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Early exposure of striped catfish (*P. hypophthalmus*) to increased salinities could elicit conditioning hormesis in juvenile fish.

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Striped catfish is a valuable species in Vietnam's growing aquaculture industry and has become a national staple good exported worldwide. Only, sea-level rise scenarios and increased frequency of extreme weather events are predicted to erode its production as freshwater ponds salinity is expected to rise and reach higher levels than those tolerated by the species. In the scope of such events, we study the potential to grow individuals adapted to these conditions through conditioning hormesis. Striped catfish larvae were treated with 5 psu water for 5 days and once in the juvenile stage, exposed to salinity increase of 10 days to reach 0 psu, 10 psu and 20 psu for 30 days followed by a Cadmium challenge. 5 psu treated juvenile fish showed slight changes in their gene expression pattern and physiological parameters (blood, glucose levels, ion concentration). These changes appeared to bring no advantages in 0 psu but made the fish slightly better adapted to higher salinities by showing an enhanced stress response. Treated fish in 10 psu grew 21.6% bigger than non-treated fish. The chronic hyperosmotic stress from 20 psu conditions drove fish into type 1 allostatic overload which eclipsed the possible benefits brought by the treatment. This method could be a viable approach to the problem of sea-level rise in the Vietnamese aquaculture industry. However, more research is needed to determine, on the one hand, which stages of the fish's life cycle allows for the best conditioning response and, on the other hand, how treated fish with salinities ranging between 10 psu and 20 psu react to hyperosmotic stress.

Introduction

Aquaculture is growing at a remarkable rate of around 9% per year for the last 80 years and now meets half of the world's demand for fish worldwide (FAO, 2018a). When properly managed, it can meet many of the sustainable development goals set by Agenda 2030. These are such as ensuring employment, health and welfare, eliminating hunger and poverty, reducing inequalities, and protecting the quality of aquatic life (Ahmed and Thompson, 2019; Belton et al., 2020; FAO, 2018b; United Nations, n.d.). The Asia-Pacific region is currently responsible for 90% of the world's aquaculture production. Vietnam ranks in 3rd position behind China and Norway in terms of export of aquaculture products with an export value reaching US\$7,320 billion in 2016 (FAO 2018). After rice and shrimp, catfish (mainly striped catfish *Pangasianodon hypophthalmus*) is the country's largest export product, at 1.42 million tons exported in 2018, generating US\$2.26 billion in exports and providing over 150,000 jobs, mainly for rural women (Berg et al., 2019; Bui et al., 2010; Phuong, 2020; Phuong and Oanh, 2010; VASEP, n.d.).

Striped catfish is a key species in Vietnam's aquaculture. With a production cycle lasting 8 to 9 months, this freshwater stenohaline, warm water, omnivorous species is tolerant to relative high stocking density and hypoxic conditions (Chowdhury et al., 2020; FishBase, n.d.; Kristanto et al., 2017; Lefevre et al., 2011; VASEP, 2014). Trade agreements, technology improvements and mastering the species' life cycle allowed for a 16-fold increase in its production in the lapse of 22 years (Berg et al., 2019; Bosma and Verdegem, 2011; Kristanto et al., 2017; Phuong and Oanh, 2010; VASEP, 2014). To meet market's demand, new fish farms have been established in the downstream provinces of the Mekong River, closer to the Gulf of Thailand and the South China Sea (Fig. 1) (Kam et al., 2012; Kuenzer et al., 2013; Phuong and Oanh, 2010). Only, as it is one of the world's lowest-lying river plains (0 m to 4 m above mean sea level), this region has been identified as very exposed to the effects of climate change, mainly the increased frequency and intensity of extreme weather events and sea-level rise (IPCC, 2019; Trieu and Phong, 2015). As a result, freshwater environments are at risk of eutrophication, increased salinity, and disease outbreaks, which are serious concerns for the aquaculture industry as these stressors have a negative effect on fish welfare and production costs (Berg et al., 2019; Crozier and Hutchings, 2014; FAO, 2014; Jahan et al., 2019; Kam et al., 2012; Kültz, 2015; P. T. H. Nguyen et al., 2014; Schmitz et al., 2016). Wendelaar Bonga define stress as 'a condition in which the animal's homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli' (Wendelaar Bonga, 1997). Allostasis is the process by which an animal reallocates energetic resources and adjusts its physiology in order to regain metabolic equilibrium. The return

to pre-stress conditions is termed as eustress, however, if the stress response is inadequate, the animal state is referred to as distress, which causes increased allostatic load. Type 1 allostatic overload (AOL1) occurs when the energy requirement exceeds the energy available to fuel the stress response, whereas Type 2 allostatic overload (AOL2) occurs when fish have enough energy available to sustain the stress response but end up having negative physiological consequences due to metabolic system wear and tear (Balasch and Tort, 2019; Madaro et al., 2020; McEwen and Wingfield, 2010; Petitjean et al., 2019; Prunet et al., 2008; Samaras et al., 2018; Wingfield et al., 2011).

This paper focuses solely on the challenges posed by rising water salinity which can cause hyperosmotic stress on striped catfish. Literature indicates that the species show lowest signs of stress in salinities between 4 psu and 6 psu and can tolerate salinities up to 10 psu, where they are closest to their iso-osmotic point (300 mOsm.kg⁻¹). These salinities require less energy from the fish to maintain homeostasis, which may then be invested in growth. Higher salinities appear to cause distress and are perceived as a threat, increasing oxidative stress, sterile inflammatory responses and, eventually, resulting in lower growth and survival rates. Jahan et al. (2019) defined striped catfish fingerling 96hr LC₅₀ to be at 14.87 psu (Hieu et al., 2021; Jahan et al., 2019; Kumar et al., 2017; Mandal et al., 2020; A. L. Nguyen et al., 2014; P. T. H. Nguyen et al., 2014; Nguyen et al., 2021, 2016; Phuc et al., 2017; Schmitz et al., 2017b, 2017a, 2016; Thanh et al., 2014; Trieu and Phong, 2015).

Several severe salinity intrusion episodes have been documented over the years and linked to declines in striped catfish production (Bal et al., 2021; Ha, n.d.; Kültz, 2015; Schmitz et al., 2016). According to the IPCC projections, the average sea level will rise by 30 cm by 2050 and by the end of the century by 43 cm to 102 cm. (IPCC, 2019). According to Trieu and Phong's (2015) model, the salt front is anticipated to increase 70 km to 80 km upstream of the delta under the IPCC SRES B2 scenario, which projects a 0.73 m sea-level rise and a 29% decrease in river water flow. With 1.424 MHA exposed to salinities over 12 psu and 0.445 MHA exposed to salinities between 5 psu and 12 psu (Fig.1) (Trieu and Phong, 2015). Hence solutions in the farming of striped catfish need to be developed to prevent possible economic and social repercussions this could have on the sector if salinity increase scenarios were to get worse.

