t* is the experimental time in days.
- Fulton's Factor Condition Index: K = $w_f/l_f^3\ x\ 10$, where w_f and l_f are the final mean weight and length, respectively.

Biochemical analyses

At 0, 2, 7, 14, and 21 DAR, 40, 15, 15, 10, and 10 fishes, respectively were euthanized with MS-222 immersion (100 mg L^{-1}), flash-freeze in liquid nitrogen, and stored (-80°C). Each sample was splitted into two sub-samples. One of the sub-samples comprissing about 70% of the total sampled biomass was homogenized (1:9-w:v) (Poly Tron, PT 2100) in ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v:v) Triton X-100, pH 7.8. After centrifugation (30,000 *g* for 30 min at 4°C) (SIGMA, 3K30), the supernatant was stored at -

80ºC until protein, enzymes, TBARS, and TEAC analyses. The other sub-sample was homogenized in 10 mM HCl and 1.3 % SSA buffer (1:9-w:v). After centrifugation (20,000 *g* for 10 min at 4°C), the supernatant was stored at -80°C until further glutathione content (GSSG and GSH) determination.

All analyses were performed in duplicate at 25±0.5°C in 96-well microplates (UVStar, Greiner Bio-One, Frickenhausen, Germany) in a microplate reader (Bio-Tek PowerWave X, USA). The optimal substrate concentration to measure the maximal specific activity for each enzyme was previously established by preliminary assays. The enzymatic reaction was initiated by the addition of homogenate.

For each enzyme activity, except for SOD, one unit or milliunit is defined as the amount of enzyme required to transform 1 µmol or nmol of substrate/min under the conditions defined for each assay. Soluble protein content was determined using bovine serum albumin as standard (Bradford, 1976) and used to estimate enzyme-specific activity.

Superoxide Dismutase (SOD) was assessed by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals (Mccord and Fridovich, 1969). DT-diaphorase (DTD) activity was measured (Sturve et al., 2005) in the reaction mixture contained DCPIP (2,6-dichlorophenol indophenol) and NADH. The control reaction contained distilled water instead of sample extract. The DTD activity was determined as the difference between sample activity and control read at 600 nm.

Catalase (CAT) activity was achieved by measuring the decrease of H_2O_2 concentration in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 10.6 mM H_2O_2 (freshly prepared) at 240 nm (Aebi, 1984).

Glucose 6-P Dehydrogenase (G6PDH) was carried out according to (Lupiañez et al., 1987) with some modifications (Barroso et al., 1994). The change in absorbance of NADPH at 340 nm was monitored to determine the enzymatic activity, NADP, and glucose-6-phosphate.

Glutathione Peroxidase (GPx) was indirectly measured according to Flohé and Günzler (1984). Using cumene hydroperoxide as substrate and monitoring spectrophotometrically NADPH consumption at 340 nm.

Glutathione Reductase (GR) was assayed according to (Carlberg and Mannervik, 1975) by measuring NADPH oxidation at 340 nm and using glutathione oxidized (GSSG) as substrate.

Glutathione-S Transferase (GST) activity was monitored spectrophotometrically at 340 nm by the formation of glutathione-CDNB-conjugate (Habig, 1974).

Glutathione (GSH) was measured according to Anderson (1985), partially modified by Baker et al. (1990), and adapted to microtiter plate by Vandeputte et al. (1994). In the sample were was measured both tGSH (total glutathione) and GSSG (oxidized glutathione). For GSSG determination, the samples were derivatized by 2-vinylpyridine. The reaction was initiated by quickly adding 40 µL GR per well. The increase of absorbance was monitored at 415 nm, as standards were prepared 0-100 mM GSH for tGSH and 0-8 mM GSSG. GSH levels were calculated by subtracting GSSG values to tGSH. Oxidative stress index (OSI) related to the amount of GSSG concerning tGSH, was calculated as follows:

$$OSI = (2GSSG/tGSH)*100$$

Trolox Equivalent Antioxidant Capacity (TEAC) was assayed by the neutralization made by the extract $ABTS^+$ at 595 nm (Erel, 2004). The value TEAC was expressed as µM equivalent of Trolox (analogous to vitamin E).

Thiobarbituric Acid Reactive Substances (TBARS) were assayed following the protocol by Buege and Aust (1978), considering that the samples utilized in the assay have malondialdehyde (MDA) that react with thiobarbituric acid (TBA). The reading was made in a microplate at 535 nm and using MDA as standard.

Statistical analysis

The data were tested for normality and homoscedasticity using Shapiro Wilk's and Levene's tests, respectively. When those conditions were not accomplished, a Rank transformation was applied (Friedman, 1937). Mean comparisons for salinity levels on survival and growth indices were analyzed by t-test. Biochemical indices were compared using factorial ANOVA, considering pH and age (DAR) as fixed factors. Significant differences in ANOVA were assessed by the Newman-Keuls test. The minimum significance level was set at 5% ($p<0.05$). Data were expressed as mean ± standard deviation. The statistical analyses were performed in Statistica 7.0 software package.

Principal component analyses (PCA) were performed in R v.3.6.1 (R Core Team 2014) to summarize and visualize graphically the results achieved. For that, factoMineR v2.3 (Husson et al., 2020), factoextra v1.0.7 (Kassambara, 2020) and corrplot v0.8.4 (Wei et al., 2017) packages in R were used. Data values were standardized (mean = 0; sd = 1).

Results

Trial 1: Effect of pH and salinity on newborns

Growth

Survivals and growth indices are provided in table 3. Final survivals (21 DAR) were extremely high in both salinity conditions (SW and BW), ranging from 86.9 to 98.9%. The highest survival was achieved at pH 6.5 in SW. Growth did not differ between pH levels in SW. However, length; weight and SGR in BW were significantly higher at pH 8.0 (Table 3).

Table 3: Trial 1 - Effects of pH on survival, growth, and condition factor (mean ± standard deviation) of newborn *Hippocampus reidi* reared in seawater (SW – salinity 33) or brackish water (BW – salinity 11) at pH 6.5 or 8.0 for 21 days (0-21 days after the male's pouch release - DAR). Within salinities, different letters indicate significant difference (P<0.05) (Student's t test).

	SW		***BW***	
	pH 6.5	**pH 8.0**	**pH 6.5**	**pH 8.0**
Final survival (%)	98.9±0.48[a]	96.9±0.96[b]	86.9±2.2[b]	92.2±2.2[a]
Final length (mm)	27.5±0.86	26.9±1.10	19.6±0.2[b]	22.8±0.7[a]
Final weight (mg)	37.8±2.7	35.8±3.5	17.6±0.4[b]	27.9±2.2[a]
SGR (%)	5.1±0.2	5.0±0.1	4.0±0.0[b]	4.8±0.1[a]
K	0.18±0.01	0.18±0.01	0.23±0.03	0.23±0.00

Biochemical oxidative stress indices

Enzymatic activities and biochemical oxidative stress indices were provided in tables 4-5 (SW) and 6-7 (BW). Differences caused by pH were only obtained for GSH (SW and BW) and OSI (BW). Except for SOD and OSI in SW, and SOD, G6PDH (BW), GST and TBARS, all other enzymatic activities and indices were significantly influenced by seahorse age.

Seawater - SW (S33)

SOD activities at pH 6.5 and 8.0 were statistically similar, and remained rather constant thorough the whole experimental period, whereas DTD levels increased with age from 2 DAR (Table 4).

CAT activity decreased in early newborns age (2 DAR) but increased afterwards, achieving the high level on 21 DAR. In addition, the increase in CAT activity at pH 6.5 occurred earlier than at pH 8.0. The G6PDH activity increased slightly and along the age, reaching the highest values in 21 DAR juveniles (Table 4).

Table 4: Trial 1 (SW) - Enzymatic activities (mean ± standard deviation) of *Hippocampus reidi* newborns reared in seawater (SW – salinity 33) at pH 6.5 or 8.0 for 21 days (0-21 DAR). Different letters indicate differences according Newman-Keuls test applied after Two-way ANOVA or One-way ANOVA (in the cases of nor dual effects neither interactions). SNA: Sample not analysed.

		Age (days after the male's pouch release - DAR)					***ANOVA (p)***		
	pH	**0**	**2**	**7**	**14**	**21**	**age**	**pH**	**age*pH**
SOD	6.5	51.5±10.2	51.6±19.9	45.7±1.8	49.9±3.2	47.4±2.5	*0.159*	*0.613*	*0.478*
	8.0		47.0±0.7	58.1±16.4	52.2±2.8	75.1±11.0			
DTD	6.5	3.1±1.0^{ab}	2.7±1.0^{b}	4.2±0.5^{ab}	4.2±0.2^{ab}	4.64±0.3^{a}	0.001	0.788	0.238
	8.0		3.4±0.2^{ab}	4.3±0.5^{ab}	3.1±0.1^{ab}	4.7±0.5^{a}			
CAT	6.5	3.6±0.6^{cd}	2.6±0.7^{d}	4.5±1.4^{c}	7.7±1.2^{ab}	11.4±0.5^{a}	*<0.001*	*0.252*	*0.438*
	8.0		2.5±0.0^{d}	3.6±0.3^{cd}	5.0±1.1^{bc}	12.1±1.5^{a}			
G6PDH	6.5	1.0±0^{b}	1.22±0.3^{b}	SNA	2.0±0.0^{a}	2.7±0.3^{a}	*<0.001*	-	*0.958*
	8.0		1.1±0.1^{b}	1.3±0.1^{b}	SNA	2.6±0.0^{a}			

Gluthation peroxidase (GPx) was not affected by pH level but the activity declined significantly from 0 DAR to 7 DAR. Subsequently, GPx levels increased until the end of the experiment. GR and GST showed a similar pattern, with progressive increases along the experimental period (Table 5).

Regarding glutathione metabolism, the GSH pattern was similar to GR and GST. Nevertheless, the increase in GSH occurred earlier at pH 6.5, leading to 18% high content in 21 DAR. On the other hand, GSSG levels increased from 2 DAR onwards. The relationship between glutathione forms (OSI) remained almost constant along the age (Table 5).

TEAC values were similar across the age at both pH levels (Table 5). Finally, TBARS was not detected in 0 DAR but the values increases with age (Table 5).

Table 5: Trial 1 (SW) - Glutathione metabolism (mean ± standard deviation) in *Hippocampus reidi* newborns reared in seawater (SW – salinity 33) at pH 6.5 or 8.0 for 21 days (0-21 DAR). Different letters indicate differences according Newman-Keuls test applied after Two-way ANOVA or One-way ANOVA (in the cases of nor dual effects neither interactions).

		Age (days after the male's pouch release - DAR)					***ANOVA (p)***		
	pH	**0**	**2**	**7**	**14**	**21**	**age**	**pH**	**age*pH**
GPx	6.5	420±240[ab]	398±45[a]	168±54[c]	227±22[abc]	351±36[ab]	*<0.001*	*0.231*	*0.934*
	8.0		344±53[ab]	129±52[c]	193±10[bc]	342±23[ab]			
GR	6.5	5.5±0.3[c]	5.2±0.8[c]	7.2±0.3[bc]	8.6±0.7[ab]	10.4±0.8[a]	<0.001	0.640	0.901
	8.0		5.2±0.2[c]	7.5±0.8[bc]	7.9±0.9[b]	10.0±1.4[a]			
GST	6.5	5.0±1.2[d]	6.3±0.1[d]	10.6±1.3[cd]	18.1±3.5[b]	17.3±2.3[bc]	<0.001	0.288	0.087
	8.0		5.7±0.1[d]	12.1±4.1[cd]	16.0±0.0[bc]	24.3±3.6[a]			
GSH	6.5	59.1±2.7[ab]	48.4±0.5[b]	57.6±4.4[ab]	68.5±11.2[a]	73.9±3.5[a]	<0.001	0.031	0.303
	8.0		45.7±0.9[b]	56.2±3.4[ab]	58.1±2.6[ab]	60.8±9.6[ab]			
GSSG	6.5	2.6±0.1[ab]	2.3±0.2[b]	3.5±0.3[ab]	3.4±0.1[ab]	3.1±0.4[ab]	0. 006	0.379	0.175
	8.0		2.7±0.0[ab]	2.9±0.7[ab]	3.6±0.8[ab]	3.9±0.2[a]			
OSI	6.5	8.7±0.7	9.7±0.8	12.3±1.9	10.0±1.4	8.4±1.4	0.379	0.095	0.171
	8.0		11.9±0.3	10.6±3.1	12.5±3.3	12.9±2.7			
TEAC	6.5	207±27[a]	183±0.7[ab]	76±43[b]	73±38[b]	99±25[b]	*<0.001*	*0.714*	*0.543*
	8.0		173±32[ab]	88±52[b]	121±47[ab]	76±24[b]			
TBARS	6.5	0.00±0.00[c]	0.02±0.02[bc]	0.06±0.05[bc]	0.18±0.11[ab]	0.14±0.05[ab]	*<0.001*	*0.918*	*0.540*
	8.0		0.0±0.0[c]	0.06±0.03[bc]	0.17±0.09[ab]	0.34±0.2[a]			

Brackish water - BW (S11)

SOD activity was affected by pH level (pH 6.5 > pH 8.0) but not by age. DTD values increases from 0 to 2 DAR but remained stable afterwards (Table 6).

CAT activity increased with age up to 7 DAR, but their raise at pH 6.5 occurred earlier than at pH 8.0 (7 and 14 DAR, respectively). It was not possible to analyse G6PDH activity in 2 DAR at pH 6.5, and no significant differences were found for pH or age changes (Table 6).

Table 6: Trial 1 (BW) - Enzymatic activities (mean ± standard deviation) of *Hippocampus reidi* newborns reared in brackish water (BW – salinity 11) at pH 6.5 or 8.0 for 21 days (0-21 DAR). Different letters indicate differences according Newman-Keuls test applied after Two-way ANOVA or One-way ANOVA (in the cases of nor dual effects neither interactions). SNA: Sample not analysed.

		Age (days after the male's pouch release -DAR)					**ANOVA (*p*)**		
	pH	**0**	**2**	**7**	**14**	**21**	**age**	**pH**	**age*pH**
SOD	6.5	39.6±17.6^{b}	68.5±4.6^{a}	100.7±32.4^{a}	91.3±14.6^{a}	89.3±33.3^{a}	*0.012*	*0.311*	*0.331*
	8.0		68.9±6.8^{a}	62.9±14.4^{a}	71.9±4.5^{a}	101.6±25.8^{a}			
DTD	6.5	1.9±2.1^{b}	4.1±0.6^{a}	4.5±0.3^{a}	4.44±0.6^{a}	4.3±0.1^{a}	*0.019*	*0.245*	*0.775*
	8.0		4.3±0.9^{a}	4.5±0.1^{a}	5.1±0.5^{a}	4.6±0.6^{a}			
CAT	6.5	4.7±0.2^{b}	5.5±1.2^{b}	8.2±2.3^{ab}	11.9±1.0^{a}	10.4±4.7^{ab}	*<0.001*	*0.288*	*0.374*
	8.0		5.0±0.4^{b}	5.3±0.5^{b}	8.8±1.8^{ab}	13.7±4.1^{a}			
G6PDH	6.5	2.0±0.4	SNA	1.8±0.3	1.8±0.5	1.6±0.6	0.147	-	0.110
	8.0		1.3±0.1	1.0±0.3	2.2±0.5	2.2±0.4			

GPx activity was higher at 0 DAR and, significantly decrease until 7 DAR, although its turn to increased afterwards. The GR activities in BW performed similarly to those in SW. There was an increase from 0 to 2 DAR and activities remained similar afterwards. GST did not show differences neither pH nor age (Table 7).

The GSH levels were significantly affected by both age and pH levels. GSH values on 14 and 21 DAR was significantly lower in the newborns kept at pH 6.5 than at pH 8.0. GSSG levels increased according age, reaching the highest values in 21 DAR juveniles. OSI was affected by both age and pH. The OSI values increased from 2 to 21 DAR for the newborn reared at pH 6.5. While, the OSI was almost stable for the newborn reared at pH 8.0 (Table 7). The TEAC were not affected by pH but decreased along the age, and on 21 DAR their value was almost negligible (Table 7). The TBARS was not affected neither by age nor by pH level (Table 7).

Table 7: Trial 1 (BW) - Glutathione metabolism (mean ± standard deviation) in *Hippocampus reidi* newborns reared in brackish water (BW – salinity 11) at pH 6.5 or 8.0 for 21 days (0-21 DAR). Different letters indicate differences according Newman-Keuls test applied after Two-way ANOVA or One-way ANOVA (in the cases of nor dual effects neither interactions). SNA: Sample not analysed.

