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Time-course of cortisol and glucose plasma concentrations in flathead grey mullet (*Mugil cephalus*) categorized according to their proactive and reactive stress coping styles

Línea de tiempo de las concentraciones plasmáticas de cortisol y glucosa en lisa (*Mugil cephalus*) categorizados de acuerdo a su estilo de afrontamiento al estrés de tipo proactivo y reactivo

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ABSTRACT

In vertebrate, cortisol is a steroid hormone related to stress that takes part in metabolic and physiological processes that prepare animals for a possible flight or confrontation. In fish farming, organisms are exposed to stressful conditions that could affect their welfare and quality. In aquaculture, fish are exposed to constant stressful situations that could affect their welfare and quality. The objective of this study was to analyze plasma concentrations of cortisol and glucose in *Mugil cephalus* during a period of 24 hours after exposure to an acute stress. Additionally, stress coping styles were characterized to determine the impact of proactive or reactive responses on cortisol production. Cortisol concentration peak appeared at 15 min post-stress (145.93 ng·mL^{-1}), and glucose plasma was observed at 30 min post-stress (43.00 mg·mL^{-1}). Homeostatic state was re-established at 120 min post-stress for both cortisol (40.03 ng·mL^{-1}) and glucose (30.00 mg·mL^{-1}) blood concentrations. Proactive fish presented a significantly lower ($p < 0.05$) cortisol level elevation than reactive fish after stress exposure and brought their homeostatic stage back down faster than reactive fish. These results may be of interest to the aquaculture industry to improve welfare and management protocols.

KEY WORDS: Corticosteroid, behaviour, fish, stress response.

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RESUMEN

En vertebrados, el cortisol es una hormona esteroidea de respuesta al estrés que interviene en procesos metabólicos y fisiológicos que preparan a los animales para huir o enfrentar el estrés. En acuicultura, los peces están sometidos a situaciones de estrés constantes que pueden afectar su salud y calidad. El objetivo de este estudio fue analizar concentraciones plasmáticas de cortisol y glucosa en *Mugil cephalus* durante un periodo de 24 horas tras la exposición a un estrés agudo. Adicionalmente, se caracterizaron los estilos de afrontamiento al estrés, y se evaluó el impacto de presentar respuestas reactivas o proactivas sobre la producción de cortisol. La concentración máxima de cortisol se observó a los 15 min post-estrés (145.93 $ng \cdot mL^{-1}$) y la de glucosa a los 30 min post-estrés (43.00 $mg \cdot mL^{-1}$). El estado homeostático se recuperó a los 120 min post-estrés, tanto para las concentraciones plasmáticas de cortisol (40.03 $ng \cdot mL^{-1}$) como de glucosa (30.00 $mg \cdot mL^{-1}$). Los peces proactivos presentaron niveles significativamente ($p < 0.05$) inferiores de cortisol que los peces reactivos y recuperaron su estado homeostático más rápido que los peces reactivos. Estos resultados pueden ser de interés para la industria acuícola para mejorar protocolos de bienestar y manejo de los organismos.

PALABRAS CLAVE: Corticosteroides, comportamiento, peces, respuestas al estrés.

Introduction

Flathead grey mullet (*M. cephalus*) is a cosmopolitan fish species that inhabits tropical, subtropical, and temperate oceans around the world. This euryhaline fish species can live in both brackish and saline waters and exhibits omnivorous and detritivorous feeding habits (Saleh, 2008). According to Crosetti & Blader (2016), the main aquaculture flathead grey mullet producers worldwide are Italy, Iran, Israel, and Egypt; which have achieved the biological cycle of this species in captivity. In Mexico, the market for flathead grey mullet is principally sustained by commercial fishing. However, captures of this fish species have significantly increased from 8,600 tons per year in 2014 (DOF, 2015) to more than 12,000 tons in 2020 (SADER, 2021), and therefore, mullets could be considered as a candidate fish species to diversify the Mexican aquaculture industry, due to its well acceptance in local markets, specifically in Nayarit, Veracruz, and Sinaloa, which are the main consumers in the country (CONAPESCA, 2018).

Aquaculture is based on the control of environmental parameters and the use of balanced feeds for the production of aquatic organisms, in open, semi-closed, and closed systems (FAO, 2024). However, actual aquaculture practices induce multiple stressful conditions (i.e., constant