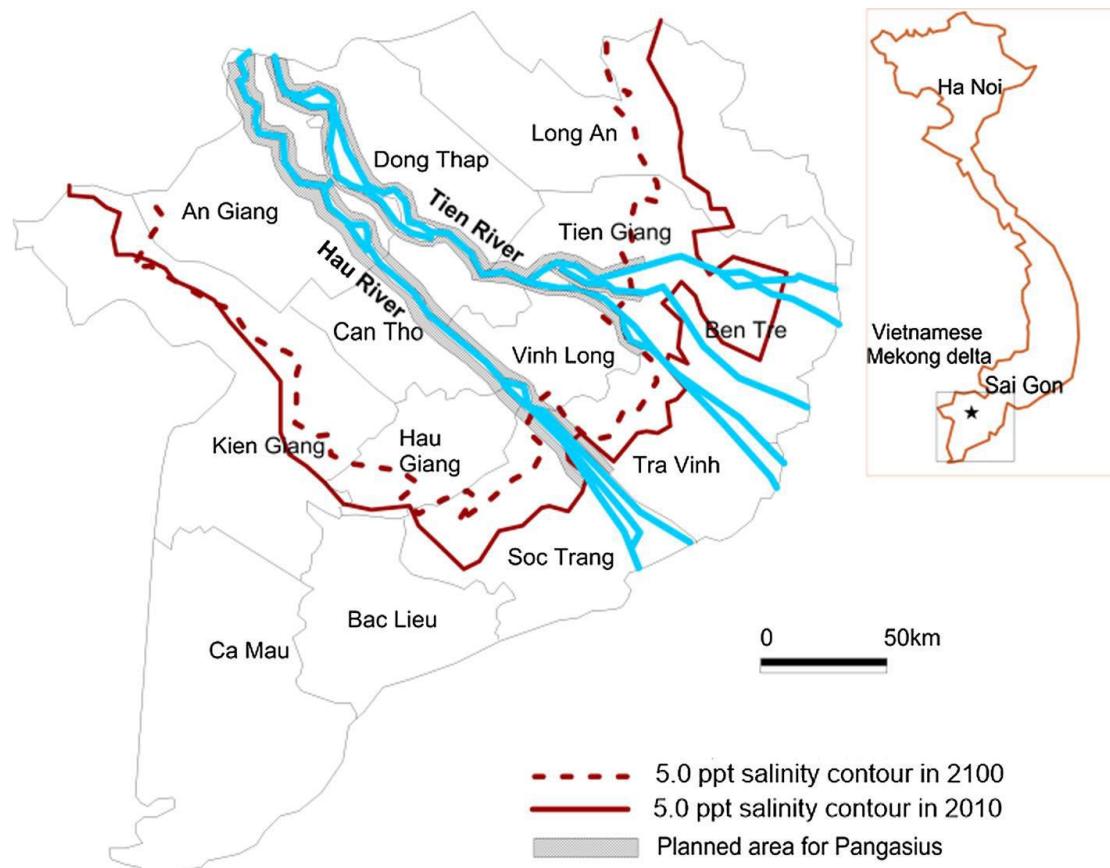


Figure 1: Projected advance of the salt front in the Mekong Delta provinces under the SRES B2 climate change scenario by 2100. The map also shows striped catfish farmable areas in gray along the branches of the Tien and Hau rivers (Trieu and Phong 2015).

Taking these factors into account, we study the possibility of triggering a conditioning hormetic response to increased salinity to develop more robust striped catfish to hyperosmotic stress. Previous research on this topic revealed that this practice has the potential to reduce stress sensitivity and allow for higher specific growth rate of aquaculture-relevant species. (Auferin and Geslin, 2008; Fevolden et al., 2002; Robinson et al., 2019; Vindas et al., 2016). Hormesis is the unifying term used for adaptation mechanisms where ‘low levels of stress activate or upregulate existing cellular and molecular pathways, improving the organism's capacity to tolerate more severe stress’ (Calabrese et al., 2007). Here we submitted striped catfish larvae (*P. hypophthalmus*) to 5 psu salinity increase for 5 days and once in the juvenile stage, allocated to tanks at 0 psu, 10 psu and 20 psu for 30 days followed by a cadmium challenge. We hypothesize that fish exposed to increased salinity in early life stages can grow tolerant to chronic exposure to this stress later in life and be fitter than non-treated individuals. Which can be assessed by higher survival and growth rates, as well as improved control of osmoregulatory processes, energy allocation capacity, and decreased oxidative stress and inflammatory response.

Material and Methods

Fish and experimental conditions

P. hypophthalmus larvae used in the experiments were obtained after reproduction of fish from Viet-Uc hatchery, An Giang province. Fertilized eggs were randomly placed in oxygenated larvae rearing tanks. Upon hatching, larvae were distributed in two rearing groups. The first, here called fresh-water group (FW) had its salinity kept constant at 0 psu, the latter, here called the salt-water group (SW), saw its tank salinity increase to 5 psu for 5 days before going back to 0 psu (fig. 2).

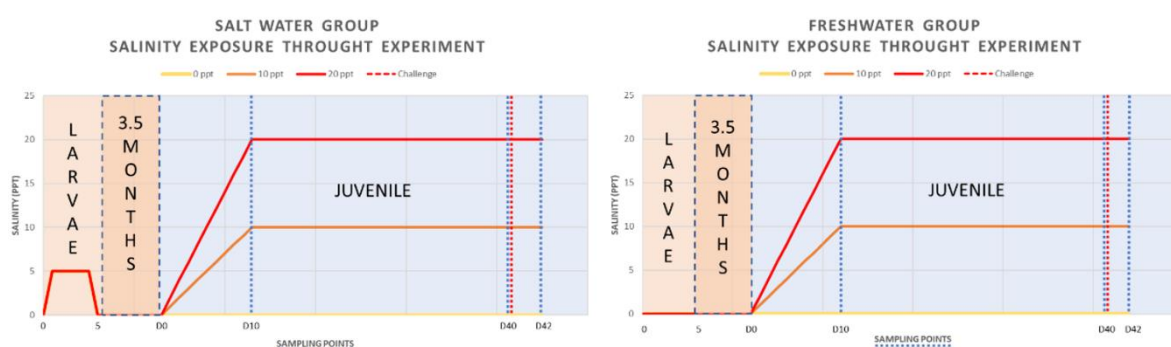


Figure 2: Graphical representation of the experimental protocol. The x-axis shows the defined sampling points (D10, D40 and D42) with blue vertical bars and the point of induction of the cadmium challenge with the red vertical bar. 3.5 months elapse between the period of exposure of the larvae to the defined salinities and the beginning of the salinity increase in the juvenile tanks. Y-axis indicates the salinity of each tank, represented by the yellow, orange, and red curve.