		Age (days after the male's pouch release - DAR)					***ANOVA (p)***		
	pH	**0**	**2**	**7**	**14**	**21**	**age**	**pH**	**age*pH**
GPx	6.5	486±110^{a}	271±41^{ab}	177±2^{b}	313±21^{ab}	226±72^{b}	<0.001	0.442	0.485
	8.0		256±39^{b}	171±31^{b}	323±128^{ab}	354±59^{ab}			
GR	6.5	7.4±1.8^{B}	7.4±0.9^{B}	10.9±2.8^{AB}	12.6±0.9^{A}	11.3±3.3^{A}	0.008	0.550	0.462
	8.0		7.1±0.3^{B}	8.3±0.6^{AB}	10.7±1.9^{A}	13.4±3.9^{A}			
GST	6.5	13.1±4.3	14.9±4.5	21.0±6.1	21.6±8.2	14.4±3.2	*0.096*	*0.777*	*0.171*
	8.0		15.3±2.1	12.7±2.7	23.2±6.0	19.7±5.7			
GSH	6.5	48±5.5^{ab}	27.3±0.1^{c}	37.2±5.7^{c}	32.9±4.3^{c}	24.0±0.7^{c}	*<0.001*	*<0.001*	*<0.001*
	8.0		31.5±3.3^{c}	32.5±3.8^{c}	56.5±7.8^{a}	56.7±0.3^{a}			
GSSG	6.5	1.1±1.5^{d}	2.5±0.5^{cd}	2.6±0.01^{cd}	2.0±0.01^{d}	4.3±0.4^{a}	<0.001	0.072	0.009
	8.0		2.5±0.2^{cd}	3.2±0.6^{abc}	2.8±0.2^{bc}	3.4±0.3^{ab}			
OSI	6.5	4.7±6.7^{e}	18.2±3.8^{bc}	15.6±1.4^{bc}	13.6±2.0^{bc}	36.0±2.1^{a}	*<0.001*	*0.001*	*<0.001*
	8.0		16.0±0.6^{bc}	19.4±1.6^{b}	10.1±1.0^{de}	11.5±0.6^{cde}			
TEAC	6.5	210±15^{a}	55±53^{abc}	40±22^{abcd}	48±39^{abcd}	2.2±3.8^{cd}	*<0.001*	*0.529*	*0.748*
	8.0		92±0^{ab}	67±48^{ab}	33±23^{bcd}	0±0^{d}			
TBARS	6.5	1.56±1.0	1.21±0.1	0.15±0.2	0.63±0.4	0.44±0.22	*0.081*	*0.164*	*0.300*
	8.0		1.25±0.4	1.26±0.3	0.38±0.3	1.23±0.94			

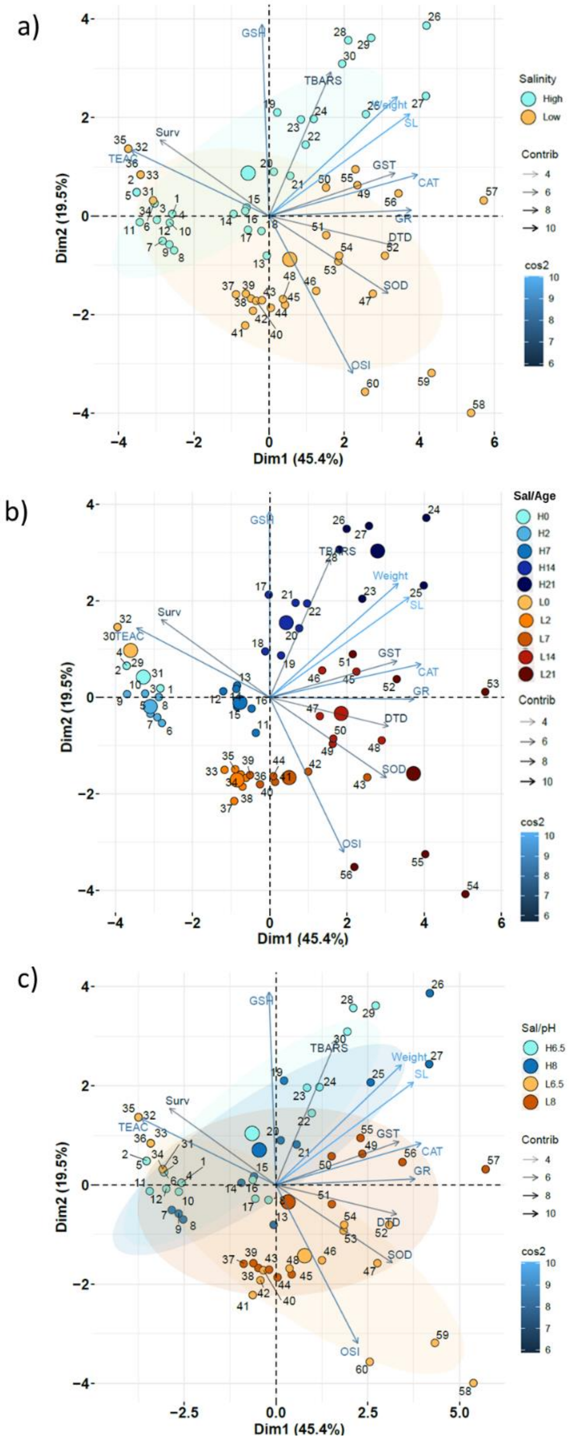
Global assessment: SW and BW

The Principal Component Analysis (PCA) performed on SW and BW samples (Figure 1a) showed significant discrimination of both salinity groups on factors 1-2 representation, which explained 45.4 and 19.5 % of total variability, respectively. Main overall differences between both salinity groups corresponded to OSI, SOD, and DTD (positively associated with BW samples), and TEAC and GSH (positively associated with SW samples) values. Globaly, TBARS and CAT levels were positively associated with age (SL and weight).

The main differences across age and salinity groups (Figure 1b) were due to development (length and weight), TBARS (especially in SW), CAT, and, to a lesser extent, GST. TEAC values were positively associated with early developmental stages, especially in BW groups.

The PCA representation on salinity-pH combinations (Figure 1c) showed smaller differences (smaller centroid distances) with pH in SW treatment compared to BW samples (see also Figure 1a). At low pH, OSI and SOD values were higher in BW samples whereas SW newborns showed higher TEAC and survivals, and lower TBARS values.

Figure 1: Trial 1 - Factor score plots for the Principal Component Analysis (PCA) on seahorse newborns. Analyses performed on a dataset including survival, enzymatic activities, and enzymatic indices. Sample IDs are provided and only the variables with the highest contributions ($cos^2 > 0.5$) are indicated. Ellipses correspond to centroid values ± 1 s.d. (shaded areas). (a): Seawater (SW = High-33) *vs* brackish water (BW = Low-11) groups. (b) All salinity levels (33 and 11) and ages (0 – 21 DAR), and (c): All salinity (SW-33 and BW-11) and pH (6.5 and 8) levels.

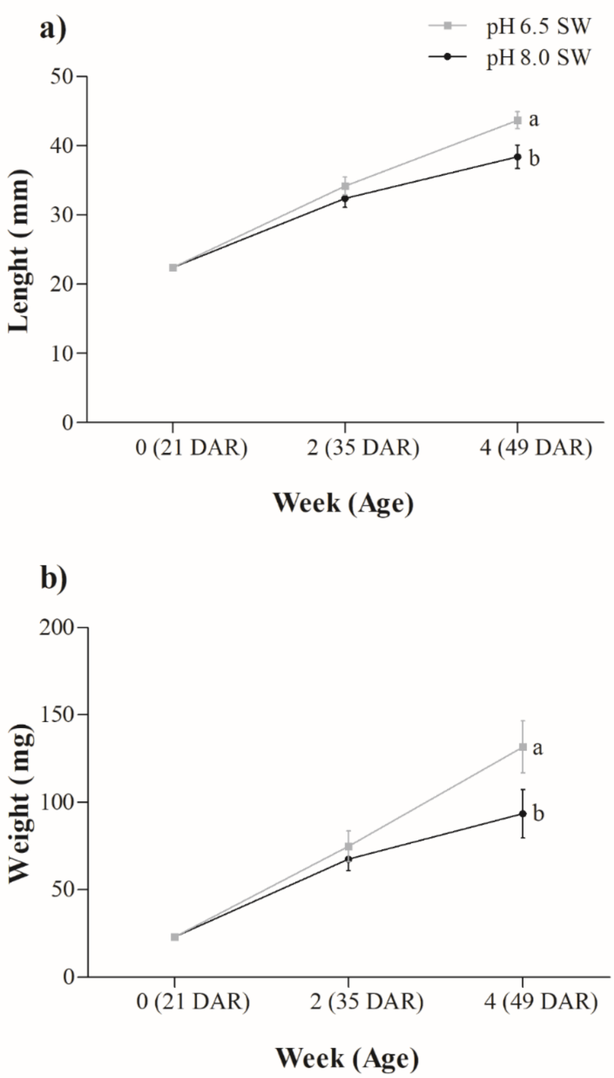


Trial 2: Effect of acidification on juveniles

Growth

The 21 DAR seahorses kept at pH 6.5 grew 12% more in length and 29% in weight than those fish maintained at pH 8.0 (Figure 2). In this sense, fish of pH 6.5 showed higher SGR, values than at pH 8.0. However, the survival of 82.4±10.6 for pH 6.5 and 72.5±7.4 for pH 8.0, as the index condition K, 0.16±0 for pH 6.5 and 0.17±0.02 for pH 8.0, was unaffected after four weeks of trial.

Figure 2: Trial 2 - Growth, (a) length and (b) weight (mean ± standard deviation) in 21 DAR seahorse juveniles, *Hippocampus reidi*, maintained for four weeks in SW at pH 6.5 and 8. Different letters indicate significant differences between pH levels at each age.



Discussion

Fishes can be exposed to significant salinity and pH alterations along their production cycle when facilities are located in unstable coastal areas or as consequence of some management practices (i.e., prophylaxis, disease treatments and transport) (Abreu et al., 2005; Cohen et al., 2018; Huang et al., 2020b; Meira-Filho et al., 2017). The impact of environmental acidification may be detrimental in early developmental stages of fishes (i.e., embryos, larvae, and early juveniles) (Heuer and Grosell, 2014; Ishimatsu et al., 2008, 2004; Lee et al., 2018). In the present study, we assessed the impact of acidic conditions on the overall fitness in newborn seahorses *Hippocampus reidi* reared at seawater (SW) and brackish water (BW), as well in older juveniles kept at SW.

The knowledge of fish response to stressors during lifespan is highly relevant for the understanding of potential physiological alterations in animals inhabiting natural habitats and for the management of *ex-situ* rearing procedures (Birnie-Gauvin et al., 2017; Gressler et al., 2014). Whereas, our global results indicate that survivals and growth were hampered in individuals kept in BW under acidic conditions. As well, opposite to BW, acidic conditions led to higher survivals and growth in newborn and older juveniles grown in seawater (SW), they triggering answers for environmental alterations (climate changes) and aquaculture facilities.

The combined effect of salinity and pH on early developing juveniles

The seahorses born from the same breeding group show similar conditions at male's pouch release (Planas et al., 2021). In captivity conditions, newborn batch size is generally 300-600 individuals (Planas et al., 2021; Randazzo et al., 2018). Since our experiment needs were much higher, the whole experimental design included two subtrials, one for BW and another one for SW. Consequently, comparisons between salinities were not performed using univariate statistics.

Acidic pH conditions were strongly associated with changes in juvenile's fitness. At pH 6.5, the survival resulted enhanced in SW compared to BW. Perhaps, newborn seahorses released at 33 salinity (at which the breeders were kept) must deal with over-challenges to perform satisfactorily the ion regulation process in BW. In spite pregnant seahorses open brooding pouches before newborn release preparing newborns to external salinity conditions (Whittington et al., 2015), the presence of ionocyte cells in *H. reidi* juveniles were only observed beyond 6 DAR (Novelli et al., 2015). In addition, the complete acclimation of ionocytes to salinity changes requires about 2 weeks (Kang et al., 2013).

For instance, cobia, *Rachycentrum canudom* (Rodrigues et al., 2015) and white seabass, *Atractoscion nobilis* (Kwan et al., 2021) face acidic exposure or ocean acidification deal with increasing ionocyties cells and NKA activity, proving the correlation with acid-base and ionic regulation, likewise nitrogen excretion (Mota et al., 2018). Thus, the capacity to deal with acidic condition is linked with physiological effects of BW and acidic pH changes, as occur with older juveniles of the same species acutely exposed to these conditions (Carneiro et al., 2021).

The acidosis compensation in marine fish is principally adjusted by differential regulation of $HCO3^{-}$ and H^{+}– effluxes, which are widely believed to be coupled to the influx of Na^{+} and Cl^{-} (Shrivastava et al., 2019; Tresguerres and Hamilton, 2017). Thus, the acidic pH is a threat likely intensified by the low concentration of Na^{+} and Cl^{-} in BW conditions. This stated is also supported by the results achieved in seabass (*Dicentrarchus labrax)* acclimatized to ocean acidification in BW after dealing with ammonia challenges (Shrivastava et al., 2019). Since the ion regulation and acid-base equilibrium are intrinsic mechanisms, these two alterations together triggered losses in the fitness of *H. reidi* newborns.

Biochemical indices: Seawater - SW (S33) and Brackish water - BW (S11)

In the present study, the growth/age of seahorses played more influence on the oxidative status than pH. For example, G6PDH and GR, showed an age-dependent increase. GSH is restored from GSSG by GR, which uses NADPH as H^{+} donator. The NADPH is produced by G6PDH activity; thus, the G6PDH function is essential to GR activity (Sánchez-Muros et al., 1995). Also, an age-dependent relationship has been reported between GPx and GR activity in fishes in order to maintain the intracellular GSH homeostasis (Gallagher et al., 1992; Otto and Moon, 1996), which is in accordance to our results. In newborn seahorses, the DTD showed the same age-dependent trend than CAT, GPx, GR, and GST, independently of salinity or acidification conditions. DTD activity is related with the reduction of quinones to hydroquinones by means of a transference of two electrons of reduced cofactors, NADH or NADPH (Winston and Di Giulio, 1991). It is likely that, DTD activity avoids the potential increase of free radicals with growth, whereas other enzymes (e.g., CAT, GPx and GST) would act to scavenge undesirable ROS levels. All of the age-dependent responses explained above might linked with food quality and ingest as the proper development.

The oxidative balance could change with fish growth or by environmental condition (Martínez-Álvarez et al., 2005). However, the antioxidant compounds received by the fish from the ingested prey may change with changes in the feeding schedule (Fernández-Díaz et al., 2006; Lushchak, 2016; Olivotto et al., 2011; Randazzo et al., 2018), influencing the oxidative status of juveniles. Moreover, it is known that alterations in the oxidative status are important to determine ontogeny events, as well as for the cell signaling involved in the development of tissues and physiological systems (Costantini, 2019; Ray et al., 2012; Y. Wang et al., 2018). Thereby, age, as well as feeding, and ontogeny changes are pivotal in the initial development of fishes, very likely contributing to support the results achieved in the present study on the oxidative status of seahorse juveniles.

Regarding growth and oxidative status, it is noteworthy that fishes face environmental challenges decrease their aerobic energy generation (G.-Toth et al., 1995; Kamler et al., 1998; Simčič et al., 2015; Sokolova et al., 2012) in order to reduce the production of free radicals (Boveris and Chance, 1973). Those findings and our results are consistent with a potential scenario of metabolism reduction in seahorse juvenile kept at pH 6.5 in BW. Furthermore, the resilient oxidative status revealed in SW at pH 6.5 lead to slight improvements in survival and growth in older juveniles (Trial 2). Even though, salinity changes trigger alterations on the immune system, which is relevant for the oxidative status, survival and growth of fishes (Biller and Takahashi, 2018; Kim et al., 2017; Lin et al., 2020b, 2020a).

Both SOD and CAT are antioxidant enzymes involved in the first line of defense against ROS. The enzyme SOD reduces the anion superoxide ($O_2^{\bullet-}$) to H_2O_2, which is further transformed by CAT into harmless molecules (H_2O and O_2). The unchanged SOD activity of Trial 1 SW and BW, independently of the salinity level, suggests that the alteration of pH played on fish did not increase the production of $O_2^{\bullet-}$ in cellular metabolism. Stable SOD activities have been also reported in flounder (*Paralichthys olivaceus*) larvae (Cui et al., 2020) and oysters (*Crassostrea gigas* and *Crassostrea angulate*) exposed to an acidic environment (Moreira et al., 2016). Additionally, the interaction bettwen H^+ and $O_2^{\bullet-}$ also were a possible way for SOD inactivation (Sampaio et al., 2018).

However, CAT activities showed an age-dependent pattern, increasing with growth, and suggesting that ontogeny is the main factor driving that activity in *H. reidi* juveniles. This statement agrees with the results achieved in the first developmental stages of sturgeon (*Acipenser naccarii*) (Díaz et al., 2010). In the same way within energy metabolism Robergs (2019, 2017) pointed out that many interactions can occur with the H^+ within the rage of pH 6.0 and 7.0. Hence, we suggest that H^+ also improved NADPH production and the stability in SOD and CAT activities that probably was the consequence of a direct interaction of H^+ with $O2^{\bullet-}$ (Sampaio et al., 2018). On the other hand, intense alterations in H^+, and other ions, can lead to mitochondrial hyperpolarization and cell death (Matsuyama and Reed, 2000), which we suggest that occurred in seahorses of pH 6.5 BW.

Acidification did not affect GPx activity in trial 1. However, Cui et al. (2020) registered an increase in that activity in flounder larvae (*Paralichthys olivaceus*) due to acidic water. However, in the present study the pH played alteration on the glutathione (GSH) that increase faster in SW and GSH, GSSG, and OSI in BW ($p < 0.05$). In the oxidative status, for example, to reduce glutathione process (GSSG - GSH), GR spends NADPH, and this process also linked to GST and GPx utilizations (Lushchak, 2016, 2011).

GSH and enzymes related with its metabolism (GPx, GR, and GST) have an important role against ROS increase. We observed that GSH content in BW conditions was lower at pH 6.5 than at pH 8.0 but did not change significantly with juveniles age, as grow. However, in other species such as sturgeon (*A. naccarii*), GSH increased with growth during the free embryo stage and drop-stabilized once juvenile started exogenous feeding (i.e., 21 days after fertilization) (Díaz et al., 2010). Ins important mention that seahorses are fishes undergoing a large and protected ontogeny, in which the embryo are nourished by males until their release (Carcupino et al., 2002; Sommer et al., 2012). Subsequently, newly released juveniles lack yolk sac and show exogenous feeding. These features might explain why GSH content did not change along with the 21 DAR age of juveniles differently that pass with *A. nacarii*.