After 3.5 months, now juvenile fish (35 g mean weight) were distributed into 6 tank triplicates with 19 fish/tank. Tank salinity was increased by 0 psu, 1 psu or 2 psu/day to reach the targeted experimental conditions of 0 psu, 10 psu and 20 psu (C0, C10 and C20) after 10 days (D10) (fig. 2). Accounting for a total of 6 treatments; C0-FW, C0-SW, C10-FW, C10-SW, C20-FW and C20-SW. Tank treatment disposition was assigned at random. Through the whole experiment, juvenile fish were fed *ad libitum* with commercial fish feed pellet and reared in recirculating systems for different salinity levels. Salinity, pH and temperature were checked on a daily basis while nitrate and total ammonia nitrogen on a weekly basis to ensure the water quality was suitable for fish development during the experiment. After 30 days (D40), the cadmium challenge test was done with 20 $\mu\text{g/L}$ cadmium (CdCl_2 , Aladdin Industrial Corporation, purity 99%) that induced approximately 50% of mortality (data not shown).

Sampling

Three juveniles/tanks were sampled at D10, D40 prior to CdCl₂ challenge and two days after CdCl₂ challenges (D42) (fig. 2). Fish were weighted at D40 to measure weight gain. Prior to blood collection needed for red blood cells, hematocrit and total white blood cell analysis, fish were euthanized with 200 mg/L⁻¹ MS-222 (Ethyl 3-aminobenzoate methane sulfonate (Sigma, USA). Blood samples were then centrifuged at 5000 g for 10 min at 4°C and the supernatant (plasma) collected and stored at -80°C for the osmolality, ion (Na⁺, Cl⁻) and glucose concentration measures. Fish gills were collected and stored in RNA lather, following Malmstrom 2015 and kept at -80°C for gene expression analysis.

Hematology

Total red blood cell (RBC, 10⁶ cells.μL⁻¹) counts were measured with a Neubauer hemocytometer using Natt-Herrick solution as a diluent stain (Natt and Herrick, 1952). Total Leukocytes (WBC, 10⁴ cells.μL⁻¹) were counted on blood smear staining with Wright's or Giemsa (Merck, MA, USA) according to (Dunning and Safo, 2011). Hematocrit (Hct, %) was determined using Microhematocrit tubes centrifuged at 12,000 rpm for 3 min and measuring ratio between the sedimented erythrocyte height to the total blood sample height.

Osmolality and Ion Concentration

Plasma osmolality (20 μL) was measured with a micro-osmometer and is reported in mOsmol.kg⁻¹ (The AdvancedTM Micro Osmometer Model 3300, Advanced Instrument Inc.). Sodium concentration (Na⁺, mM) was measured with a sodium-potassium analyzer (420 Dual Channel Clinical Flame Photometer, Sherwood) following the manufacturer's instruction and determined using 5μL plasma and 2 mL buffer (100 mM Na⁺Cl⁻; 10 mM K⁺Cl⁻). Chloride concentration (Cl⁻, mM) was measured with a chloride analyzer (MK II Chloride Analyzer 926S, Sherwood) using 20μL plasma and buffer (0.99 mL of nitric acid 65%, 10 mL acetic acid 99-100%, 3 drops of Tween 20 completed with pure water to reach 100 mL, pH=0.89) titration.

Glucose Concentration

Prior to glucose analysis, 20μL plasma samples were treated with perchloric acid (40μL, 0.33M) for protein removal. Followed by mixing and centrifuging for 10 min at 3000 rpm, 25°C. 1.25 μL of the supernatant was diluted in 1.5 mL of reactive solution and incubated at 38°C for 15 min. Glucose concentration (g.L⁻¹) could then be determined via a standard curve from 0 g.L⁻¹ to 100 g.L⁻¹ read at 436 nm spectrophotometry and calculated according to the following formula:

$$\text{Glucose final concentration} = \left(\frac{(\text{absorbance} - b)}{a} \right) * 100$$

Where a and b are the coefficients of the standard curve (Huggett and Nixon, 1957).

Relative Gene expression

RNA extraction

Gill samples were transferred into RNase free tubes along with beads and 750µL of cold Extract all® before being homogenized for 3 min and left at room temperature (rt) for 5 min in order break down the cell components. 200µL of β-mercaptoéthanol was then added to the mix for protein breakdown, before mixing and being left at rt for 3 min. Samples were centrifuged at 12 000 g for 15 min at 4°C. The supernatant containing the samples RNA was collected, transferred to new RNase free tubes and mixed with 500µL of isopropanol before being kept at rt for 5-10 min. Samples were centrifuged at 12 000 g at 4°C for 10 min for the RNA to precipitate. The liquid was removed with RNase free filtered tips pipette and washed with 750µL of ethanol 75% before vortexing and centrifuging at 7500 g at 4°C for 5 min. After doing this step twice in a row, tubes were left open under a clean air vent for 10-15 minutes for the remaining ethanol to evaporate. Precipitate was washed if 50µL of cold RNase free water for the dissolution of RNA. Concentration and quality of the RNA extraction were done via Nanodrop™ (OD260/OD280 and OD260/OD230 nm absorption ratio) and Gel electrophoresis (1.2% agarose gel). Lab best practices were applied at every step to avoid any possible contamination of the samples and RNA degradation.

Reverse Transcription

Samples were treated with rDNase I (Thermo Fischer Scientific, Waltham, MA, USA) before the reverse transcription to remove any trace amounts of gDNA. RNA was retrotranscribed into double stranded cDNA with RevertAid H Minus First Strand cDNA Synthesis Kit. RT minus control was done to control for any possible gDNA contamination. Samples were diluted 30 times for RT-qPCR analysis.

Primer Design

The primers for the selected genes were designed on NCBI and were to span over an Exon-Exon junction to avoid any gDNA replication and checked on Amplifix. The primers used for housekeeping genes (HKG) and genes of interest are listed in Annex 4. Primer Mix preparation was done by adding 50µL of 0,1 nmol/L Forward and 50µL of 0,1 nmol/L Reverse primers to 900µL of RNase free water. Their efficiency was confirmed with pooled cDNA samples prior to

analysis. The RT-qPCR master mix was done with duplicates of 2.5µL of cDNA samples, 2.5µL of primer mix and 5µL of SYBR®green. The thermocycler protocol for the RT-PCR was divided into a hold phase (10 min at 95°C), 45 cycles of PCR at with 15s at 95°C, a step -1.6°C.s-1 until reaching 60°C for 30s followed by Melt curve stage consisting of 95°C for 15s, -1.6°C.s-1 till 60°C for 1 min and 0.075°C.s-1 till reaching 95°C for 15s. A standard curve of all pooled cDNA samples was done to calculate for efficiency, transcript normalization and relative PCR quantification. The presence of dimers was controlled through melt curves and eventually gel electrophoresis. A GOI/HKG ratio was calculated to assess the difference in expression levels between experimental groups. No difference could be observed between expression levels of selected HKG among the samples.