Oxidative Stress Index (OSI) relates the proportion of GSSG with the total amount of GSH (tGSH), and reveals a status of low stress in seahorses reared in SW, independently of water acidification. On the other hand, pH 6.5 was a stressful situation in seahorses kept at BW. On the other side, OSI levels represent an inverse relation with total antioxidant capacity (TEAC). As mitochondrial respiration, (i.e., ROS production) increased with growth, TEAC levels dropped dramatically in seahorses kept in BW and the oxidation degree (OSI) reached its highest levels by the end of the experimental period (Trial 1).

The yellow seahorse *Hippocampus kuda* showed the similar antioxidant properties independently of their origin (wild or rearing) (Sanaye et al., 2014). In our study, glutathione levels improved with age in juveniles kept at pH 6.5 in SW. Hence, it is feasible that both juveniles and adults kept in acidic environments have a better oxidative status than individuals kept in a natural environment (pH 8.0). Thus, the results of the present study are profitable for seahorses rearing for Chinese traditional medicine uses.

We did not observe differences in TBARS. However, considering the overall alterations, seahorses kept at pH 6.5 BW probably is undergo a metabolic depression. Thereby, this context is associated with the reduction in growth and increase in mortality. For instance, low LPO values were achieved in fishes submitted to stress (Hermes-lima et al., 2015; Maulvault et al., 2018), reinforcing our hypothesis.

Global assessment (PCA)

The multivariate approach (PCA analysis) revealed a global scenery on the acidic effects in juveniles submitted to both SW and BW conditions. In newborn from experiment 1, BW provided low survival and growth compared to SW. This finding disagrees with the optimum growth among 10-20 salinity, previously achieved for the same species (Hora et al., 2016; Tseng et al, 2020).

The differences observed in survivals across trials and treatments might be associated to differences in offspring quality as suggested by some biochemical indices in 0 DAR juveniles (e.g., TBARS = 0±0 and 1.56±1.0 nmol MDA mg protein^{-1} for SW and BW, respectively). However, previous studies reported small differences in newborn quality in *H. reidi* (Planas et al., 2021).

The Principal Component Analysis revealed that juveniles kept in BW were more susceptible to SOD increases, reinforcing the hypothesis of lower resilience in fishes grown under acidic conditions at low salinity. Similarly, SOD activities increased in sand smelt (*Atherina presbyter*) larvae when the environment was slightly acidified but dropped with more drastic acidification (Silva et al., 2016). Since SOD, OSI and DTD were associated to BW, the interactions of these parameters could be responsible of the mortalities and lower growth observed in BW at pH 6.5.

TEAC levels were positively correlated with survival in SW whereas OSI was negatively correlated with survival in BW. Increases in GSH could help in reducing the harmful effect of ROS and intracellular homeostasis maintenance in stressing conditions such as acidic environments (Srikanth et al., 2013). However, the levels of GSH in BW were almost two-fold higher in seahorses reared at pH 8.0 compared to pH 6.5. Hence, even in an estuarine species such as *H. reidi*, acidification conditions accompanied by reduced salinities would be hardly tolerated. This statement was already recognized by Shrivastava et al. (2019) in *D. labrax*.

Concerning fish damage, TBARS levels were correlated with age/growth. Similar results were reported in *D. labrax* larvae by Malvault et al. (2018), who observed increases in malondialdehyde (MDA) in warm and acidification conditions, accompanied by a growth improvement. However, we found that juvenile in SW showed lower TBARS values than those in BW and acidic conditions. It is likely that survivals in the former higher be enhanced by higher TEAC and GSH levels in SW.

Trial 2: Effect of pH on growth of seahorse juveniles

Since age and size are intrinsic factors to consider for the understanding environmental stress in fishes (Almroth et al., 2010; Barcellos et al., 2012), we also performed a second experiment in older juveniles. Given that trial 1 revealed lower resilience in juveniles to acidic conditions in BW, the second trial was carried out only under a SW environment.

Explanations supporting growth improvement in juveniles kept at pH 6.5 in SW should consider parasitology and energy generation. About the former, the effect of acidic conditions on ectoparasites is well documented. Ectoparasite growth is impaired under acidic conditions. Consequently, parasitology threats would be reduced in seahorses reread at low pH (Halmetoja et al., 2000; Meira-Filho et al., 2017).

Regarding cell energy, pH levels may alter the matrix membrane potential in mitochondria, which may enhance ATP-synthase function (Jain and Nath, 2000) and generate more energy (ATP). It is important to highlight that G6PDH activity is linked to growth factors (Stanton, 2012). Although enzymatic analyses were not performed in this trial, the age increasing results achieved in trial 1 to G6PDH activity suggest that the growth would be enhanced in older juveniles reared under acidic conditions (second trial). This assumption agrees with the growth increase induced at pH 6.3 in the turbot *Psetta maxima*, while compared to upper pH levels tested (up to pH 8.8) (Wang et al., 2018). In addition, H^+ is involved in aerobic and non-aerobic energy metabolism, affecting mitochondrial proton leak and interacting with metabolites of non-mitochondrial energy generation (Kuno et al., 2016; Robergs, 2017), which can trigger benefits for cell energy and oxidative status.

Conclusion

In summary, the rearing of *H. reidi* juveniles held in low salinity (BW) caused SOD, DTD, OSI increases, and TEAC consumption whereas both survival and growth were hampered at pH 6.5. Consequently, our results indicate that pH oscillations should be avoided in rearing performed in BW, since seahorses showed low resilience to acidic conditions. We suggest that *Hippocampus reidi* juveniles be reared in SW at acidic conditions (pH 6.5), which increased survival and growth.

References

Abreu, P. c, Robaldo, R.B., Sampaio, L.A., Bianchini, A., Odebrecht, C., 2005. Recurrent Amyloodiniosis on Broodstock of the Brazilian Flounder *Paralichthys orbignyunus*: Dinospore Monitoring and Prophylactic Measures. J. World Aquac. Soc. 36, 42–50.

Aebi, H., 1984. [13] Catalase in vitro, in: Methods in Enzymology. Academic Press, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3.

Almroth, B.C., Johansson, A., Förlin, L., Sturve, J., 2010. Early-age changes in oxidative stress in brown trout , *Salmo trutta*. Comp. Biochem. Physiol. Part B 155, 442–448. https://doi.org/10.1016/j.cbpb.2010.01.012

Anderson, M.E., 1985. [70] Determination of glutathione and glutathione disulfide in biological samples. In: Methods in Enzymology. Academic Press, pp. 548–555. https://doi.org/10.1016/S0076-6879(85)13073-9.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem. 190, 360–365. https://doi.org/10.1016/0003-2697(90)90208-Q

Barcellos, J.L.G., Kreutz, L.C., Koakoski, G., Oliveira, T.A., Rosa, J.G.S. da, Fagundes, M., 2012. Fish age , instead of weight and size , as a determining factor for time course differences in cortisol response to stress. Physiol. Behav. 107, 397–400. https://doi.org/10.1016/j.physbeh.2012.09.008

Barroso, J.B., García-Salguero, L., Peragón, J., de la Higuera, M., Lupiáñez, J.A., 1994. The influence of dietary protein on the kinetics of NADPH production systems in various tissues of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 124, 47–59. https://doi.org/10.1016/0044-8486(94)90353-0

Biller, J.D., Takahashi, L.S., 2018. Oxidative stress and fish immune system: phagocytosis and leukocyte respiratory burst activity. An. Acad. Bras. Cienc. 90, 3403–3414. https://doi.org/10.1590/0001-3765201820170730

Birnie-Gauvin, K., Costantini, D., Cooke, S.J., Willmore, W.G., 2017. A comparative and evolutionary approach to oxidative stress in fish: A review. Fish Fish. 18, 928–942. https://doi.org/10.1111/faf.12215

Blanco, A., Chamorro, A., Planas, M., 2014. Implications of physical key factors in the early rearing of the long-snouted seahorse *Hippocampus guttulatus*. Aquaculture 433, 214–222. https://doi.org/10.1016/j.aquaculture.2014.06.019

Boveris, A., Chance, B., 1973. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. 134, 707–716. https://doi.org/10.1042/bj1340707

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.

Calado, R., 2017. Location, in: Marine Ornamental Species Aquaculture. Wiley, pp. 75–79. https://doi.org/10.1002/9781119169147.ch5

Carcupino, M., Baldacci, A., Mazzini, M., Franzoi, P., 2002. Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: A morphological and ultrastructural comparative study on three anatomically different pouches. J. Fish Biol. 61, 1465–1480. https://doi.org/10.1111/j.1095-8649.2002.tb02490.x

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480.

Cohen, F.P., Valenti, W.C., Calado, R., 2013. Traceability Issues in the Trade of Marine Ornamental Species. Rev. Fish. Sci. 21, 98–111. https://doi.org/10.1080/10641262.2012.760522

Cohen, F.P.A., Planas, M., Valenti, W.C., Lillebø, A., Calado, R., 2018. Optimizing packing of live seahorses for shipping. Aquaculture 482, 57–64. https://doi.org/10.1016/J.AQUACULTURE.2017.09.024

Cohen, F.P.A., Valenti, W.C., Planas, M., Calado, R., 2017. Seahorse Aquaculture, Biology and Conservation: Knowledge Gaps and Research Opportunities. Rev. Fish. Sci. Aquac. 25, 100–111. https://doi.org/10.1080/23308249.2016.1237469

Copatti, C.E., Baldisserotto, B., Souza, C. de F., Monserrat, J.M., Garcia, L., 2019. Water pH and hardness alter ATPases and oxidative stress in the gills and kidney of pacu (*Piaractus mesopotamicus*). Neotrop. Ichthyol. 17, 1–10. https://doi.org/10.1590/1982-0224-20190032

Costantini, D., 2019. Understanding diversity in oxidative status and oxidative stress: The opportunities and challenges ahead. J. Exp. Biol. 222. https://doi.org/10.1242/jeb.194688

Cui, W., Cao, L., Liu, J., Ren, Z., Zhao, B., Dou, S., 2020. Effects of seawater acidification and cadmium on the antioxidant defense of flounder *Paralichthys olivaceus* larvae. Sci. Total Environ. 718. https://doi.org/10.1016/j.scitotenv.2020.137234

Díaz, M.E., Furné, M., Trenzado, C.E., García-Gallego, M., Domezain, A., Sanz, A., 2010. Antioxidant defences in the first life phases of the sturgeon *Acipenser naccarii*. Aquaculture 307, 123–129. https://doi.org/10.1016/j.aquaculture.2010.06.026

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358. https://doi.org/10.1016/j.aquaculture.2006.03.019

Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem. 37, 277–285. https://doi.org/10.1016/j.clinbiochem.2003.11.015

Faleiro, F., Baptista, M., Santos, C., Aurélio, M.L., Pimentel, M., Pegado, M.R., Paula, J.R., Calado, R., Repolho, T., Rosa, R., S, S., D, Q., M, Manning, Z, C., M, Marquis, KB, A., M, T., HL, M., 2015. Seahorses under a changing ocean: the impact of warming and acidification on the behaviour and physiology of a poor-swimming bony-armoured fish. Conserv. Physiol. 3, 1–7. https://doi.org/10.1093/conphys/cov009

Fernández-Díaz, C., Kopecka, J., Cañavate, J.P., Sarasquete, C., Solé, M., 2006. Variations on development and stress defences in *Solea senegalensis* larvae fed on live and microencapsulated diets. Aquaculture 251, 573–584. https://doi.org/10.1016/j.aquaculture.2005.06.014

Flohé, L., Günzler, W.A., 1984. Assays of Glutathione Peroxidase. Methods Enzymol. 105, 114–120.

Fonseca, T., David, F.S., Ribeiro, F.A.S., Wainberg, A.A., 2015. Technical and economic feasibility of integrating seahorse culture in shrimp / oyster farms 1–10. https://doi.org/10.1111/are.12912

Foster, S., Wiswedel, S., Vincent, A., 2016. Opportunities and challenges for analysis of wildlife trade using CITES data - seahorses as a case study. Aquat. Conserv. Mar. Freshw. Ecosyst. 26, 154–172. https://doi.org/10.1002/aqc.2493

Foster, S.J., Kuo, T.C., Wan, A.K.Y., Vincent, A.C.J., 2019. Global seahorse trade defies export bans under CITES action and national legislation. Mar. Policy. https://doi.org/10.1016/j.marpol.2019.01.014

Friedman, M., 1937. The Use of Ranks to Avoid the Assumption of Normality Implicit in the Analysis of Variance. J. Am. Stat. Assoc. 32, 675–701. https://doi.org/10.1080/01621459.1937.10503522

G.-Toth, L., Szabo, M., Webb, D.J., 1995. Adaptation of the tetrazolium reduction test for the measurement of the electron transport system (ETS) activity during embryonic development of medaka. J. Fish Biol. 46, 835–844. https://doi.org/10.1006/jfbi.1995.0076

Gallagher, E.P., Hasspieler, B.M., Di Glulio, R.T., 1992. Effects of buthionine sulfoximine and diethyl maleate on glutathione turnover in the channel catfish. Biochem. Pharmacol. 43, 2209–2215. https://doi.org/10.1016/0006-2952(92)90180-Q

Gressler, L.T., Riffel, A.P.K., Parodi, T.V., Saccol, E.M.H., Koakoski, G., da Costa, S.T., Pavanato, M.A., Heinzmann, B.M., Caron, B., Schmidt, D., Llesuy, S.F., Barcellos, L.J.G., Baldisserotto, B., 2014. Silver catfish *Rhamdia quelen* immersion anaesthesia with essential oil of *Aloysia triphylla* (L'Hérit) Britton or tricaine methanesulfonate: effect on stress response and antioxidant status. Aquac. Res. 45, 1061–1072. https://doi.org/10.1111/are.12043

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Halliwell, B., Gutteridge, J.M.C., 2015. Free Radicals in Biology and Medicine. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780198717478.001.0001

Halmetoja, A., Valtonen, E.T., Koskenniemi, E., 2000. Perch (*Perca fluviatilis* L .) parasites reflect ecosystem conditions : a comparison of a natural lake and two acidic reservoirs in Finland. Int. J. Parasitol. 30, 1437–1444.

Hermes-lima, M., Moreira, D.C., Rivera-ingraham, G.A., Giraud-billoud, M., Genaro-mattos, T.C., Campos, É.G., 2015. Preparation for oxidative stress under hypoxia and metabolic depression : Revisiting the proposal two decades later. Free Radic. Biol. Med. 89, 1122–1143.

Heuer, R.M., Grosell, M., 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. Am. J. Physiol. Integr. Comp. Physiol. 307, R1061–R1084. https://doi.org/10.1152/ajpregu.00064.2014

Hora, M. dos S.C. da, Joyeux, J., 2009. Closing the reproductive cycle : Growth of the seahorse *Hippocampus reidi* (Teleostei , Syngnathidae) from birth to adulthood under experimental conditions. Aquaculture 292, 37–41. https://doi.org/10.1016/j.aquaculture.2009.03.023

Hora, M. dos S.C. da, Joyeux, J.-C., Rodrigues, R.V., Sousa-Santos, L.P. de, Gomes, L.C., Tsuzuki, M.Y., 2016. Tolerance and growth of the longsnout seahorse *Hippocampus reidi* at different salinities. Aquaculture 463, 1–6. https://doi.org/10.1016/j.aquaculture.2016.05.003

Huang, J., Qin, G., Zhang, B., Tan, S., Sun, J., Lin, Q., 2020a. wrong Effects of food, salinity, and ammonia-nitrogen on the physiology of juvenile seahorse (*Hippocampus erectus*) in two typical culture models in China. Aquaculture 118214. https://doi.org/10.1016/j.apcatb.2019.118214

Huang, J., Qin, G., Zhang, B., Tan, S., Sun, J., Lin, Q., 2020b. Effects of food, salinity, and ammonia-nitrogen on the physiology of juvenile seahorse (*Hippocampus erectus*) in two typical culture models in China. Aquaculture 520, 734965. https://doi.org/10.1016/j.aquaculture.2020.734965

Ishimatsu, A., Hayashi, M., Kikkawa, T., 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. 373, 295–302. https://doi.org/10.3354/meps07823

Ishimatsu, A., Kikkawa, T., Hayashi, M., Lee, K.-S., Kita, J., 2004. Effects of CO_2 on Marine Fish: Larvae and Adults. J. Oceanogr. 60, 731–741. https://doi.org/10.1007/s10872-004-5765-y

Jain, S., Nath, S., 2000. Kinetic model of ATP synthase: pH dependence of the rate of ATP synthesis. FEBS Lett. 476, 113–117. https://doi.org/10.1016/S0014-5793(00)01716-6

Kalaimani, N., Chakravarthy, N., Shanmugham, R., Thirunavukkarasu, A.R., Alavandi, S. V., Santiago, T.C., 2008. Anti-oxidant status in embryonic, post-hatch and larval stages of Asian seabass (*Lates calcarifer*). Fish Physiol. Biochem. 34, 151–158. https://doi.org/10.1007/s10695-007-9155-4

Kamler, E., Keckei, H., Bauer-Nemeschkal, E., 1998. Temperature-induced changes of survival, development and yolk partitioning in *Chondrostoma nasus*. J. Fish Biol. 53, 658–682. https://doi.org/10.1111/j.1095-8649.1998.tb01009.x

Kang, C.-K., Yang, W.-K., Lin, S.-T., Liu, C.-C., Lin, H.-M., Chen, H.-H., Cheng, C.-W., Lee, T.-H., Hwang, P.-P., 2013. The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 181–91. https://doi.org/10.1016/j.cbpa.2012.08.010

Kassambara, A., 2020b. factoextra: Extract and visualize the results of multivariate data analyses. https://CRAN.R-project.org/package=factoextra [Accessed February 23, 2020].

Kikkawa, T., Kita, J., Ishimatsu, A., 2004. Comparison of the lethal effect of CO 2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Mar. Pollut. Bull. 48, 108–110. https://doi.org/10.1016/S0025-326X(03)00367-9

Kim, J., Park, H., Kim, K., 2017. Growth performance , oxidative stress , and non-specific immune responses in juvenile sablefish, *Anoplopoma fimbria* , by changes of water temperature and salinity. Fish Physiol. Biochem. 1421–1431. https://doi.org/10.1007/s10695-017-0382-z

Koldewey, H.J., Martin-Smith, K.M., 2010. A global review of seahorse aquaculture. Aquaculture 302, 131–152. https://doi.org/10.1016/j.aquaculture.2009.11.010

Kuo, T.C., Vincent, A., 2018. Assessing the changes in international trade of marine fishes under CITES regulations – A case study of seahorses. Mar. Policy 88, 48–57. https://doi.org/10.1016/j.marpol.2017.10.031

Kwan, G.T., Shen, S.G., Drawbridge, M., Checkley, D.M., Tresguerres, M., 2021. Ion-transporting capacity and aerobic respiration of larval white seabass (*Atractoscion nobilis*) may be resilient to ocean acidification conditions. Sci. Total Environ. 791, 148285. https://doi.org/10.1016/j.scitotenv.2021.148285

Lee, C., Kwon, B.-O., Hong, S., Noh, J., Lee, J., Ryu, J., Kang, S.-G., Khim, J.S., 2018. Sub-lethal and lethal toxicities of elevated CO_2 on embryonic, juvenile, and adult stages of marine medaka *Oryzias melastigma*. Environ. Pollut. 241, 586–595. https://doi.org/10.1016/j.envpol.2018.05.091

Lemos, C.H. da P., Ribeiro, C.V.D.M., de Oliveira, C.P.B., Couto, R.D., Copatti, C.E., 2018. Effects of interaction between pH and stocking density on the growth, haematological and biochemical responses of Nile tilapia juveniles. Aquaculture 495, 62–67. https://doi.org/10.1016/j.aquaculture.2018.05.037

Lin, G., Zheng, M., Gao, D., Li, S., Fang, W., Huang, J., Xie, J., Liu, J., Liu, Y., Li, Z., Lu, J., 2020a. Hypoosmotic stress induced tissue-specific immune responses of yellow fi n seabream (*Acanthopagrus latus*) revealed by transcriptomic analysis. Fish Shellfish

Immunol. 99, 473–482. https://doi.org/10.1016/j.fsi.2020.02.028

Lin, G., Zheng, M., Li, S., Xie, J., Fang, W., Gao, D., Huang, J., Lu, J., 2020b. Response of gut microbiota and immune function to hypoosmotic stress in the yellowfin seabream (*Acanthopagrus latus*). Sci. Total Environ. 745, 140976. https://doi.org/10.1016/j.scitotenv.2020.140976

Lourie, S.A., Foster, S.J., Cooper, E.W.T., Vincent, A.C.J., 2004. A Guide to the Identification of Seahorses. University of British Columbia and World Wildlife Fund. Cover, Washington D.C.

Lupiañez, J.A., Adroher, F.J., Vargas, A.M., Osuna, A., 1987. Differential behaviour of glucose 6-phosphate dehydrogenase in two morphological forms of *Trypanosoma cruzi*. Int. J. Biochem. 19, 1085–1089. https://doi.org/10.1016/0020-711X(87)90310-7

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish : a mechanistic approach. Fish Physiol. Biochem. 42, 711–747. https://doi.org/10.1007/s10695-015-0171-5

Martínez-Álvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant Defenses in Fish: Biotic and Abiotic Factors. Rev. Fish Biol. Fish. 15, 75–88. https://doi.org/10.1007/s11160-005-7846-4

Martinez-Cardenas, L., Purser, J.G., 2016. Effect of Direct Transfer to Different Salinities on Early Juvenile Pot-bellied Seahorse, *Hippocampus abdominalis* , Survival in Culture Conditions. J. World Aquac. Soc. 47, 201–206. https://doi.org/10.1111/jwas.12268

Matsuyama, S., Reed, J.C., 2000. Mitochondria-dependent apoptosis and cellular pH regulation. Cell Death Differ. 7, 1155–1165. https://doi.org/10.1038/sj.cdd.4400779

Maulvault, A.L., Barbosa, V., Alves, R., Anacleto, P., Camacho, C., Cunha, S., Fernandes, J.O., Ferreira, P.P., Rosa, R., Marques, A., Diniz, M., 2018. Integrated multi-biomarker responses of juvenile seabass to diclofenac, warming and acidification co-exposure. Aquat. Toxicol. 202, 65–79. https://doi.org/10.1016/j.aquatox.2018.06.016

Mccord, J., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.

Meira-Filho, M.R.C., Ramirez, J.R.B., Vianna, R.T., Júnior, J.P., 2017. Efficacy of glacial acetic acid in the control of *Trichodina sp*. and *Apiosoma sp*. associated with *Mugil liza*.

Aquaculture 479, 7–12. https://doi.org/10.1016/j.aquaculture.2017.05.014

Moreira, A., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. The effects of arsenic and seawater acidification on antioxidant and biomineralization responses in two closely related *Crassostrea* species. Sci. Total Environ. 545–546, 569–581. https://doi.org/10.1016/j.scitotenv.2015.12.029

Mota, V.C., Hop, J., Sampaio, L.A., Heinsbroek, L.T.N., Verdegem, M.C.J., Eding, E.H., Verreth, J.A.J., 2018. The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac. Res. 49, 3456–3467. https://doi.org/10.1111/are.13812

Mourente, G., Tocher, D.R., Diaz, E., Grau, A., Pastor, E., 1999. Relationships between antioxidants, antioxidant enzyme activities and lipid peroxidation products during early development in *Dentex dentex* eggs and larvae. Aquaculture 179, 309–324. https://doi.org/10.1016/S0044-8486(99)00167-2

Novelli, B., Socorro, J.A., Caballero, M.J., Otero-Ferrer, F., Segade-Botella, A., Domínguez, L.M., 2015. Development of seahorse (*Hippocampus reidi*, Ginsburg 1933): histological and histochemical study. Fish Physiol. Biochem. 41, 1233–1251. https://doi.org/10.1007/s10695-015-0082-5

Olivotto, I., Avella, M.A., Sampaolesi, G., Piccinetti, C.C., Ruiz, P.N., Carnevali, O., 2008. Breeding and rearing the longsnout seahorse *Hippocampus reidi* : Rearing and feeding studies. Aquaculture 283, 92–96. https://doi.org/10.1016/j.aquaculture.2008.06.018

Olivotto, I., Di Stefano, M., Rosetti, S., Cossignani, L., Pugnaloni, A., Giantomassi, F., Carnevali, O., 2011. Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larval development and metamorphosis: Molecular and biochemical implications. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 159, 207–218. https://doi.org/10.1016/j.cbpa.2011.02.004

Otto, D.M.E., Moon, T.W., 1996. Endogenous antioxidant systems of two teleost fish, the rainbow trout and the black bullhead, and the effect of age. Fish Physiol. Biochem. 15, 349–358. https://doi.org/10.1007/BF02112362

Park, M.S., Shin, H.S., Choi, C.Y., Na Kim, N., Park, D.-W., Kil, G.-S., Lee, J., 2011. Effect of hypoosmotic and thermal stress on gene expression and the activity of antioxidant enzymes in the cinnamon clownfish, *Amphiprion melanopus*. Animal Cells Syst. (Seoul). 15, 219–225. https://doi.org/10.1080/19768354.2011.604941

Pellegrin, L., Nitz, L.F., Maltez, L.C., Copatti, C.E., Garcia, L., 2019. Alkaline water improves the growth and antioxidant responses of pacu juveniles (*Piaractus mesopotamicus*). Aquaculture 734713. https://doi.org/10.1016/j.aquaculture.2019.734713

Planas, M., Burhans, R., Simões, N., 2017a. Seahorses and Pipefish, in: Marine Ornamental Species Aquaculture. Wiley, pp. 299–326. https://doi.org/10.1002/9781119169147.ch17

Planas, M., Chamorro, A., Quintas, P., Vilar, A., 2008. Establishment and maintenance of threatened long-snouted seahorse, *Hippocampus guttulatus*, broodstock in captivity. Aquaculture 283, 19–28. https://doi.org/10.1016/j.aquaculture.2008.06.023

Planas, M., Olivotto, I., González, M.J., Laurà, R., Angeletti, C., Amici, A., Zarantoniello, M., 2021. Pre-breeding Diets in the Seahorse *Hippocampus reidi*: How Do They Affect Fatty Acid Profiles, Energetic Status and Histological Features in Newborn? Front. Mar. Sci. 8. https://doi.org/10.3389/fmars.2021.688058

Planas, M., Silva, C., Quintas, P., Chamorro, A., Piñero, S., 2017b. Ongrowing and enhancement of n-3 HUFA profile in adult Artemia: short- vs long-time enrichment. J. Appl. Phycol. 29, 1409–1420. https://doi.org/10.1007/s10811-016-1016-z

R Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org [Accessed February 23, 2018].

Randazzo, B., Rolla, L., Ofelio, C., Planas, M., Gioacchini, G., Vargas, A., Giorgini, E., Olivotto, I., 2018. The influence of diet on the early development of two seahorse species (*H. guttulatus* and *H. reidi*): Traditional and innovative approaches. Aquaculture 490, 75–90. https://doi.org/10.1016/j.aquaculture.2018.02.029

Ray, P.D., Huang, B.W., Tsuji, Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell. Signal. 24, 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008

Regoli, F., Nigro, M., Benedetti, M., Fattorini, D., Gorbi, S., 2005. Antioxidant efficiency in early life stages of the Antarctic silverfish, *Pleuragramma antarcticum*: Responsiveness to pro-oxidant conditions of platelet ice and chemical exposure. Aquat. Toxicol. 75, 43–52. https://doi.org/10.1016/j.aquatox.2005.07.003

Robergs, R.A., 2017. Competitive cation binding computations of proton balance for reactions of the phosphagen and glycolytic energy systems within skeletal muscle. PLoS One 1–24. https://doi.org/https://doi.org/10.1371/journal.pone.0189822

Robergs, R.A., 2019. Invited review: Quantifying proton exchange from chemical reactions - Implications for the biochemistry of metabolic acidosis. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 235, 29–45. https://doi.org/10.1016/j.cbpa.2019.04.024

Rodrigues, R.V., dos Santos Pedron, J., Romano, L.A., Tesser, M.B., Sampaio, L.A., 2015. Acute responses of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) to acid stress. Aquac. Res. 46, 1241–1247. https://doi.org/10.1111/are.12282

Sampaio, E., Lopes, A.R., Francisco, S., Paula, J.R., Pimentel, M., Maulvault, A.L., Repolho, T., Grilo, T.F., Pousão-Ferreira, P., Marques, A., Rosa, R., 2018. Ocean acidification dampens physiological stress response to warming and contamination in a commercially-important fish (*Argyrosomus regius*). Sci. Total Environ. 618, 388–398. https://doi.org/10.1016/j.scitotenv.2017.11.059

Sanaye, S. V., Pise, N.M., Pawar, A.P., Parab, P.P., Sreepada, R.A., Pawar, H.B., Revankar, A.D., 2014. Evaluation of antioxidant activities in captive-bred cultured yellow seahorse, *Hippocampus kuda* (Bleeker, 1852). Aquaculture 434, 100–107. https://doi.org/10.1016/j.aquaculture.2014.08.007

Sánchez-Muros, M.J., García-Rejón, L., Lupiáñez, J.A., De la Higuera, M., 1995. Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout, *Oncorhynchus mykiss* (Walbaum): adaptive response to a high-protein/non-carbohydrate diet and starvation of glucose 6-phosphate dehydrogenase activity. Aquac. Nutr. 1, 213–220. https://doi.org/10.1111/j.1365-2095.1995.tb00046.x

Sanz, A., López-rodríguez, M.J., García-mesa, S., Trenzado, C.E., Ferrer, R.M., Figueroa, J.M.T.D.E., 2017. Are antioxidant capacity and oxidative damage related to biological and autecological characteristics in aquatic insects? J. Limnol. 76, 170–181. https://doi.org/10.4081/jlimnol.2016.1581

Shrivastava, J., Ndugwa, M., Caneos, W., De Boeck, G., 2019. Physiological trade-offs, acid-base balance and ion-osmoregulatory plasticity in European sea bass (*Dicentrarchus labrax*) juveniles under complex scenarios of salinity variation, ocean acidification and high ammonia challenge. Aquat. Toxicol. 212, 54–69. https://doi.org/10.1016/j.aquatox.2019.04.024

Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability , development and biochemical responses of sand smelt larvae. Sci. Total Environ. 563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091

Simčič, T., Jesenšek, D., Brancelj, A., 2015. Metabolic Potential, Respiration Rate and Their Relationship in Offspring of Different Sizes of Marble Trout (*Salmo marmoratus* Cuvier). Turkish J. Fish. Aquat. Sci. 15, 39–48. https://doi.org/10.4194/1303-2712-v15

Sokolova, I.M., 2013. Energy-Limited Tolerance to Stress as a Conceptual Framework to Integrate the Effects of Multiple Stressors. Integr. Comp. Biol. 53, 597–608. https://doi.org/10.1093/icb/ict028

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Solorzano, L., 1969. Determination of Ammonia in Natural Waters by the Phenolhypochlorite. Method. Limnol. Oceanogr. 14, 799–801.

Sommer, S., Whittington, C.M., Wilson, A.B., 2012. Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family Syngnathidae). BMC Dev. Biol. 12, 12–15. https://doi.org/10.1186/1471-213X-12-39

Srikanth, K., Pereira, E., Duarte, A.C., Ahmad, I., 2013. Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish—a review. Environ. Sci. Pollut. Res. 20, 2133–2149. https://doi.org/10.1007/s11356-012-1459-y

Stanton, R.C., 2012. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. IUBMB Life 64, 362–369. https://doi.org/10.1002/iub.1017

Stoliar, O.B., Lushchak, V.I., 2012. Environmental Pollution and Oxidative Stress in Fish, in: Lushchak, V. (Ed.), Oxidative Stress - Environmental Induction and Dietary Antioxidants. InTech, pp. 131–166.

Sturve, J., Stephensen, E., Förlin, L., 2005. Effects of redox cycling on DT diaphorase activity in the liver of rainbow trout (*Oncorhynchus mykiss*). Comp. Hepatol. 8, 1–8. https://doi.org/10.1186/1476-5926-4-4

Tlusty, M., 2002. The benefits and risks of aquacultural production for the aquarium trade.

Aquaculture 205, 203–219. https://doi.org/10.1016/S0044-8486(01)00683-4

Tresguerres, M., Hamilton, T.J., 2017. Acid-base physiology, neurobiology and behaviour in relation to CO_2-induced ocean acidification. J. Exp. Biol. 220, 2136–2148. https://doi.org/10.1242/jeb.144113

Tseng, C., Hun, J., Chu, C.T., Liu, Y.S.T.H.C., 2020. Effects of food type , temperature and salinity on the growth performance and antioxidant status of the longsnout seahorse , *Hippocampus reidi*. Aquac. Res. 4793–4804. https://doi.org/10.1111/are.14826

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biol. Toxicol. 10, 415–421. https://doi.org/10.1007/BF00755791

Wang, S., Jiang, Z., Mao, M., Mao, S., Sun, Y., Sui, Y., 2018. Effects of seawater pH on survival, growth, energy budget and oxidative stress parameters of juvenile turbot *Scophthalmus maximus*. Iran. J. Fish. Sci. 17, 675–689. https://doi.org/10.22092/ijfs.2018.116814

Wang, Y., Branicky, R., Noë, A., Hekimi, S., 2018. Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J. Cell Biol. jcb.201708007. https://doi.org/10.1083/jcb.201708007

Wei, T., Simko, V., Levy, M., Xie, Y., Jin, Y., Zemla, J., 2017. corrplot: Visualization of a correlation matrix. https://CRAN.R-project.org/package=corrplot [Accessed March 6, 2020].

Whittington, C.M., Griffith, O.W., Qi, W., Thompson, M.B., Wilson, A.B., 2015. Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. Mol. Biol. Evol. 32, 3114–3131. https://doi.org/10.1093/molbev/msv177

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137–161. https://doi.org/10.1016/0166-445X(91)90033-6

8. CAPÍTULO 5: Chronic effects of pH and salinity on juvenile orange clownfish *Amphiprion percula*: Survival, growth, coloration, and oxidative status

Artigo formatado para a revista Aquaculture.

Authors: M.D. Dias Carneiro[a,b*], A. F. F de Medeiros[a], F. M. Weber[a], R. V. Rodrigues[a], J. M. Monserrat[c,d], M. Planas[b], L. A. Sampaio [a].