The standard curve for each gene was drawn on excel from the following formula:

$$y = a. \overline{Ct_{standard\ curve}} + b$$

Standard curve parameter values were used to calculate the relative gene expression.

$$k = \frac{\overline{Ct_{sample}} - b}{a}$$

$$Normalised\ Gene\ Expression = \frac{10^{k_{GOI}}}{10^{k_{HKG}}}$$

Statistical analysis

Results were analyzed on RStudio software and studied under a linear mixed model to predict the effect of our independent variables (rearing groups, salinity conditions and sampling day) and their interaction on our dependent variable of choice. The model included tanks and individuals as random factors. The data was log10 transformed to fit testing assumptions and a post hoc test of type Tukey (95% CI) was led on the least square means of the dependent variables prior to being back transformed to report the values on tables (mean± SEM). Results were also reported on a scaled heatmap and PCA which were clustered with a Euclidian method.

Results

Throughout this work, when only the salinity condition (C0, C10 or C20) is mentioned, we include both rearing groups (FW or SW) in the discussed item, and vice versa. Detailed statistical data from the results can be found in annexes 1 to 3.

Survival rate

Previous research observed no mortality in fingerlings exposed to salinities up to 13 psu up to 60 days. While survival rate dropped to 0 % after 20 days exposure to such conditions (Jahan et al., 2019; Mandal et al., 2020). In juveniles, increased salinity appeared to induce earlier onset of mortality in fish infected with enteric septicemia causing bacteria, *E. ictaluri*. With a survival of 33 % and 8 % after 10 days of exposure to 10 psu and 20 psu (Schmitz et al., 2016). In our study, no deaths were recorded in C0 throughout the whole experiment and remained unchanged post Cadmium challenge. In C10 the first death was observed 4 days post-challenge, with a similar decrease in survival rates (SR) for both rearing groups, which reached $22.81 \pm 10.96\%$ in FW and $26.32 \pm 9.12\%$ in SW 17 days post challenge. Fish in C20 had their SR drop from $93.3 \pm 3.3\%$ and $97.8 \pm 1.9\%$ on the first sampling points to $33.3 \pm 8.1\%$ and $41.9 \pm 8.5\%$ in the second for FW and SW groups respectively. No fish in C20 survived the challenge.

Weight Gain

Rearing groups ($F=7.06$, $p=0.02$) and salinity conditions ($F=4.93.4$, $p<0.0001$) showed a significant effect on the fish's growth. Moreover, their growth under different salinity conditions was modulated by their rearing group ($F=4.51$, $p=0.03$). Individuals in C0 had a mean weight gain (WG) of 44.29 ± 1.69 g and 45.59 ± 1.69 g for FW and SW groups, with no significant difference observed in C10-FW treatment. Fish in C10-SW had a significantly higher weight gain, at 53.5 ± 1.69 g over the same period. The observed difference suggests that the WG of fish in increased salinities is positively modulated by rearing in SW. C20 individuals showed a near zero weight gain (0.76 ± 1.69 g), with no significant difference between the two rearing groups (fig. 3).

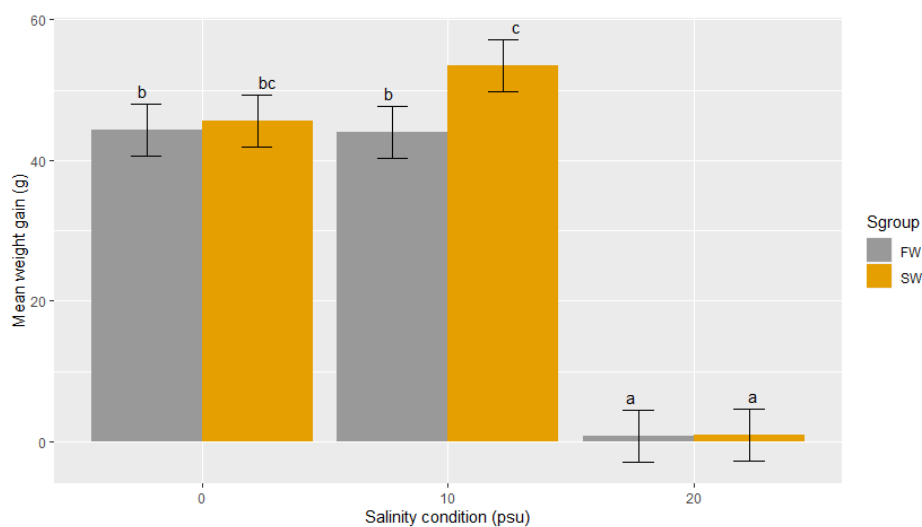


Figure 3: Weight gain in grams (mean $\pm$ CI) for striped catfish by treatment in D40. Different letters denote statistically significant differences between treatments ($p < 0.05$).

Effect of Treatment on Studied Parameters

To better visualize any changes between treatments, which factors have the biggest influence on these differences, and how they correlate, a Principal Component Analysis was performed to data acquired from physiological measurements and assessed genes of interest. In D10, four clusters can be identified, the first consists of individuals from treatments C0-FW, C0-SW and C10-FW, The second by C10-SW, third by C20-FW and finally C20-SW (fig. 4a). Together principal components one and two captures 49% of the variance among samples in the first sampling point (PC3=7,9%). Upon closer look into the data via linear mixed model analysis, only some of the trends distinguishing SW from FW and C20 from C0 and C10 picked by the PCA appeared to present significative difference. Here these differences are indicated with a “*” indicating a p-value < 0.05.

Fish in C20 appear as showing lower values of Insulin Like Growth Factor 1* (*igf-1*), Growth hormone (*gh*) (growth metabolism), aquaporin* (*aqp*) and Na⁺K⁺ATPase-beta 2 subunit (*nkab2*) (osmoregulation pathway), glutathione peroxidase* (*gpx1a*), catalase (*cat*) (oxidative stress response pathways) which appeared as inversely correlated with lysozyme (*lyz*), interleukin-1-beta* (*il-1b*), Tumor Necrosis Factor (*tnfa*) (inflammatory response pathway), Heat-shock Protein-70* (*hsp70*), *hsp60** (oxidative stress response pathways), Na⁺K⁺ATPase* (*nka1a1*) (osmoregulation pathway) and Cytochrome oxidase (*cox4*) (energy metabolism) along with a non-correlated increase of their osmolality* and Ion concentration*.

The main variables appearing to explain the variance between rearing groups were Na⁺K⁺2Cl⁻ cotransporter (*slc12a1*), Inositol Monophosphatase (*impa1*), *aqp* (osmoregulation pathway), *tnfa* (inflammatory response pathway), *cox* (energy metabolism), *cat* (oxidative stress response pathways), *gh* and *igf* (growth metabolism) along with WBC count, osmolality, sodium, Chloride and Glucose concentrations, which showed generally lower, but non-significant values in SW than FW fish in D10 according to the PCA and linear mixed model.

In the second sampling point, the four clusters could still be identified. The PCA could only capture 38,4% of the samples variance with the measured variables (PC3=12.5%). The explanatory variables that appeared to be high in C20 now appear under-expressed or on the same level as C0 and C10. Only *ghrb** exhibits higher values. From the PCA projection, SW can still be distinguished from FW mostly due to *il-1b*, *hsp70*, *hsp60*, *cox4*, *nka1a1*, Osmolality and Na⁺, Cl⁻ ions concentrations which present a non-significative increase in their expression in these treatments and low RBC and Hct (fig. 4b).

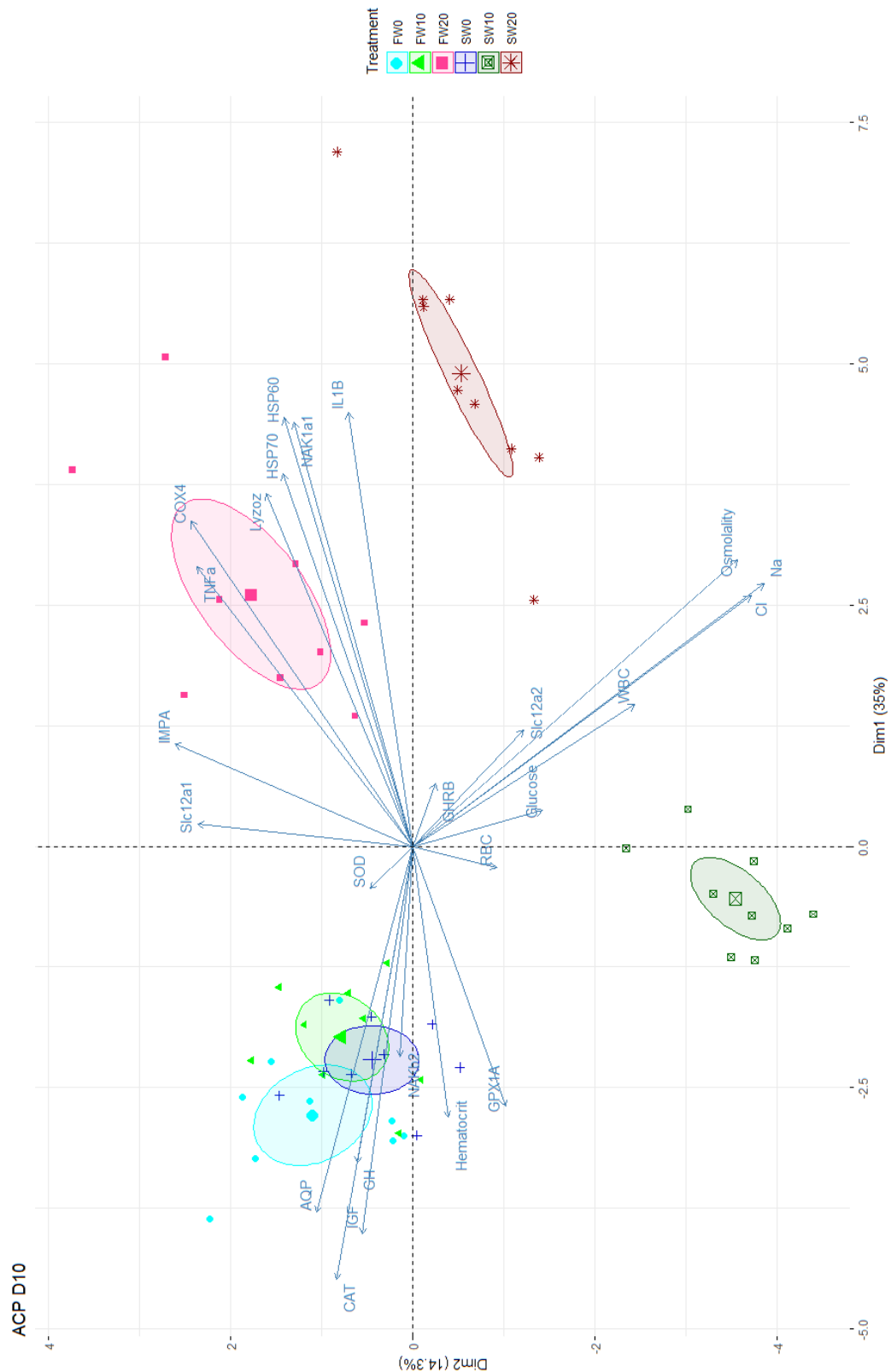


Figure 4a: Principal component analysis plot projecting samples and measured variables on D10. Ellipses are confidence type. Treatments can be distinguished by color. C0-FW (Light blue), C0-SW (Dark blue), C10-FW(Light green), C10-SW(Dark green), C20-FW(Light red), C20-SW (Dark red).

Osmolality and ion concentration

A detailed examination of these characteristics reveals that Osmolality, Na^+ , and Cl^- were all strongly impacted by salinity conditions and sampling day ($p < 0.001$). Moreover, the values in different conditions appeared modulated by the sampling day ($p < 0.01$). These parameters showed increased values as salinity increased. Though no significant differences between SW and FW could be noted and differences dampened in later sampling points.

The fish's osmolality in C0 showed no significant difference through the whole experiment and ranged around $281.77 \pm 2.02 \text{ mOsmol.kg}^{-1}$ in FW and $278.4 \pm 2.02 \text{ mOsmol.kg}^{-1}$ in SW. In C10, the fish's osmolality was of $306.13 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (FW) and $294.91 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (SW) in D10 and showed a non-significant decrease in D40 to $291.89 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (FW) and $294.42 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (SW). Post-challenge, osmolality, Na^+ and Cl^- values remained unchanged for individuals in C0 but all rose for individuals in C10. The recorded osmolality from fish in C20 is far higher than other conditions leveling at $417.87 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (FW) and $414.02 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (SW) in D10 and in D40 394.59 ± 2.02 (FW) and $393.5 \pm 2.02 \text{ mOsmol.kg}^{-1}$ (SW).