[a] Laboratório de Piscicultura Estuarina e Marinha, Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil

[b] Department of Ecology and Marine Resources, Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain

[c] Laboratório de Bioquímica Funcional de Organismos Aquáticos (BIFOA), Instituto de Oceanografia, Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil

[d] Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande – FURG, Rio Grande, RS, Brazil

[*]Author for correspondence (mariioiddc@gmail.com)

Abstract

Recirculating aquaculture system (RAS) are frequently used in the production of ornamental fish in captivity. For RAS located far away from the coast reduced salinity can be an economical option. However, in RAS acidification of the water can occur. Although, effects of acidification and low salinity on production of anemonefishes is not completely understood, and as such, in this study we evaluated the production of juvenile orange clownfish *Amphiprion percula* in RAS, while exposed to different pH (6.5 and 8.0) in brackish (BW – salinity 11) and seawater (SW – salinity 30) for 45 days. Orange clownfish production was compromised when they were reared in BW at low pH (6.5).

However, our results confirmed that it is possible to produce orange clownfish in BW, as long as the pH is kept at 8.0. We also demonstrated that acidification does not harm its production, as long as the fish be maintained in SW. Nevertheless, the fishes exposed to BW at pH 6.5 showed lower survival and growth, as well a series of biochemical alterations. The activity of glutathione-S-transferase (GST) and the total antioxidant competence (ACAP) were reduced. Furthermore, protein damage (P-SH) was higher at pH 6.5 BW, whereas lipid peroxidation (TBARS) increased in BW. It is noteworthy that 38% of the clownfish reared in BW, independently of the pH, developed a partial melanism typical of the onyx variety, which is more valuable than the normal fish coloration in the ornamental fish market. The fishes produced under pH 6.5 and 8.0 in SW or BW were transferred to the control condition (pH 8.0 and SW), and their colouration was recovered in 7 days. The oxidative damage disappeared in transferred fishes previously reared in at pH 6.5 BW, as TBARS and P-SH returned to levels similar to those in fish from the control condition (SW, pH 8.0). In conclusion, the combined effects of low pH and low salinity compromised growth, survival, and oxidative status of juvenile orange clownfish.

Keywords: Anemonefish; Ornamental Aquaculture; Oxidative status; Reef-fish; Coloration

Introduction

The ornamental fish trade is an important industry worldwide, with the USA being responsible for more than half of that market (Tissot et al, 2010). About 11 million marine fishes (1,802 species from 125 families) are imported every year into the USA, including the famous “Nemo”, belonging to the Pomacentridae Family. The named “Nemo effect” increased the demand for clownfish after Pixar movie (Pouil et al., 2019); thus *Amphiprion ocellaris* and *Amphiprion percula* ranked the fifth position of fish imports by the USA (Rhyne et al., 2012). Both species are quite similar, and they are often mistaken one for another. Hence, they may be referred to as a complex (*i.e. A. ocellaris/percula*). The annual global market for *A. percula* accounts for about 100,000 individuals, 80% of which originate from aquaculture (Maison and Graham, 2015). Pomeroy et al. (2006) have already suggested that aquaculture should be considered a good alternative to reduce fishing pressure on coral reefs. That assertion also applies to some clownfish species currently available in the market (Rhyne et al., 2017).

Marine ornamental fish aquaculture is not a large-scale industry and as such it does not necessarily occupy large areas in coastal fragile ecosystems (Tlusty, 2002). Despite large companies are located in the USA (Maison and Graham, 2015), small-scale operations are considered economically feasible (Kodama et al., 2011). The production of marine fish in recirculating aquaculture system (RAS) allows its location inland, where the cost of land is lower compared to coastal sites. An *ex-situ* facility also offers other advantages, such as proximity of commercialization points, biosecurity, and fewer license issues. Even though RAS demands skilled employees and high energy costs, the production of ornamental fish in RAS can be economically profitable due to the recent optimization and intensification of rearing techniques (Calado, 2017b). Fish production in low salinity environments can also reduce the costs of adding salt to the water, or to transport saltwater from the coast, avoid pathologies and allow better growth.

Water acidification is a negative consequence of fish production in RAS. Further the CO_2 accumulation(Aslam et al., 2019), it occur due to the nitrification process, which is responsible for the oxidation of ammonia into nitrate in biofilters (van Kessel et al., 2015). This process includes consumption of bicarbonate reserves by microorganisms (Ebeling et al., 2006) and thus triggers acidification. Salinity plays a role in the nitrifying process, which is more efficient at low salinities (Timmons & Ebeling, 2010). However, nitrification is negatively influenced by acidic pH (Boyd, 2015).

The disruption of acid-base balance and ion regulation by the reallocation of metabolic resources to deal with stress and homeostasis maintenance can affect other processes including antioxidant and oxidative stress responses as well as growth and survival (Silva et al., 2016). The imbalance between the production of pro oxidants molecules and the antioxidant defenses, in favor of oxidizing agents can lead to damage in macromolecules (lipids, protein, and DNA) or disturbed cellular metabolism and regulatory pathways, landmarks of what is called oxidative stress (Lushchak, 2016).

Ion regulation and acid-base regulation are interrelated physiological processes (Claiborne et al., 2002; Evans et al., 2005). Salinity and pH variations are important factors influencing stress responses in fishes (Lemos et al., 2018; Pellegrin et al., 2019). Salinity plays a main role in many physiological pathways, which can influence fish growth and survival. Interestingly, some species can benefit from the comfort of the isosmotic environment to improve their overall health, including growth (Bœuf and Payan, 2001; Patterson et al., 2012).

Clownfish studies have shown that it is possible for *A. percula* to reproduce in brackish water (Kottila Veettil Dhaneesh et al., 2012). Furthermore, a salinity level of 25 is recommended for larviculture of *A. ocellaris* (Evangelista et al., 2020). However, it has been reported that the activity of antioxidant enzymes in *Amphiprion melanopus* decreased after an acute transfer from 35 to 17.5 salinity (Park et al., 2011a).

Acidification has not been well documented regards growth and biochemical indexes of reef fishes (Clark et al., 2020; Heuer and Grosell, 2014). However, *A. percula* acutely exposed to acidic seawater showed protein damage and high mortality at pH 5 (Carneiro et al., 2021). Furthermore, it has been shown that the reproduction output of some species, including *A. melanopus* (Miller et al., 2013) and *A. percula* (Welch & Munday, 2015), can be improved when facing small pH reduction (-ΔpH of 0.3-0.4) compared with control conditions (pH 8.1).

Beyond physicochemical alterations, environmental fluctuations may play effects on fish phenotype. Environmental diversity including temperature, food composition, water depth, population density, or background color, might lead to color changes in anemonefishes (Frédérich et al., 2010; Leimar, 2007; Olivotto et al., 2011; Sugimoto, 2002). For example, Fautin and Allen (1992) observed limited melanism, named Onix variety, in *A. percula* living in symbiosis with the sea anemone *Stichodactyla*.

Little is known about the production of reef fishes at simultaneously low salinity and pH. Thus, in this study we aimed to evaluate survival, growth, oxidative status, and color patterns of *A. percula* juveniles produced in acidic environments at both high and low salinity.

Material and methods

This study was conducted at the Laboratory of Marine Fish Culture (LAPEM) of the Federal University of Rio Grande - FURG. The fishes used in the experiments were reared at LAPEM according to the methodology described by Hoff (1996). The experiments were approved by the Ethics Committee on Animal Use (CEUA) of FURG under 23116.003734/2018-86 protocol number.

Exposure to different pH and salinities

A factorial design experiment (2 x 2) evaluated the effect of two salinities (11 and 33, BW- brackish water and SW - sea water, respectively) combined with two pH levels (6.5 and 8.0) on survival, growth performance, and oxidative status of juvenile orange clownfish *Amphiprion percula* (Lacepède, 1802). All treatments (BW-6.5, BW-8.0, SW-6.5, and SW-8.0) were conducted in triplicate.

Twenty fish (40 days post-hatching, 73.0±15.0 mg and 15.6 ±1.3 mm) were randomly distributed in 12 tanks (20 L each, density of 1 fish/L) distributed in four RAS. The fishes were produced with seawater (pH 8.0 - 8.5; 30 -35 salinity) and then abruptly transferred from the production tanks, to the experimental units and maintained under the experimental salinity and pH levels desired. Those levels were permanently stabilized by means of pH controllers (Tecna Evo 603, Seko, Brazil). The water was acidified by adding HCl 3% (0.36 M). Salinity levels were controlled by adding local tap water, after salinity adjustment. The water was chlorinated (sodium hypochlorite, 10 ppm) and dechlorinated (thiosulfate, 10 ppm) before use.

During the experiment, the fish were fed on a commercial diet (Orange Grow Large, 500-800 µm, Inve, USA) three times a day until apparent satiation.

After 50 days, four fish were sampled of each tank for further biochemical analyses, rendering twelve organism per treatment.

Water quality

Water quality parameters were monitored every morning, including: salinity (refractometer ATAGO S/Milli-E, Japan); temperature and oxygen (Oximeter 550A, YSI, USA), total ammonia nitrogen (TAN) (UNESCO, 1983), nitrite (Bendschneider and Robinson, 1952), pH (pH meter EcoSense pH 100A, YSI, USA), and alkalinity (Eaton et al., 2005). Nitrate levels were measured once a week (Aminot and Chaussepied, 1983). Considering that the nitrifying process was hampered in some treatments, water was exchanged equally in all treatments when total ammonia nitrogen (TAN) in any of the units reached 1 mg L^{-1}. Water quality parameters and their statistical *P* values are shown in Table 1.

Table – 1: Water quality parameters in the rearing of orange clownfish *Amphiprion percula* juveniles grown in SW (salinity 33) or BW (salinity 11) at pH 6.5 or pH 8.0. Different letters indicate significant differences between experimental groups ($p<0.05$) (mean ± SD, n =3).

Treatment	**pH 6.5**	**pH 8.0**	**pH 6.5**	**pH 8.0**	*P pH*	*P Salinity*	*P pH*Salinity*	
	BW		*SW*					
Salinity (‰)	11±1	11±1	33±1	33±1	-	-	-	‡
pH	6.42±0.003	8.40±0.010	6.43±0.009	8.26±0.008	-	-	-	‡
Temperature (°C)	26.74±0.02[a]	26.60±0.01[b]	26.69±0.06[a]	26.68±0.01[a]	0.005	0.471	0.007	
Oxygen (mg O_2 L^{-1})	7.14±0.004[a]	7.15±0.002[a]	6.31±0.008[c]	6.33±0.005[b]	0.006	1.00	<0.001	
Alkalinity (mg $CaCO_3$ L^{-1})	18±5[d]	178±5[b]	20±5[c]	187±5[a]	<0.001	1.00	0.001	
TAN (mg N-NH_4^++NH_3^- L^{-1})	0.89±0.003[b]	0.13±0.008[d]	1.03±0.016[a]	0.25±0.007[c]	<0.001	1.00	0.001	
Ammonia (mg NH_3^- L^{-1})	0.0011±0.000009[d]	0.017±0.0007[a]	0.0012±0.00001[c]	0.013±0.0006[b]	<0.001	1.00	0.001	
Nitrite (mg NO_2 L^{-1})	0.03±0.001[d]	0.27±0.004[c]	0.52±0.008[b]	0.59±0.004[a]	<0.001	1.00	<0.001	
Nitrate (mg NO_3 L^{-1})	0.26±0.08[d]	0.81±0.09[c]	1.45±0.06[b]	1.51±0.01[a]	0.044	0.268	<0.001	

Growth parameters

At the beginning (30 fishes) and at the end of the experiment (all survivors), the fish were fasted for 12 h and anesthetized with benzocaine (50 ppm) to measure the total length and weight. Survival and growth parameters were calculated as follows:

Survival (S, %) = *Initial number of fish/Final number of fish x 100*;

Specific growth rate (SGR, %) = *(ln) average final weight – (ln) average initial weight/days of experiment x 100*;

Feed conversion rate (FCR, %) = *Feed offered/weight gain X 100*;

Weight gain (WG) = *Average final weight – Average initial weight;*

Fulton's Condition Factor (K) = *Weight/Lenght*^{3} *X 100.*

Fish color

At the end of the trial, photographs of seven fishes were taken (camera: Nikon, P900, Japan) on a white background at 589±29 lx (Luxmeter Chauvin Arnoux, C.A 810, France) to analyze the presence of black areas near the white stripes characteristic of the *Onix* variety. When present, black areas were calculated as:

Percentage of fish with black area (%) = *(Number of fishes with a black area/total number of fish)*100*.

The total fish area and the black area were measured with Image J software. The relative black area was calculated as:

Relative black area (%) = *black area/whole body area*.

Survival and oxidative status after returning fish to control conditions

In ornamental trade, the fishes are usually transported from the fish farm/collection site to the retail stores and from there to the final consumer aquarium. It is assumed that a fish surviving seven days after the transport can be considered in good condition for commercialization (Lim et al., 2003b). Therefore, we examined survival and oxidative status of survivors at the end of the growth experiment both at 1 and 7 days after they were returned to the control condition (salinity 33, pH 8.0). The first day, about 95% of the water in each RAS was replaced in order to achieve the control condition. All survivors were maintained in their original tanks, four fish from each tank were sampled at 1 and 7 days after the water exchange. During that period, feeding and water quality control followed the protocol apllied during the growth experiment.

Biochemical analyses

The fishes were sacrificed within benzocaine (300ppm) batches, flash-frozen in liquid nitrogen and stored at -80°C. Each fish were homogenized (1:5 – w/v) for 60 s at 100% of potency sonication (QSonica, Q55, 50W and 20 kHz, USA) in an ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, pH 7.80 and 1% Triton X-100 (v/v) (Castro et al., 2012). Then the samples were centrifuged at 10.000 x *g* for 30 minutes at 4 °C (SOLAB SL-703, Brazil). The supernatant was removed and stored at -80 °C until biochemical measurements. The total protein level of each sample was determined by Biuret assay using commercial kits (Doles, Brazil) at 550 nm. All assays were performed in a microplate reader (BioTek, Synergy HT, USA).

The glutathione-S-transferase (GST) activity was measured according to Habig et al. (1974). Supernatant volume samples (10 µL) reacted with 1 mM of reduced glutathione (GSH) and 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB) and the produced conjugate read at 340 nm at each 1 min across 5 min.

The non-proteic (NP-SH) and proteic thiols (P-SH) were measured according to Sedlak and Lindsay (1968), using DTNB (5,5- dithiobis-(2-nitrobenzoic acid; Sigma) reactions. First, 50 µL samples were added to 40 µL of distilled water and deproteinized with 10 µL trichloroacetic acid (TCA 50% w/v). After adding TCA, samples were incubated on ice (15 min) and then centrifuged (3,000 x *g*, at 4°C for 15 min). After adding DTNB, the supernatant absorbance was measured at 405 nm for estimation of NP-SH content.

The total antioxidant capacity against peroxyl radicals (ACAP) was determined according to the method described by Amado et al. (2009). Previously all samples were diluted with homogenization buffer to achieved 2.0 mg protein mL^{-1}. The reaction of 10 µL of diluted samples in 127.5 µl of reaction buffer (pH 7.2, 30 mM HEPES, 200 mM KCl, and 1 mM $MgCl_2$) and 7.5 µl of 20 µM 2,2-azobis-2-methylpropionamidine dihydrochloride solution (ABAP) at three of the six wells within 10 µl 2′, 7′ dichlorofluorescein diacetate (H_2DCF-DA) at 40 µM. At 37 °C, ABAP thermolysis generated the peroxyl radicals that reacted with H_2DCF-DA l, leading the production of the fluorescent molecule DCF, which was read (excitation 485 nm; emission 520 nm) at every 5 min for 30 min. For interpretation of the results, a higher relative area means a lower antioxidant capacity.

The protein thiols (P-SH) were measured according to Sedlak and Lindsay (1968), following the NP-SH step. After supernatant removed for NP-SH determination, the protein pellet was re-suspended with 50 µL homogenization buffer and 40 µL of 0.2 M Tris-Base at pH 8.2. From this solution, 30 µL were removed and incubated at 50 °C by 30 min after addition of 10.8 µL of 0.15 phosphate buffer pH 7.5 and 4.5 µL of ethanol. After, to 30 µL of this extract it was added 150 µL of 0.2 M Tris-Base at pH 8.2 and 50 µL of DTNB, ice incubated for 15 min and after centrifugation (3,000 x *g*, at room temperature for 15 min) read at 405 nm absorbance.

For the evaluation of lipoperoxidation (LPO), we applied the protocol of reactive substances to thiobarbituric acid (TBARS) determination (Oakes & Van Der Kraak, 2003). Firstly, 20 µl of butylated hydroxytoluene solution (BHT, 67 µM), 150 µL of 20 % acetic acid solution, 150 µL of thiobarbituric acid (TBA) solution (0.8%), 50 µl of distilled water, and 20 µL of sodium dodecyl sulfate (SDS, 8.1%) were added to supernatant sample (20 µL). Then, the mixture was heated at 95 °C for 30 min. Afterwards, 100 µL of distilled water and 500 µL of n-butanol were added to the solution, which was centrifuged (3,000 g for 10 min at 15°C). A 150 µL volume of the supernatant was used for reading at 515 nm (excitation) and 580 nm (emission). As standard for the construction of a calibration curve, 1,1,3,3-tetramethoxypropane (TMP) was employed.

Statistical analysis

Exposure to different pH and salinities

The normality and homoscedasticity of data were verified using Shapiro-Wilk and Levene’s test, respectively. When at least one of these two conditions was not fulfilled, the Rank transformations were applied (Friedman, 1937) and then a factorial ANOVA was performed. Significant ANOVA was followed by the Newman-Keuls test to identify differences among treatments.