Glucose

Salinity conditions ($F = 8.87$, $p < 0.001$) and sampling day ($F = 43.59$, $p < 0.0001$) appeared to have a significant effect on blood glucose levels. Moreover, the values of glucose in given conditions were modulated by the sampling day ($F = 14.46$, $p < 0.0001$). In D10, individuals in SW all showed lower levels of glucose than FW, although non-significant. Levels appeared to slightly increase with higher salinities. Glucose of FW individuals going from $70.62 \pm 2.08 \text{ g.L}^{-1}$ in C0, $73.9 \pm 2.08 \text{ g.L}^{-1}$ in C10 and $84.54 \pm 2.08 \text{ g.L}^{-1}$ in C20 and SW individuals going from $59.67 \pm 2.08 \text{ g.L}^{-1}$ in C0, $66.68 \pm 2.08 \text{ g.L}^{-1}$ in C10 and $65.46 \pm 2.08 \text{ g.L}^{-1}$ in C20.

In D40, this trend was no longer seen and individuals in C20 had significantly lower glucose levels than individuals at C0 and C10 (C20: $35.82 \pm 2.08 \text{ g.L}^{-1}$, C0: $54.85 \pm 2.08 \text{ g.L}^{-1}$) and close to no difference could be noted between rearing groups. Post challenges, the levels of this parameter showed close to no difference from D40.

Hematological parameters

Erythrocyte concentration and Hematocrit

Salinity conditions appeared to have a significant effect on RBC ($F = 7.5$, $p = 0.005$ RBC) while Hct was affected by salinity conditions ($F = 61.4$, $p < 0.001$) and sampling day ($F = 19.45$, $p < 0.0001$).

Rearing groups did not show to cause significant effects on these parameters ($F=0.14$, $p=0.71$ RBC, $F=3.2$, $p=0.09$ Hct).

In D10, no significant differences could be observed in RBC ($1.71 \pm 0.21 \times 10^6$ cells. μL^{-1} C0-FW). Hct of individuals in C0 appeared higher than individuals in saltier conditions, with individuals in C10 having a significantly lower value at $29.94 \pm 2.06\%$ ($p=0.001$), compared to $34.83 \pm 2.06\%$ and $38.86 \pm 2.06\%$ for individuals in C20 and C0 respectively.

In D40, Hct from individuals rose from $38.86 \pm 2.06\%$ to $50.61 \pm 2.06\%$ in C0 and $29.94 \pm 2.06\%$ to $44.94 \pm 2.06\%$ in C10. At that sampling points Hct and RBC from fish in C20 ($25.49 \pm 2.06\%$, $0.99 \pm 0.21 \times 10^6$ cells. μL^{-1}) were significantly lower than those in C0 and C10 ($1.85 \pm 0.21 \times 10^6$ cells. μL^{-1} for FW-C0) ($p<0.01$). Post challenges, Hct in C0 and C10 leveled at similar values as the ones from D10 with non-significant changes in RBC count.

White Blood Cells

Rearing groups, salinity conditions and sampling day showed a significant effect on the fish's WBC ($p<0.0001$). Moreover, the value of WBC for fish in different salinity conditions was modulated by their rearing group ($F=3.5$, $p=0.03$) and sampling day ($F=3.87$, $p=0.01$).

In D10, SW individuals displayed non-significant lower WBC concentration than FW individuals for C0 and C10 conditions with close to no differences between rearing groups in C20. Also in D10, individuals in C10 showed the lowest WBC count at $4.82 \pm 0.22 \times 10^4$ cells. μL^{-1} (FW) and $3.93 \pm 0.22 \times 10^4$ cells. μL^{-1} (SW). C20 showed significantly higher values at $9.4 \pm 0.22 \times 10^4$ cells. μL^{-1} (FW) and $9.55 \pm 0.22 \times 10^4$ cells. μL^{-1} (SW) and C0 showed intermediate values at 7.93×10^4 cells/ μL (FW) and $5.14 \pm 0.22 \times 10^4$ cells. μL^{-1} (SW).

In D40, WBC dropped and showed no significant differences among all treatments with values ranging from $4.56 \pm 0.22 \times 10^4$ cells. μL^{-1} in C0-FW and $2.34 \pm 0.22 \times 10^4$ cells. μL^{-1} in C10-SW. No significant changes were observed in SW-C10 post challenge whereas WBC in all other treatments rose. FW-C0 had the steepest reaction observed with $11.64 \pm 0.22 \times 10^4$ cells. μL^{-1} , followed by FW-C10 and SW-C10 individuals at $6.29 \pm 0.22 \times 10^4$ cells. μL^{-1} and $5.59 \pm 0.22 \times 10^4$ cells. μL^{-1} respectively.

Discussion

The aim of this experiment was to determine if treatment of striped catfish larvae to higher salinities would allow for conditioning hormesis and adapt to hyperosmotic chronic stress. Adaptive animals can develop superior change capabilities for a specific environment and thus resist it better, which is relevant in the case of sea level rise (Sae-Lim et al., 2017). This was done by exposing fish larvae to 5 psu conditions, based on findings from Hieu et al. (2021) that found it to be the optimal concentrations for hormetic dose response pattern (Hieu et al., 2021). Which Calabrese et al. (2007) supports as being the optimal dose to be used in conditioning treatments (Calabrese et al., 2007). As we wanted to assess the effect of chronic stress on this species, we focused on variables under the influence of secondary (hematology, ion concentration, glucose and mRNA abundance) and tertiary (growth and survival) stress responses. These take part in an individual's long-term stress response and occur as a result of the quick primary stress response characterized by the release of catecholamines and corticosteroids from the hypothalamic-pituitary-adrenal (HPI) axis (Fiol and Kültz, 2007; Seibel et al., 2021).

As chronic hyperosmotic stress intensifies, eustress turns to distress.

Before discussing the effects of early exposure to increased salinities, we need to understand the effect each condition had on striped catfish juveniles.

Phuc et al. (2017) found that juvenile striped catfish achieve eustress despite being exposed to increased salinities of up to 12 psu and temperatures of up to 35°C (Phuc et al., 2017). Moghadam et al. (2017) reported a similar trend in which gene expression levels of *S. salar* subjected to extended periods of stress during embryogenesis and post-hatching stages returned to levels equivalent to control fish, suggesting the existence of an adaptive response to stress in these fish (Moghadam et al., 2017). In our study, genes whose expression appeared to be significantly altered in higher salinity conditions in D10 seemed to be dampened in D40. This is apparent in the heatmap (fig. 5), where the color intensity of gene expression decreases from D10 to D40. Following Phuc's and Moghadam's research line of thought, this would thus be indicative of adaptation in fish in from our experiment. These adaptations, however, could only be attributed to eustress in individuals from C10, while fish from C20 display typical indications of maladaptation and distress, such as a high death rate and a poor growth rate by D40 and could not tolerate the additional burden posed by the Cadmium challenge.

The purpose of the Cadmium challenge was to understand how fish would cope with and additional source of stress and to elicit a reaction from the immune system of individuals. This

heavy metal was prioritized over a bacterial challenge, as bacteria might show different virulence for different salinity control that we cannot account for. Wingfield suggests that once a stress threshold is crossed, gene expression is ablated in order to decrease the maladaptive stress response associated with allostatic overload (AOL) (Wingfield et al., 2011). This is in line with the theory proposed by Petitjean et al. (2019), which states that when fish cannot maintain their aerobic metabolism, which is comparable to an AOL1, they adopt a "conservation strategy" rather than a "compensation strategy" similar to an AOL2 in which they face the harsh environmental conditions without setting up response mechanisms to fight against the stress (Petitjean et al., 2019). This has been detected in a number of species (Sopinka et al., 2016).

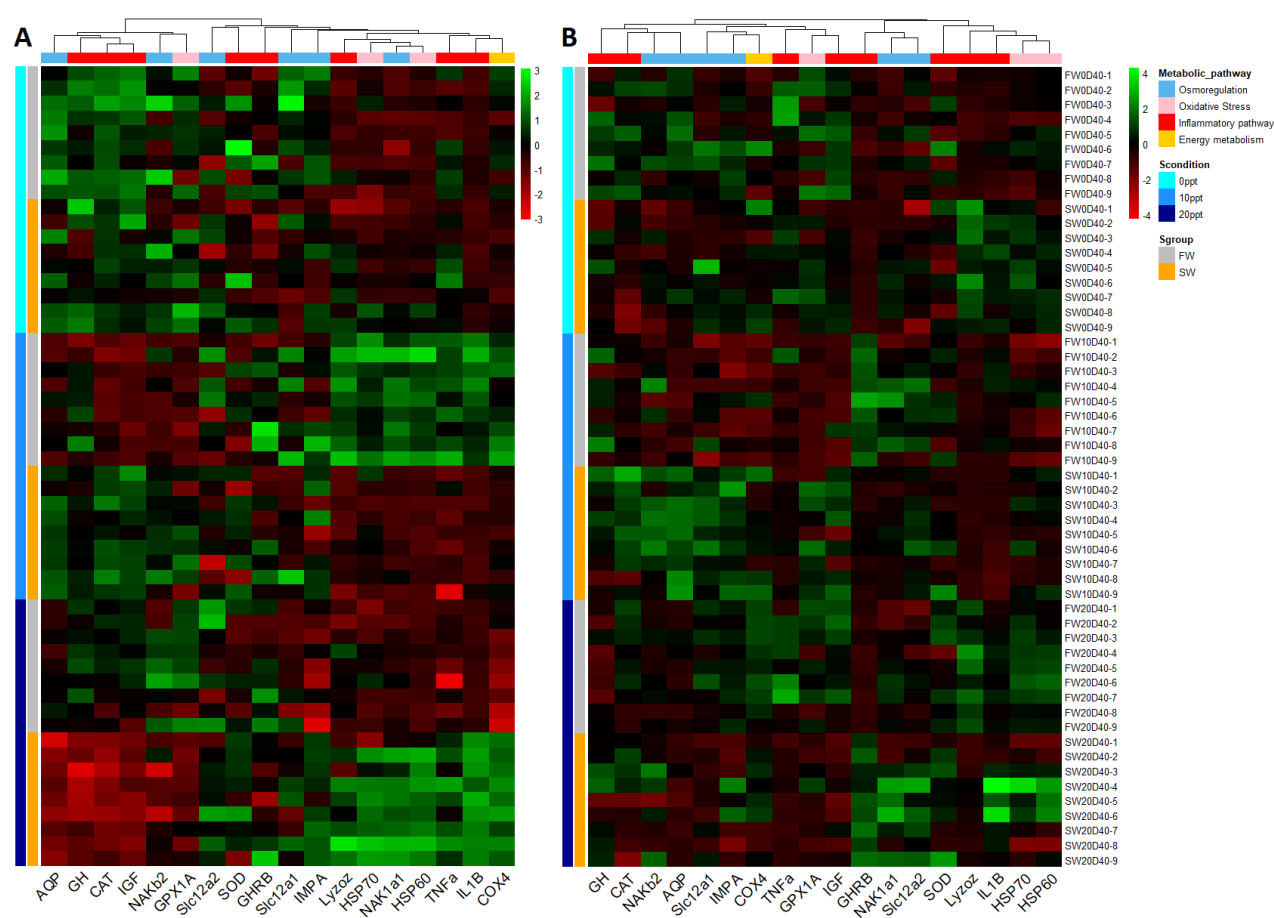


Figure 5: Clustering of gene expression per treatment. Heatmap of differential gene expression in individuals exposed to distinct treatments in D10 (A) and D40 (B). Done following scaling and correlation clustering. Columns show genes studied and individuals are displayed by rows.

Madara notes that stressful situations release cortisol that inhibits the circulation of growth hormones such as *gh* and *igf* (Madara et al., 2020). This decrease is also associated with a switching away from growth toward coping with stress (Robinson et al., 2019). In our experiment,

the abundance of mRNA in C20 was low in *gh* (lower for SW) and very low for *igf* in comparison to C10 and C0. When exposed to the cadmium challenge, the value for these parameters significantly dropped in C0 and C10 indicating the perceived stress from these individuals and the stressed state of C20 fish.

It has been established that striped catfish growth rate is highly influenced by water salinity. Fish under 4 psu to 10 psu show increased specific growth rate compared to freshwater and higher salinities which demand increased energy allocation for regulatory volume increase (Bœuf and Payan, 2001; Hieu et al., 2021; Jahan et al., 2019; Mandal et al., 2020; A. L. Nguyen et al., 2014). The gene expression analyses revealed changes in the expression level of many genes in response to the experimental conditions. They are part of the stress response set up to regain a homeostatic level but appear to be not sufficient. Indeed, we note a decrease in the expression of freshwater type chloride cells solute transporter *slc12a1*, and *nkab2* while sea water type chloride cells *slc12a2*, and *nka1a1* expression tends to increase in individuals with higher salinity. Similarly, levels of *impa*, which takes part in the biosynthesis of myo-inositol, an important osmolyte in the process of regulatory volume increase (RVI), appears higher in D40 (although it shows a negative correlation with increasing salinities, which is contrary to other findings such as noted by Kültz (2015); thus, further attention needs to be carried to understand this behavior). The *aqp* expression levels responded to salinity by decreasing its levels, fish in SW also tended to show an even lower level. This same reaction has been observed in the literature and similar experiments lead o this species and is reckoned as a mechanism allowing fighting against the passive loss of water between cell membranes but also important osmolytes needed to maintain cell volume in hyperosmotic conditions (Alfieri and Petronini, 2007; Jumah Yashier Upling, 2020; Ruhr et al., 2016; Schmitz et al., 2017b).