Recovery after returning to control conditions

After checking the assumptions of normality and homoscedasticity, factorial ANOVA followed by Newman-Keuls test was applied to analyze differences in the biochemical variables from fishes maintained at the different salinities and pH and subsequently restored to control conditions after 1 and 7 days. These results were compared with those for the basal control group (pH 8.0 SW) using the Dunnett's test.

All data were expressed as mean ± 1 standard deviation. In all tests, the significance level of 5 % was applied.

Results

Exposure to different pH and salinities

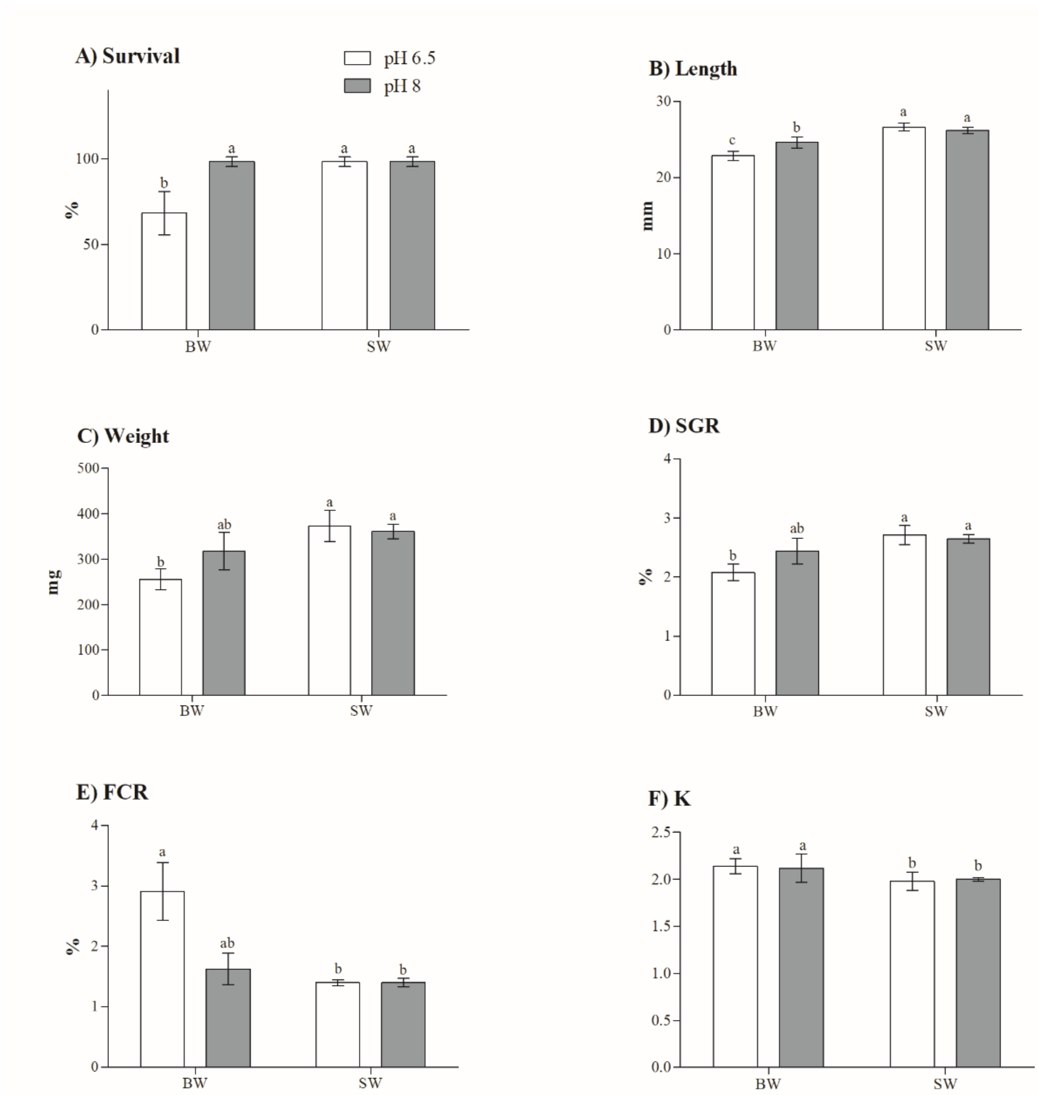
Survival and growth

There was no difference about mortality (less than 2%) in orange clownfish reared in SW, regardless of the pH level applied. The same finding was observed in fishes reared in BW- 8.0, but only 68% of the fish survived when reared in BW- 6.5 (P_{pH}= 0.007; $P_{salinity}$= 0.007; $P_{pH*salinity}$= 0.007) (Fig. 1A).

The combination of low pH and low salinity was also harmful for the growth of orange clownfish. Final lengths, final weights, and specific growth rates were lower that scenario compared to fish reared in SW at both pH levels. Fish reared in SW reached 26.6 mm after 50 days, while total length in those reared in BW-6.5 was significantly smaller (22.8 mm) (P_{pH}=0.077; $P_{salinity}$<0.001; $P_{pH*salinity}$=0.011) (Fig 1B). Regarding weight, fish reared in SW weighed 30 % more than those reared in BW-6.5 (P_{pH}=0.187; $P_{salinity}$=0.001; $P_{pH*salinity}$=0.062) (Fig. 1C). The specific growth rate (SGR) was 22 % lower in fish reared in BW-6.5 compared to SW-6.5, being the latter statistically similar to other treatments. There was no significant effect of pH on SGR for fish reared in SW (P_{pH}=0.210; $P_{salinity}$=0.002; $P_{pH*salinity}$=0.059) (Fig. 1D).

The FCR were influenced by both pH and salinity (P_{pH}= 0.004; $P_{salinity}$<0.001; $P_{pH*salinity}$= 0.004). FCR was two-fold higher when fish were reared in BW-6.5 (Fig. 1E). Salinity also influenced the condition factor, being 7 % lower in SW (Fig. 1F) (P_{pH}=0.936; $P_{salinity}$= 0.036; $P_{pH*salinity}$= 0.707).

Figure 1: Survival and growth of orange clownfish *Amphiprion percula* juveniles reared in SW (salinity 33) or BW (salinity 11) at pH 6.5 or pH 8.0 pH. Different letters indicate significant differences between experimental groups (P<0.05) (mean ± SD, n =3).



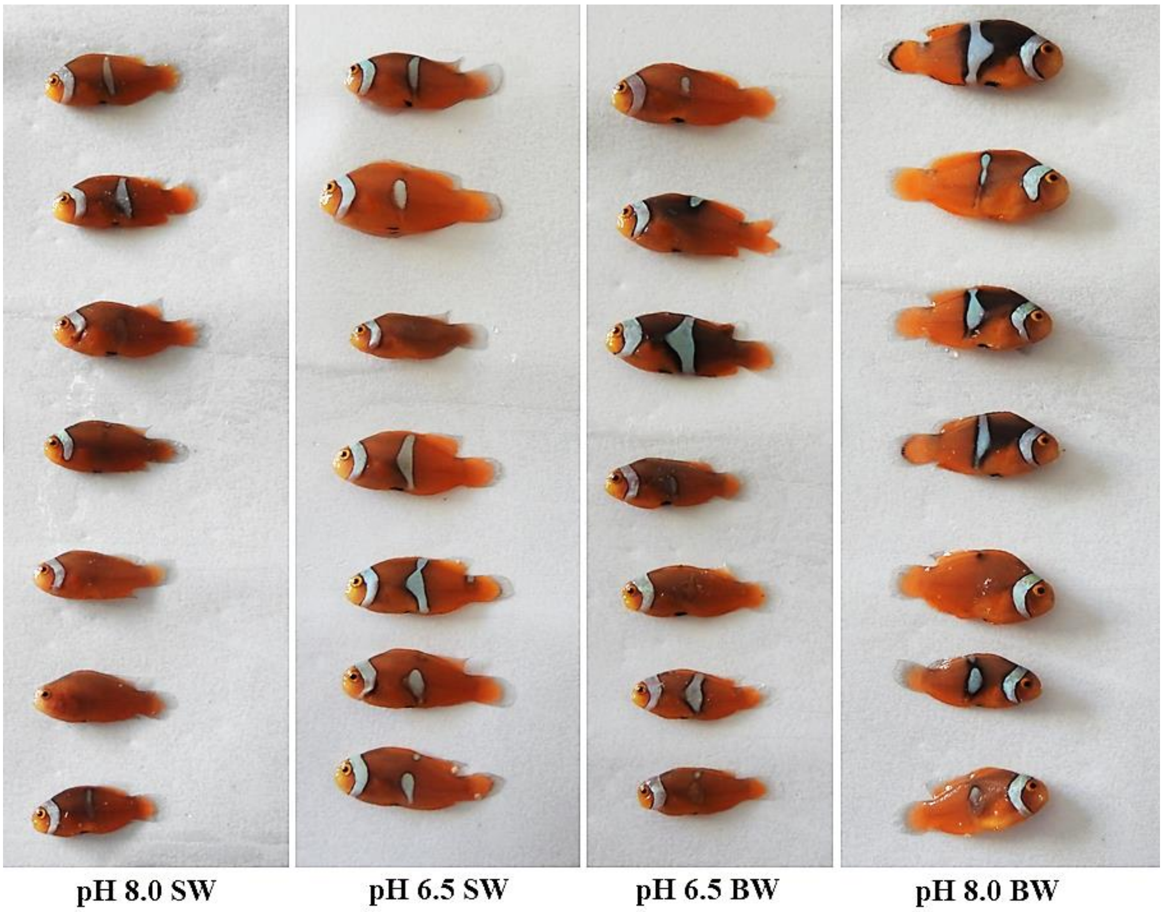
Fish color

The *Onix* variety, characterized by the presence of limited melanism, reached 40% of the fish reared in BW. The relative black body area was 15% higher in BW, irrespective of pH. Two fishes with a small black area along the white stripe were observed only in one tank in SW-6.5. However, pH did not influence (P>0.05) the melanism of fishes (Table 3 and Fig. 2).

Table – 2: Color changes in orange clownfish *Amphiprion percula* juveniles reared in SW (salinity 33) or BW (salinity 11) at pH 6.5 or pH 8.0 pH. Different letters indicate significant differencesbetween salinity treatments. Differences between pH levels were not significant (mean ± SD, n =3).

Treatment	**pH 6.5**	**pH 8.0**	**pH 6.5**	**pH8.0**	*P*
	BW		*SW*		
Presence of Onix (%)	38.1±8.2[a]	38.1±21.8[a]	0.0[b]	0.0[b]	0.002
Black body area (%)	14.6±6.1[a]	16.4±15.2[a]	0.5±0.9[b]	0.0[b]	0.003

Fig. – 2: Color phenotypes in orange clownfish *Amphiprion percula* juveniles reared for 50 days in SW (salinity 33) or BW (salinity 11) at pH 6.5 or pH 8.0.



Biochemical analyses

Biochemical responses and their statistical *P* values are shown in Table 2. No differences were found for protein levels across treatments. GST activity was 18 % higher in SW, irrespective of pH level. NP-SH content was not influenced by pH or salinity. Total antioxidant capacity (ACAP) was three-fold higher (lower relative area) at in SW-6.5 when compared to responses observed in BW, irrespective of pH. Higher P-SH levels were observed at pH 8.0-SW when compared with the other treatments, which were 34 % lower. TBARS levels were two-fold higher in BW-8.0 compared to SW.

Return to control conditions

No mortality was observed 7 days after fish were moved to the control conditions: 8.0-SW.

Biochemical analyses during the return period

Protein did not differ across pH, salinity or time, even when the interaction of pH and salinity was significant after transferring fish to control conditions (P_{pH}=0.728; $P_{salinity}$=0.059; P_{time}=*0.756;* $P_{pH*salinity}$=0.011; $P_{pH*time}$=*0.172;* $P_{salinity*time}$=*0.344;* $P_{pH*salinity*time}$=*0.151*). Also, no differences (*P*>0.05) were found compared to production control condition (SW-8.0) (Fig. 3A).

GST activity was higher in SW-6.5 after the transference (P_{pH}= 0.003; $P_{salinity}$<0.001; P_{time}*<0.001;* $P_{pH*salinity}$<0.001; $P_{pH*time}$=*0.104;* $P_{salinity*time}$=*0.038;* $P_{pH*salinity*time}$=*0.567*). The GST activity showed differences on day 1 for BW-6.5, BW-8.0 and SW-8.0, as well as in BW–6.5 on day 7 in respect to control conditions (*P*<0.05) (Figure 3B).

NP-SH levels were similar in all treatments, even when the interaction between pH and salinity was significant (P_{pH}=0.204; $P_{Salinity}$=0.104; P_{time}=*0.307;* $P_{pH*salinity}$=0.038; $P_{pH*time}$=*0.155;* $P_{salinity*time}$=*0.779;* $P_{pH*salinity*time}$=0.255) and for the control production condition according the Dunnett's test (*P*>0.05) (Fig. 3C).

Total antioxidant capacity was lower (higher relative area) in SW-6.5, being different from the other treatments (P_{pH}=0.733; $P_{salinity}$=0.004; P_{time}=*0.001;* $P_{pH*salinity}$=0.493; $P_{pH*time}$=*0.023;* $P_{salinity*time}$=*0.003;* $P_{pH*salinity*time}$=*0.025*). Comparing to control conditions, Dunnett's test revealed lower antioxidant capacity (*P<0.05*) in SW-6.5 at day 1, and for BW at day 7 (Figure 3D).

Differences in P-SH were observed between days 1 and 7 (P_{pH}= 0.469; $P_{salinity}$ = 0.219; P_{time} = *0.042;* $P_{pH*salinity}$ <0.001; $P_{pH*time}$=*0.349;* $P_{salinity*time}$ = *0.190;* $P_{pH*salinity*time}$ = 0.553). Additionally, P-SH in BW-6.5 and SW-8.0 were lower at day 1 in comparison to production control condition conforming Dunnett's test (Figure 3E).

TBARS was reduced along the recovery period, being lower at day 7 than at day 1 (P_{pH}=0.188; $P_{salinity}$=0.788; P_{time}=*0.019;* $P_{pH*salinity}$=0.631; $P_{pH*time}$=*0.819;* $P_{salinity*time}$=*0.222;* $P_{pH*salinity*time}$=*0.335)*. However, treatments and basal control treatment (SW-8.0) did not differ significantly (Figure 3F).

Discussion

In the present study, we investigated the effects of pH and salinity on the production of an important marine ornamental species, the orange clownfish *A. percula*. Previous studies (Carneiro et al., 2021) pointed out that oxidative stress responses due to acidification, which is an important factor to consider regarding fish quality and welfare in fish aquarium trade.

Growth parameters

Considering the different environmental conditions applied in our study, the most undesirable scenario for the production of orange clownfish was achieved in BW-6.5. Mortality and reduced growth parameters (e.g., weight, length, FCR, SGR) revealed tertiary stress responses in fishes (Barton, 2002). On the other hand, the growth was not hampered in fishes maintained in BW-8.0 or SW regardless of pH, Similarly, *Psetta maxima* juveniles did not showed a negative response in growth when reared at pH 5.7 and salinity 15 (Mota et al., 2018). The growth of *A. melanopus* larvae was either not impaired when exposed to high pCO_2 (pH 7.77) (Miller et al., 2012). This species also showed an improvement in their reproductive output, namely higher fecundity, under the acidic condition (Miller et al., 2013). Similarly, a high reproductive performance was observed in *A. percula* kept under a scenario of limited ocean acidification (pH 7.8) by a GABA neurotransmitter disorder and overproducing of reproductive hormone (Welch and Munday, 2015).

Cobia *Rachycentron canadum* juveniles reared at low salinity showed low FCR, which increasing with food salt supplementation (7.5-10% NaCl), although unaffected SGR (Santos et al., 2014). However, SGR of *Centropomus parallelus* was enhanced when reared at 5 salinity compared to 20 and 30 salinity (Rocha et al., 2007). Those species, *Rachycentron canadum* and *Centropomus parallelus* are, respectively, marine and estuarine fishes. Even the *A. percula* was a reef species, their food consumption changes seems to estuarine species, increasing with salinity decreases.

The fish condition factor K, can reveal growth disruptions (e.g., water or fat accumulation) likely related to ionic and acid-base regulation (Freire et al., 2008; Rocha et al., 2005). *A. percula* juveniles of the present study showed higher condition factor when reared in BW, irrespective of pH, compared to juveniles from the other treatments, suggesting impairments in growth and/or nutrient accumulation/utilization what lead to shorter fishes in both pH and lighter at pH 6.5 in BW

Biochemical responses

The antioxidant system comprises antioxidant enzymes and low molecular-mass antioxidants produced by the organism or incorporated from the diet to scavenge ROS and their toxic byproducts (Lushchak, 2016).

Glutathione-S-transferase (GST) deactivates hydrophobic xenobiotic compounds, which may result in cytotoxic and or genotoxic species (Blanchette et al., 2007). In our study GST activity was high in SW, indicating a higher detoxificatory capacity, as previously reported in fish *Fundulus heteroclitus* maintained under high salinity (Loro et al., 2012). Additionally, the GST activity in the white sturgeon *Acipenser transmontanus* increased when acclimated to SW (Donham et al., 2006).

The total antioxidant capacity against peroxyl radicals (ACAP) decreased in low salinity, principally in acidic condition. Other studies also reported reduced antioxidant capacity in fishes submitted to acidic (Copatti et al., 2019b, 2019a; Pellegrin et al., 2019) or low salinity conditions(Loro et al., 2012), indicating an overload in the antioxidant system.

In BW-8.0 treatment, the fish showed low P-SH compared with SW-8.0. The reduction in P-SH levels indicates protein oxidation, and the oxidation of cysteine SH groups can trigger intermolecular protein cross-linking and enzyme inactivation (Colombo et al., 2020; Mitton et al., 2016). Observation of protein oxidation in BW-6.5 suggests it is a useful biomarker to evaluate the effects of acidification as pass with Atlantic halibut juveniles(Carney Almroth et al., 2019b). Indeed, at this study protein oxidation also is important for salinity changes, and for salinity and pH changes, confirming the roles of protein comprises in these challenges.

Low salinity decreased antioxidant capacity, increased lipid peroxidation, and triggered immunological impairment in yellowfin seabream (Lin et al., 2020c). This finding is in accordance with our results regarding BW-8.0 treatment, in which the growth did not result affected but physiological impacts were observed, including lower GST activity, and higher protein and lipid damage.

The high level of TBARS in BW-8.0, where the energetic costs for osmorregulation should be reduced due to the isosmotic environment, might favor LPO due to the higher storage of nutrients (Bœuf and Payan, 2001; Sokolova et al., 2012). Energy expenditure can both affect the oxidative status and damage in fish, as pointed out in *Solea senegalesis* fed on high-lipid diets increase the liver and muscle TBARS, (Rueda-Jasso et al., 2004). This finding would explain the high level of TBARS in BW-8.0, where the energy demand should be low, but aerobic, favoring increased LPO. In this regard, it could be possible to reduce LPO by antioxidant supplementation in diets of marine fishes reared in low salinity.

Fish color

An unexpected different coloration occurred in fish maintained in BW. The phenotype was characterized by a limited melanism (Fautin and Allen, 1992) that led to the production of another variety of *A. percula* named *onix*. It is known that environment (background alteration, light intensity) and nutritional (i.e., dietary carotenoid supplementation) characteristics may promote color alterations in clownfish *A. ocellaris* resulting in an intensification of the orange color in the body or in the brightness of some fins (Yasir and Qin, 2010, 2009a, 2009b). However, up to now the effect of reduced salinity in generating *onix* variety of *A. percula* had not been studied.

Color changes occur by the activities of specific "effector" pigmentary cells in the skin, named chromatophores. These cells contain characteristic pigmentary organelles named chromatosomes. Melanophores are a type of chromatophores that contain melanized organelles, the melanosomes, which are responsible for the dark colors of the skin (Sugimoto, 2002). Our results suggest that melanosomes might have been influenced by BW in some way, leading to the transformation of fish into the *onix* variety. This variety is also found in nature, being more valued in the ornamental fish market. Therefore, more investigations are needed to understand the mechanisms underlying color changes in clownfishes as consequence of environmental changes. Indeed the melanocytes under hypoosmotic environment may respond as do with some anemones venoms, since only *A. percula* in symbiosis relation with *Stichodactyla gigantean* show melanism (Fautin and Allen, 1992).

Restored experimental control conditions

In ornamental trade, seven days is considered as the time limit to death occurrence by commercialization (Lim et al., 2003a). Since, acidic or low salinity challenges can occur in managements as transfers and transport in present study time-span effects after control condition reestablished has been understood.

Ion channels and ionocity cells have one acute regulatory phase to environmental changes. The regulation may occur between 24 h and 15 days (Kang et al., 2013), and can be linked with cellular changes to new contexts. Since, P-SH changes may cause signaling, division, growth and death of cells, as the alterations in protein channels function (Mailloux et al., 2020; Ray et al., 2012), we suggest that ionocity types alterations might have been signalized by P-SH changes in favor to deal with recovered control conditions levels.

The mechanisms that induced oxidative stress due to salinity and pH changes are poorly understood, but it may be closely related with changes in antioxidant enzymes activity (Zeng et al., 2017). However, low P-SH levels occurred in juveniles from group SW-8.0 on the day following the transfer to control conditions.

The GST also reduced at day 1 in SW-8.0, what probably is intrigued with these unexpected result P-SH. Additionally, in SW-6.5, where ACAP is low (high relative area) on day 1, the GST activity is high. This finding reinforces the role of GST to maintain the oxidative status after the transfer management. Thus, we suggest that the management of water exchange may trigger this unexpected alteration in the same condition of production control.

After recovery the ACAP values remains low in BW fishes, as the GST activity was low in BW-6.5 even after 7 days. In a situation where the antioxidant potential is not high enough to cope with increased ROS levels, the organism may need to increase its antioxidant potential before it can effectively decrease ROS levels, which can lead to the following mechanisms: (i) ROS steady-state concentration return to the initial steady-state range (chronic oxidative stress); or (ii) a new steady-state ROS level, named quasi-stationary, can be established (Lushchak, 2016). Then, low ACAP and GST at BW and

BW-6.5should be in a quasi-stationary phase or indicate that more days are needed for the complete stabilization of biochemical variables (Kang et al., 2013; Lushchak, 2016). Nevertheless, oxidative status, as protein and NP-SH, and cellular/tissue damage, as TBARS and P-SH, in all challenged clownfish juveniles was restored to normal levels in one week after their return to normal environmental conditions (SW-8.0).

Conclusion

The combined effects of low pH and low salinity compromised the growth of orange clownfish juveniles. The biochemical indices tested indicate that the juveniles were capable to cope with a less oxidative condition at high salinity, independently of pH, as evidenced by changes in their antioxidant capacity and/or by the lower levels of oxidative damage. The BW-8.0 treatment promoted, at the same time, good growth and lipid peroxidation, as well, for unknown reasons, induced melanism in fish producing higher price *onix* variety. Finally, our results also demonstrate that orange clownfish juveniles can be reared in acidic pH or brackish water. Thereby, we encourage production in brackish water avoiding pH fluctuations, since brought benefits in coloration, possibilities of production far from the coast as recovery the damages after return to control conditions.

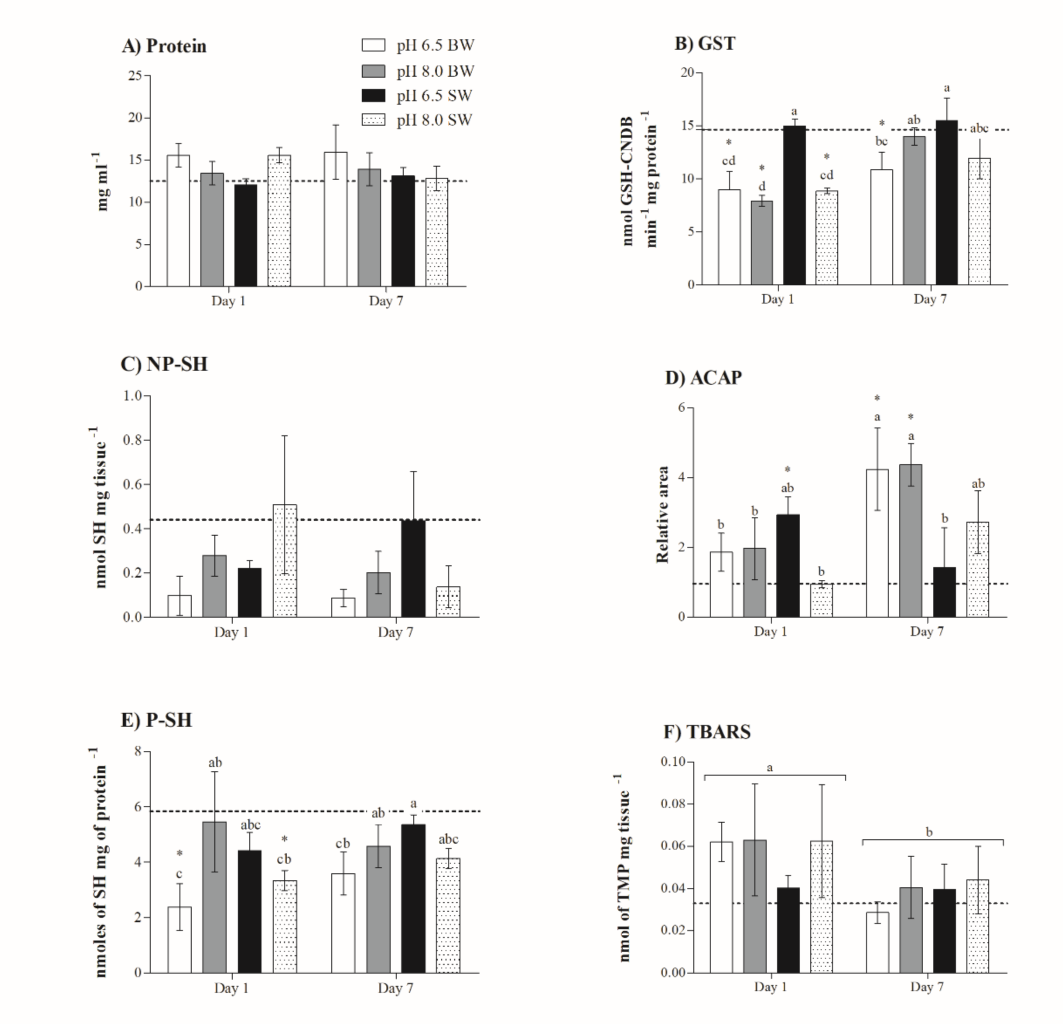
Acknowledgments

The Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil) provided access to the Portal de Periódicos and financial support through Programa de Excelência Acadêmica-PROEX (Brazil). L.A. Sampaio and J.M Monserrat are recipient of research fellowship provided by Brazilian CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and also M.D.D. Carneiro was granted by CAPES. We acknowledge the co-tutelage agreement between FURG (Brazil) and Universidade de Vigo (Spain) for M.D.D. Carneiro, which is within the scope of the Capes PrInt Program (public notice 041/2017). We also are thankful to Ane Medeiros, Diego Alba and João Timm for help in biochemical analyses.

Table – 3: Biochemical responses of orange clownfish *Amphiprion percula* juveniles reared in SW (salinity 33) or BW (salinity 11) at pH 6.5 or pH 8.0 pH. Different letters indicate significant differences between experimental groups($p<0.05$). Protein: $mg.ml^{-1}$; Glutathione-S-transferase (GST) activity: nmoles of GSH-CNDB. min^{-1}. mg of $protein^{-1}$; non-proteic thiol groups (NP-SH): nmoles of SH.mg of $tissue^{-1}$; total antioxiant capacity (ACAP): relative area; proteic thiol groups (P-SH): nmoles of SH.mg of $protein^{-1}$; thiobarbituric acid substances (TBARS): nmoles of TMP.mg of $tissue^{-1}$ (mean ± SD, n =3).

Treatment	**pH 6.5**	**pH 8.0**	**pH 6.5**	**pH 8.0**	*P pH*	*P Salinity*	*P pH*Salinity*
	BW		*SW*				
Protein	15.60±5.64	14.53±2.79	14.51±2.71	12.53±2.26	0.382	0.232	0.076
GST	$11.65±3.43^{b}$	$10.95±2.06^{b}$	$13.11±3.44^{a}$	$14.64±3.39^{a}$	0.640	0.016	0.226
NP-SH	0.46±0.17	0.28±0.23	0.15±0.11	0.44±0.10	0.552	0.439	0.032
ACAP	$2.01±0.94^{a}$	$1.41±0.26^{ab}$	$0.69±0.13^{c}$	$0.96±0.36^{bc}$	0.797	0.002	0.222
P-SH	$4.17±1.10^{b}$	$3.70±0.64^{b}$	$3.68±0.52^{b}$	$5.84±0.62^{a}$	0.089	0.096	0.016
TBARS	$0.042±0.007^{ab}$	$0.069±0.009^{a}$	$0.031±0.003^{b}$	$0.033±0.005^{b}$	0.053	0.002	0.421

Figure 3: Whole-body biochemical variables in orange clownfish *Amphiprion percula* juveniles at 1 and 7 days after their transference from the rearing experimental conditions (SW and BW; pH 6.5 and 8.0), to control condition (SW and pH 8.0). Different letters indicate significant differences ($P<0.05$) between treatments. Factorial ANOVA (factors: pH, salinity and days) followed by Newman-Keuls test. Asterisks indicate significant differences ($P<0.05$) between the experimental conditions and production control groups (SW-8.0; dotted lines) by Dunnett's test (mean±SD, n=3).



References

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes, J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity. Sci. Total Environ. 407, 2115–2123. https://doi.org/10.1016/j.scitotenv.2008.11.038

Aminot, A.; Chaussepied, M. Manuel des analyses chimiques en milieu marin. Brest, E: C.N.E.X.O., 1983. 395 p.

Aslam, S.N., Navada, S., Bye, G.R., Mota, V.C., Terjesen, B.F., Mikkelsen, Ø., 2019. Effect of CO_2 on elemental concentrations in recirculating aquaculture system tanks. Aquaculture 511, 734254. https://doi.org/10.1016/j.aquaculture.2019.734254

Barton, B.A., 2002. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 42, 517–525. https://doi.org/10.1093/icb/42.3.517

Bendschneider, K., Robinson, R.J., 1952. A new spectrophotometric method for the determination ofnitrite in sea water. JMar Res 11:87–96

Blanchette, B., Feng, X., Singh, B.R., 2007. Marine glutathione S-transferases. Mar. Biotechnol. 9, 513–542. https://doi.org/10.1007/s10126-007-9034-0

Bœuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 130, 411–423. https://doi.org/10.1016/S1532-0456(01)00268-X

Calado, R., 2017. Location, in: Marine Ornamental Species Aquaculture. Wiley, pp. 75–79. https://doi.org/10.1002/9781119169147.ch5

Carney Almroth, B., Bresolin de Souza, K., Jönsson, E., Sturve, J., 2019. Oxidative stress and biomarker responses in the Atlantic halibut after long term exposure to elevated CO_2 and a range of temperatures. Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol. 238, 110321. https://doi.org/10.1016/j.cbpb.2019.110321

Castro, C., Pérez-Jiménez, A., Guerreiro, I., Peres, H., Castro-Cunha, M., Oliva-Teles, A., 2012. Effects of temperature and dietary protein level on hepatic oxidative status of Senegalese sole juveniles (*Solea senegalensis*). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 163, 372–378. https://doi.org/10.1016/j.cbpa.2012.07.003

Choi, C.Y., Shin, H.S., Choi, Y.J., Kim, N.N., Lee, J., Kil, G-S., 2012. Effect of LED light spectra on starvation-induced oxidative stress in the cinnamon clownfish *Amphiprion melanopus*. Comp. Biochem. Physiol. A163, 357-363. http://dx.doi.org/10.1016/j.cbpa.2012.07.005

Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002. Acid – base regulation in fishes: Cellular and molecular mechanisms. J. Exp. Zool. 293, 302–319. https://doi.org/10.1002/jez.10125

Clark, T.D., Raby, G.D., Roche, D.G., Binning, S.A., Speers-roesch, B., Jutfelt, F., Sundin, J., 2019. Ocean acidification does not impair the behaviour of coral reef fishes. Nature. https://doi.org/10.1038/s41586-019-1903-y

Colombo, G.M., Simião, S., Schmitz, M.J., Pedrosa, V.F., Romano, L.A., Tesser, M.B., Baptista, P., Wilson, R., Monserrat, J.M., 2020. The role of açaí (*Euterpe oleracea* Mart . 1824) as a chemoprotective agent in the evaluation of antioxidant defence , oxidative damage and histology of juvenile shrimp *Litopenaeus vannamei* (BOONE, 1931) exposed to ammonia. Aquac. Res. 1–16. https://doi.org/10.1111/are.14503

Copatti, C.E., Baldisserotto, B., Souza, C. de F., Garcia, L., 2019a. Protective effect of high hardness in pacu juveniles (*Piaractus mesopotamicus*) under acidic or alkaline pH: Biochemical and haematological variables. Aquaculture 502, 250–257. https://doi.org/10.1016/j.aquaculture.2018.12.028

Copatti, C.E., Baldisserotto, B., Souza, C. de F., Monserrat, J.M., Garcia, L., 2019b. Water pH and hardness alter ATPases and oxidative stress in the gills and kidney of pacu (*Piaractus mesopotamicus*). Neotrop. Ichthyol. 17, 1–10. https://doi.org/10.1590/1982-0224-20190032

Donham, R.T., Morin, D., Tjeerdema, R.S., 2006. Salinity effects on activity and expression of glutathione S-transferases in white sturgeon and Chinook salmon. Ecotoxicol. Environ. Saf. 63, 293–298. https://doi.org/10.1016/j.ecoenv.2005.01.007

Eaton, A. D., Clesceri L.S., Rice, E.W., Greenberg, A.E., 2005. Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington, D.C.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358. https://doi.org/10.1016/j.aquaculture.2006.03.019

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177. https://doi.org/10.1152/physrev.00050.2003.

Fautin D., Allen, G.R., 1992. Anemonefishes and their host sea anemones. Western Australian Museum, Perth, 160 p.