The costs of such metabolic changes are usually compensated by an increased expression of genes related to the Krebs cycle as well as the mitochondrial respiratory chain (Thanh et al., 2014). Nguyen et al. (2016) observed upregulation of *cox* in striped catfish when exposed to increased salinity. In this study, *cox* showed a similar tendance although not significant in D10. In D40, *cox* expression appeared significantly higher than in D10. Individuals in C20 had the lowest expression and C10 highest and SW fish showed increased expression compared to FW, although these differences were not significative. The low expression from *cox* coupled with the lowered levels of glucose for individuals in C20 further points to a possible exhaustion of energetic reserves following adaptation attempts from these individuals.

Hyperosmotic stress is known to generate higher levels of reactive oxygen species that the organism detoxifies with increased production of antioxidant enzymes such as *gpx1a*, *sod* and *cat* (Birnie-Gauvin et al., 2017; Seibel et al., 2021). In our study, *sod* remained unchanged for individuals in C10 and showed a slight increase in fish exposed to C20 in D40 while *gpx1a* and *cat* showed a negative trend with increasing salinities that flattened in D40. A heat shock type reaction could be noted in D10 as individuals in C20 showed significantly higher expression for *hsp70* and *hsp60* which are chaperone produced to cope with stress (Deane et al., 2002). Their expression leveled with other conditions in D40 possibly indicating the adoption of a conservation strategy.

Chronic hypertonicity was found to trigger the release of pro-inflammatory cytokines which can cause excessive inflammation and immunopathological conditions in striped catfish (Chen and Nuñez, 2010; Schmitz et al., 2017b, 2016; Seibel et al., 2021). Schmitz et al. 2017, found exposing juvenile striped catfish to a slight increase of salinity stimulates immune factors and leads to excessive inflammatory response after 20 days in 20 psu (Schmitz et al., 2017a). Here, in D10, we observed a progressive but non-significant increase of lysozyme, *tnf-a*, a significantly higher count of WBC and a 10-fold peak in *il-1b* expression in gills from fish in C20 which points to an increased inflammatory reaction triggered by the hyperosmotic stress. By the 30th day of exposure to these conditions, in D40, the expression of these genes and WBC in C20 fish had leveled down to values close to those unstressed fish as it did for *hsp*. Given the high mortality of fish in this condition, this leveling recall more the adoption of a conservation strategy, done to halt the possible excessive inflammatory reaction than signs of adaptation to such strong hyperosmotic conditions (Petitjean et al., 2019).

Phuc et al. (2017) found a significant decrease in Hct in fish exposed to 12 psu. This happens following the increased loss of water to hyperosmotic external environment affecting the plasma volume and Hct ratio. Our results showed significantly lower levels for these Hct and RBC in C20 compared to C0 and C10 following a 30-day chronic exposure.

Early salinity exposure group show signs of conditioning hormesis

Analyzing WG and survival can give us a good understanding of the fish's general condition and inform on its allostatic load (Madaro et al., 2020). Fish in 10 psu seemed to be in a eustress state. What stands out is that only fish in C10-SW showed an increased WG averaging to be 21.6 % greater than for fish in C10-FW and C0. Individuals in C20 showed clear signs of distress and AOL1 with close to zero WG and high mortality rates, which were steeper in FW. Rearing fish in

SW positively modulated WG in all conditions but only shown to lead to significant differences with SW in C10.

The PCA performed clearly showed a distinction between fish from SW-C10, SW-C20 projected on the one side and FW-C10, FW-C20 on the other, and only a little one for FW-C0 and SW-C0. This might be indicative that the fish's metabolism changed depending on whether the fish had undergone early exposure to increased salinities or not. Additionally, that when individuals were exposed to freshwater conditions, the changes brought by rearing groups are less expressed, and becomes more pronounced when exposed to hyperosmotic conditions. This phenomenon could be at the origin of the positive effect SW had on weight gain and led to a more gradual mortality rate in C20 which was of $33,3 \pm 8.1$ % in FW and $41,9 \pm 8.5$ % in SW in D40. The lack of a significant difference in growth across rearing groups in C0 could be explained by the fact that the changes brought on by the rearing group allow the fish to present an increased fitness when faced with the stress again, but do not provide a competitive advantage in non-stressful environments. Whereas the lack of a significant difference in growth across rearing groups in C20 might be attributed to the negative effects of AO1 outweighing the positive effects of early exposure to increased salt. This inference must be approached with care as no significant differences of measured variables were identified by the statistical model between rearing groups, only tendencies at best. However, an increasing body of research appears to support this hypothesis. First, Calabrese (2008) argues that threshold models based on standard hypothesis usually underestimate the frequency of hormesis events (Calabrese et al., 2008). Second, research led on rainbow trout (*O. mykiss*) found, on the one hand, that individuals exposed to stress in early life stages had long-lasting effect on the HPI-axis sensitivity to stress (Auperin and Geslin, 2008) and in the other hand that rainbow trout selected for low cortisol response show increased specific growth ratio compared to high cortisol selected (Fevolden et al., 2002). Atlantic salmon (*S. salar*) exposed to stress in the smolt phase presented a greater growth during usually stress triggering periods of smoltification and transfer to sea water and had reduced hypothalamic catecholaminergic and brainstem serotonergic responses to stress (Vindas et al., 2016). All these observations, including ours, fit the definition of hormesis as individuals show signs of differentially expressed molecular pathways that lead to better stress coping. Here, the cumulative sum of these small alterations in the expressivity of the variables, despite their low level of significance, could then be sufficient to influence the overall individual's fitness.

Conclusion

Increasing salinities triggered an allostatic reaction in striped catfish that allowed for individuals to reach eustress in 10 psu and created an allostatic overload in 20 psu. Exposing larvae to 5 psu led to multiple small changes in their physiology that brought no advantage in a non-stressful environment but could be the reason that made them appear more adapted to increased salinities. Fish treated with 5 psu got to present a better response to 10 psu conditions and achieved a growth 21,6% higher than non-treated fish. The possible advantages brought by this treatment appeared to be insufficient in the face of the serious burden posed by the 20 psu conditions that created a type 1 allostatic load. It would therefore be relevant to test this in salinities slightly lower than 20 psu so that the burden caused by hyperosmotic stress would not create such a strong type 1 allostatic overload. Climate models predicting sea-level rise does not yet indicate that the salt front will reach such elevated salinities. Considering these facts, we propose to perform this experiment with salinities between 15 psu and 18 psu.

Moghadam et al. found that the potential for stress conditioning is dependent on which early life stages the animal is exposed. He observed that *S. salar* showed better fitness to stress when exposed to stress before and after larval hatching than individuals that were exposed only before or after hatching. Furthermore, he observed changes in the epigenome of these stressed individuals in mapping regions close to genes related to stress response mechanisms suggesting the involvement of epigenetic mechanisms in responses to these conditions (Moghadam et al., 2017; Robinson et al., 2019). So, in future experiments, along with an exposure to a broader range of salinity, testing which time of treatment to increased salinities have the best outcome and the epigenetic signatures it creates could be of great interest.

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