Frédérich, B., Mills, S.C., Denoël, M., Parmentier, E., Brié, C., Santos, R., Waqalevu, V.P., Lecchini, D., 2010. Colour differentiation in a coral reef fish throughout ontogeny: habitat background and flexibility. Aquat. Biol. 9, 271–277. https://doi.org/10.3354/ab00257

Freire, C. a., Amado, E.M., Souza, L.R., Veiga, M.P.T., Vitule, J.R.S., Souza, M.M., Prodocimo, V., 2008. Muscle water control in crustaceans and fishes as a function of habitat, osmoregulatory capacity, and degree of euryhalinity. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 149, 435–446. https://doi.org/10.1016/j.cbpa.2008.02.003

Friedman, M., 1937. The use of ranks to avoid the assumption of normality implicit in the analysis of variance. J. Am. Stat. Assoc. 32, 675–701. https://doi.org/10.1080/01621459.1937.10503522

Genz, J., Taylor, J.R., Grosell, M., 2008. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. J. Exp. Biol. 211, 2327–2335. https://doi.org/10.1242/jeb.016832

Gilmour, K.M., Perry, S.F., 2009. Carbonic anhydrase and acid-base regulation in fish. J. Exp. Biol. 212, 1647–1661. https://doi.org/10.1242/jeb.029181

Habig, W.H., Pabs,t M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130-7139.

Halliwell B., Gutteridge, J.M. 2015. Free radicals in biology and medicine. Oxford University Press, USA

Kang, C.-K., Yang, W.-K., Lin, S.-T., Liu, C.-C., Lin, H.-M., Chen, H.-H., Cheng, C.-W., Lee, T.-H., Hwang, P.-P., 2013. The acute and regulatory phases of time-course changes in gill mitochondrion-rich cells of seawater-acclimated medaka (*Oryzias dancena*) when exposed to hypoosmotic environments. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 181–91. https://doi.org/10.1016/j.cbpa.2012.08.010

Leimar, O., 2009. Environmental and genetic cues in the evolution of phenotypic polymorphism. Evol Ecol 125–135. https://doi.org/10.1007/s10682-007-9194-4

Lemos, C.H. da P., Ribeiro, C.V.D.M., de Oliveira, C.P.B., Couto, R.D., Copatti, C.E.,

2018. Effects of interaction between pH and stocking density on the growth, haematological and biochemical responses of Nile tilapia juveniles. Aquaculture 495, 62–67. https://doi.org/10.1016/j.aquaculture.2018.05.037

Lim, L.C., Dhert, P., Sorgeloos, P., 2003. Recent developments and improvements in ornamental fish packaging systems for air transport 923–935.

Lin, G., Zheng, M., Li, S., Xie, J., Fang, W., Gao, D., Huang, J., Lu, J., 2020. Response of gut microbiota and immune function to hypoosmotic stress in the yellowfin seabream (*Acanthopagrus latus*). Sci. Total Environ. 745, 140976. https://doi.org/10.1016/j.scitotenv.2020.140976

Loro, L.V., Jorge, M.B., Silva, K.R., Wood, C.M., 2012. Oxidative stress parameters and antioxidant response to sublethal waterborne zinc in a euryhaline teleost *Fundulus heteroclitus*: Protective effects of salinity. Aquat. Toxicol. 110–111, 187–193. https://doi.org/10.1016/j.aquatox.2012.01.012

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish: a mechanistic approach. Fish Physiol. Biochem. 42, 711–747. https://doi.org/10.1007/s10695-015-0171-5

Maison, K.A, Graham, K.S., 2015. Status review report: Orange clownfish (*Amphiprion percula*). Report to National Marine Fisheries Service, Office of Protected Resources. July, 2015. 67 p.

Miller, G.M., Watson, S.A., Donelson, J.M., McCormick, M.I., Munday, P.L., 2012. Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nat. Clim. Chang. 2, 858–861. https://doi.org/10.1038/nclimate1599

Miller, G.M., Watson, S.A., Mccormick, M.I., Munday, P.L., 2013. Increased CO_2 stimulates reproduction in a coral reef fish. Glob. Chang. Biol. 19, 3037–3045. https://doi.org/10.1111/gcb.12259

Mitton, F.M., Ribas, J.L., Gonzalez, M., Miglioranza, K.S.B., Monserrat, J.M., 2016. Antioxidant responses in soybean and alfalfa plants grown in DDTs contaminated soils: Useful variables for selecting plants for soil phytoremediation? Pestic. Biochem. Physiol. 130, 17–21. https://doi.org/10.1016/j.pestbp.2015.12.005

Mota, V.C., Hop, J., Sampaio, L.A., Heinsbroek, L.T.N., Verdegem, M.C.J., Eding, E.H., Verreth, J.A.J., 2018. The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac. Res. 49, 3456–3467. https://doi.org/10.1111/are.13812

Olivotto, I., Di Stefano, M., Rosetti, S., Cossignani, L., Pugnaloni, A., Giantomassi, F., Carnevali, O., 2011. Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larval development and metamorphosis: Molecular and biochemical implications. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 159, 207–218. https://doi.org/10.1016/j.cbpa.2011.02.004

Patterson, J., Bodinier, C., Green, C., 2012. Effects of low salinity media on growth, condition, and gill ion transporter expression in juvenile Gulf killifish, *Fundulus grandis*. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 161, 415–421. https://doi.org/10.1016/j.cbpa.2011.12.019

Pellegrin, L., Nitz, L.F., Maltez, L.C., Copatti, C.E., Garcia, L., 2019. Alkaline water improves the growth and antioxidant responses of pacu juveniles (*Piaractus mesopotamicus*). Aquaculture 734713. https://doi.org/10.1016/j.aquaculture.2019.734713

Pimentel, M.S., Faleiro, F., Machado, J., Pousão-Ferreira, P., Rosa, R., 2020. Seabream larval physiology under ocean warming and acidification. Fishes 5, 1–13. https://doi.org/10.3390/fishes5010001

Rhyne, A.L., Tlusty, M.F., Szczebak, J.T., Holmberg, R.J., 2017. Expanding our understanding of the trade in marine aquarium animals. PeerJ 5, e2949. https://doi.org/10.7717/peerj.2949

Rocha, A.J. da S., Gomes, V., Van Ngan, P., Passos, M.J. de A.C.R., Furia, R.R., 2005. Metabolic demand and growth of juveniles of *Centropomus parallelus* as function of salinity. J. Exp. Mar. Bio. Ecol. 316, 157–165. https://doi.org/10.1016/j.jembe.2004.11.006

Rocha, A.J.S., Gomes, V., Ngan, P. V., Passos, M.J.A.C.R., Furia, R.R., 2007. Effects of anionic surfactant and salinity on the bioenergetics of juveniles of *Centropomus parallelus* (Poey). Ecotoxicol. Environ. Saf. 68, 397–404. https://doi.org/10.1016/j.ecoenv.2006.10.007

Rueda-Jasso, R., Conceição, L.E., Dias, J., Coen, W. De, Gomes, E., Rees, J.F., Soares, F., Dinis, M.T., Sorgeloos, P., 2004. Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture 231, 417–433. https://doi.org/10.1016/S0044-8486(03)00537-4

Santos, R., Bianchini, A., Jorge, M.B., Romano, L., Sampaio, L., Tesser, M.B., 2014.

Cobia Rachycentron canadum L. reared in low-salinity water: does dietary sodium chloride affect growth and osmoregulation? Aquac. Res. 45, 728–735. https://doi.org/10.1111/are.12017

Sedlak, J., Lindsay R.H., 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman’s reagent. Anal Biochem 25:192–205. https://doi.org/10.1016 /0003-2697(68)90092-4

Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability, development and biochemical responses of sand smelt larvae. Sci. Total Environ. 563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091

Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Sugimoto, M., 2002. Morphological color changes in fish: Regulation of pigment cell density and morphology. Microsc. Res. Tech. 58, 496–503. https://doi.org/10.1002/jemt.10168

Timmons, M.B, Ebeling, J.M., 2010. Recirculating Aquaculture. 2° Ed. NRAC Publications. 948p.

Tissot, B.N., Best, B.A., Borneman, E.H., Bruckner, A.W., Cooper, C.H., Agnes, H.D., Fitzgerald, T.P., Leland, A., Lieberman, S., Mathews, A., Sumaila, R., Telecky, T.M., Mcgilvray, F., Plankis, B.J., Rhyne, A.L., Roberts, G.G., Starkhouse, B., Stevenson, T.C., 2010. How U.S. ocean policy and market power can reform the coral reef wildlife trade. Mar. Policy 34, 1385–1388. https://doi.org/10.1016/j.marpol.2010.06.002

Tlusty, M., 2002. The benefits and risks of aquacultural production for the aquarium trade. Aquaculture 205, 203–219. https://doi.org/10.1016/S0044-8486(01)00683-4

Tornhill, D.J., 2012. Ecological impacts and practices of the coral reef wildlife trade. Defenders of Wild Life. 179p.

UNESCO (1983) Chemical methods for use in marine environmen- tal monitoring. Manual and Guides 12. Intergovernamental Oceanographic Commissiony, Paris, France

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single

microorganism. Nature 528, 555–559. https://doi.org/10.1038/nature16459
Welch, M.J., Munday, P.L., 2015. Contrasting effects of ocean acidification on reproduction in reef fishes. Coral Reefs 35, 1–9. https://doi.org/10.1007/s00338-015-1385

9. DISCUSSÃO GERAL

O crescimento e a sobrevivência levam a crer que o efeito do baixo pH seja piorado em baixa salinidade. Apesar de este fato ser verdadeiro pela ótica de estresse oxidativo e pelos próprios resultados de desempenho de peixes palhaço *Amphiprion percula* e cavalos marinhos *Hippocampus reidi* é possível sugerir que estes animais sofrem impactos similares, e que apesar de muito distintos e distantes filogeneticamente, responderam de maneira similar aos efeitos do pH ácido e salinidade, porém em níveis de intensidade diferentes. Portanto, uma vez que formas jovens dos peixes tendem a ser mais afetadas pela acidificação (Ishimatsu et al., 2008; Lee et al., 2018) e que a sobrevivência dos juvenis de *H. reidi* são maiores (86%) do que a de juvenis de *A. percula* (68%) quando expostos a pH 6.5 BW, aparentemente o cavalo marinho é mais resiliente a acidificação em baixa salinidade que os peixes palhaço.

Como introduzidos, os efeitos conhecidos da acidificação nos peixes são contraditórios, sendo que algumas espécies apresentam respostas negativas como o caso do bacalhau do atlântico (*Gadus morhua*) e atum (*Thunnus albacares*) (Frommel et al., 2016, 2012) e outras não são prejudicadas como o linguado (*Psetta maxima*) (Mota et al., 2018) apresentam respostas positivas como em smolts de salmão da atlântico (*Salmo salar*) (McCormick and Regish, 2018) e outras apesar de alterações bioquímicas negativas expõem um melhor crescimento como no linguado (*Scophthalmus maximus*) e no sand smelt (*Atherina presbyter*) (Silva et al., 2016; S. Wang et al., 2018). Adiciona-se a isto o fato de que os trabalhos geralmente são bastante diversificados quanto a idade, habitat, salinidade e outros fatores que podem ser importantes para a interpretação das respostas como o estado de nutrição dos peixes. Inclusive as próprias respostas estudadas são muito diferentes perpassando desde a etologia, histologia, fisiologia/ bioquímica e o crescimento.

Esta realidade de diversidade experimental, somada as respostas espécie-específicas, faz com que também, se instigue a necessidade de trabalhos mais aprofundados para cada espécie. Por exemplo, peixes-palhaço apresentam uma interação de protocooperação com as anêmonas que pode revelar uma importante adaptação ao ambiente ácido, promovido por um evento indireto: a capacidade de evitar a ação de venenos das anêmonas nos chamados “Acid sense ion chanels - ASIC” decorrentes da co-evolução com as anêmonas (Cristofori-Armstrong and Rash, 2017).

Entretanto é curioso pensar que cavalos marinhos, que apesar de não apresentarem uma "aptidão" fisiológica co-evolutiva, tenham a capacidade não só de tolerar, mas também de melhorar seu desempenho em ambientes ácidos marinhos. Mesmo que não possam ser estatisticamente comparados, os cavalos marinhos respondem de uma maneira mais eficaz que peixes-palhaço em ambientes ácidos salobros. Um fator que pode contribuir para esta resiliência dos cavalos marinhos é que eles vivem em ambientes estuarinos, mais instáveis e sujeitos a ação de corpos hídricos dulcícolas e oscilações de maré e provavelmente este ambiente faz com que tenham características de maior plasticidade não só a salinidade como já era conhecido (Hora et al., 2016) mas como nesta tese foi testado, também a o pH.

9.1 Referências

Cristofori-Armstrong, B., Rash, L.D., 2017. Acid-sensing ion channel (ASIC) structure and function: Insights from spider, snake and sea anemone venoms. Neuropharmacology 1–12. https://doi.org/10.1016/j.neuropharm.2017.04.042

Frommel, A.Y., Maneja, R., Lowe, D., Malzahn, A.M., Geffen, A.J., Folkvord, A., Piatkowski, U., Reusch, T.B.H., Clemmesen, C., 2012. Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. Nat. Clim. Chang. 2, 42–46. https://doi.org/10.1038/nclimate1324

Frommel, A.Y., Margulies, D., Wexler, J.B., Stein, M.S., Scholey, V.P., Williamson, J.E., Bromhead, D., Nicol, S., Havenhand, J., 2016. Ocean acidification has lethal and sub-lethal effects on larval development of yellowfin tuna, *Thunnus albacares*. J. Exp. Mar. Bio. Ecol. 482, 18–24. https://doi.org/10.1016/j.jembe.2016.04.008

Hora, M. dos S.C. da, Joyeux, J.-C., Rodrigues, R.V., Sousa-Santos, L.P. de, Gomes, L.C., Tsuzuki, M.Y., 2016. Tolerance and growth of the longsnout seahorse *Hippocampus reidi* at different salinities. Aquaculture 463, 1–6. https://doi.org/10.1016/j.aquaculture.2016.05.003

Ishimatsu, A., Hayashi, M., Kikkawa, T., 2008. Fishes in high-CO_2, acidified oceans. Mar. Ecol. Prog. Ser. 373, 295–302. https://doi.org/10.3354/meps07823

Lee, C., Kwon, B.-O., Hong, S., Noh, J., Lee, J., Ryu, J., Kang, S.-G., Khim, J.S., 2018. Sub-lethal and lethal toxicities of elevated CO_2 on embryonic, juvenile, and adult stages of marine medaka *Oryzias melastigma*. Environ. Pollut. 241, 586–595. https://doi.org/10.1016/j.envpol.2018.05.091

McCormick, S.D., Regish, A.M., 2018. Effects of ocean acidification on salinity tolerance and seawater growth of Atlantic salmon *Salmo salar* smolts. J. Fish Biol. 93, 560–566. https://doi.org/10.1111/jfb.13656

Mota, V.C., Hop, J., Sampaio, L.A., Heinsbroek, L.T.N., Verdegem, M.C.J., Eding, E.H., Verreth, J.A.J., 2018. The effect of low pH on physiology, stress status and growth performance of turbot (*Psetta maxima* L.) cultured in recirculating aquaculture systems. Aquac. Res. 49, 3456–3467. https://doi.org/10.1111/are.13812

Silva, C.S.E., Novais, S.C., Lemos, M.F.L., Mendes, S., Oliveira, A.P., Gonçalves, E.J., Faria, A.M., 2016. Effects of ocean acidification on the swimming ability , development and biochemical responses of sand smelt larvae. Sci. Total Environ. 563–564, 89–98. https://doi.org/10.1016/j.scitotenv.2016.04.091

Wang, S., Jiang, Z., Mao, M., Mao, S., Sun, Y., Sui, Y., 2018. Effects of seawater pH on survival, growth, energy budget and oxidative stress parameters of juvenile turbot *Scophthalmus maximus*. Iran. J. Fish. Sci. 17, 675–689. https://doi.org/10.22092/ijfs.2018.116814

10. CONCLUSÕES GERAIS

Peixes palhaços *Amphiprion percula* e cavalos-marinhos *Hippocampus reidi* sobrevivem a exposição agudo a ambientes tão ácidos quanto pH 5, ainda que seus parâmetros bioquímicos pareçam revelar uma tolerância a pH tão ácido quanto 6, e que as exposições em ambiente salobro congreguem um maior número de alterações dos parâmetros bioquímicos avaliados. Apesar de mostrar algumas alterações bioquímicas o ambiente ácido (pH 6,5) não causa prejuízos nas respostas zootécnicas para nenhuma das espécies quando produzidas em água marinha, sendo inclusive a melhor opção para cavalos marinhos *H. reidi*. Para o peixe-palhaço *A. percula* a produção em baixa salinidade demonstrou ser a melhor opção, considerando a produção distante da costa, o benefício de coloração alcançado e que os as alterações bioquímicas vistas no ambiente salobro são normalizadas após o retorno a salinidade marinha. Entretanto a conjuntura de respostas bioquímicas e de crescimento e sobrevivência são bastante comprometidas na exposição conjunta de pH ácido e ambiente salobro para ambas as espécies. Assim a como conclusão geral, podemos dizer que para ambas as espécies a exposição ao ambiente ácido é mais prejudicial em baixa salinidade.

11. ANEXOS

Análise de componentes principais do capítulo (PCA) iii: **Primary, secondary, and tertiary stress responses of juvenile seahorse *Hippocampus reidi* exposed to acute acid stress in brackish and seawater**

Figure 7: Factor score plots for the Principal Component Analysis (PCA) on seahorse juveniles. Analyses performed on dataset including survival, enzymatic activities and enzymatic indices. Only the variables with the highest contributions ($cos^2 > 0.5$) are indicated. Ellipses correspond to centroid value ± 1 s.d. (shaded areas). (a) and (b): Sea water (High-33) *vs* brackish water (Low-11) salinity groups – Factors 1-2 and 3-4, respectively. (c) and (d): All salinities (33 and 11) and pH (5 – 8) groups – Factors 1-2 and 3-4, respectively.