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handling, stocking densities) that could affect important productive parameters, such as growth or reproduction (Sopinka *et al.,* 2016). Furthermore, long-term chronic stress could lead to physiological alterations with detrimental effects on fish productivity (Schreck & Tort, 2016). Stress is defined as the physiological cascade of events that occurs when an organism tries to resist death or restore its homeostasis during aversive situations (Schreck & Tort 2016). According to Bordin & Freire (2021), the three main stages of stress responses are: i) *Primary reaction*, which is the activation of the hypothalamic-pituitary-interrenal axis (HPI) through the secretion and synthesis of corticosteroid hormones and catecholamines; ii) *Secondary reaction*, which includes cardiovascular and respiratory alterations, by promoting the mobilization of oxygen and energetic substrates towards the bloodstream, such as glucose; and iii) *Tertiary reaction,* which is related to chronic stress situations, affecting productivity (growth, resistance to diseases, or reproduction). Fish adaptive capacity to variations in physical, chemical and biological environmental parameters, has been referred as Stress Coping Styles (SCS), defined as the coherent set of behavioural and physiological responses to stress, which are consistent over time and across contexts and range between two extremes of behaviour defined as: proactive (or bold), and reactive (or shy) (Koolhaas et al., 1999). Compared to reactive organisms, proactive ones are more prone to an explorative and highly active behaviour, risk-taking in unknown environments and they produce less cortisol (Øverli *et al.,* 2007; Alfonso *et al.,* 2020). In aquaculture, SCS has an impact on important production parameters such as reproduction, immune response, growth, adaptability, and fingerling quality, among others (see the review by Castanheira et al., 2017).

Cortisol is considered as the principal biomarker of stress (Sopinka *et al.,* 2016). In fishes, its production starts with the activation of the HPI axis, with the release of two hormones by the hypothalamus: corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH), which enhance the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland. Cortisol impacts several body tissues and physiological processes, particularly on inflammation and on lipid and carbohydrate metabolism, by promoting gluconeogenesis, which, successively, promotes the production of energy that is necessary for escaping or fighting in stressful situations (James & Tomas, 2023). Besides, higher cortisol concentrations may affect biological processes of fish such as reproduction (Faught & Vijayan, 2019) or disease resistance (Guo and Dixon, 2021). Moreover, it is well known that cortisol is released in the circulatory system several minutes after a stress situation, however, its regulation and duration in the system varies among fish species in accordance to age, sex, and stress responsiveness (Samaras, 2023). Hence, this study aimed to evaluate the regulation of plasma concentrations of cortisol and glucose over a period of 24 h after an acute stress situation in flathead grey mullet (*M. cephalus*) juveniles. Fish were also subjected to different stress coping style tests to characterize proactive and reactive behaviour and to compare the regulation of cortisol homeostasis between both behavioural profiles. The results of the present study will contribute to the understanding of stress management in flathead grey mullet to optimize rearing protocols.

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Material and methods

Fish handling and rearing system management

A total of 100 mullets were captured from the wild in Mazatlán, Mexico and transported to the Nayarit Centre for Innovation and Technological Transference (CENITT-UAN) in Nayarit, México. Fish have been adapted to rearing conditions for six months. A total of 25 organisms, with a mean initial weight of 257.40 ± 110.00 g and total length of 29.15 ± 4.00 cm, were used for the present experiment. All fish were tagged (FRD, 0.5mmØ; China) for identification. Fish were maintained in three 220 L (80 cm length, 68 cm high, 48 cm width) rectangular tanks, connected into a recirculation aquaculture system (RAS) and water parameters were maintained as follows: pH (7.3 ± 0.2), salinity (25.3 ± 0.7 ppm), oxygen (6.5 ± 0.2 $mg \cdot L^{-1}$), temperature (26.4 ± 0.5 °C), ammonium (<0.05 $mg \cdot L^{-1}$), nitrites (<10 $mg \cdot L^{-1}$), nitrates (<100 $mg \cdot L^{-1}$) and alkalinity (>100 $mg \cdot L^{-1}$). All parameters were measured every morning, except ammonium, nitrites and nitrates, which were measured twice a week (Multiparameter HI 83300, HANNA). Three months prior to the experiment, RAS photoperiod was adjusted to 10 h light and 14 h darkness and lights were turned on at 08:00 h and turned off at 18:00 h (Figure 1), with an electronic dimmer (MyTouchSmart, General Electric®), to regulate flathead grey mullet circadian rhythm along the experiment, following recommendations of Kitagawa *et al.* (2015). Flathead grey mullets were fed three times a day until apparent satiety with a commercial diet (protein: 35 %, lipid: 16 %; Skretting®).

Morphometric measurements

Fish were measured (ichthyometer Biologika®) and weighted (electronic balance Rhino® model BAPRE-3; 3000g-resolution 0.01g) two weeks after their acclimatization (initial point) and three months after (final point), once blood extractions were performed on all fish. Specific Growth Rate (SGR) was calculated with the following formula:

$$SGR = \frac{\ln(\mathrm{Wf}) - \ln(\mathrm{Wi})}{\mathrm{t}} * 100$$

Wi: initial weight (in g), Wf: final weight (in g), t: duration between both measurements (in days).

Time-course of cortisol and glucose plasma concentrations.

Firstly, basal levels of cortisol and glucose of unstressed fish were measured after one month of acclimatization and two months before the beginning of the experiment. For this, blood was extracted from the caudal vein (0.5 mL) of 5 unstressed flathead grey mullets, selected randomly from the different holding tanks, with an insulin syringe (23G; Corporate DL) coated with heparin (Inhepar -Pisa®- 5000 IU/mL). Once blood samples were extracted, all fish (n=25) were distributed randomly into five tanks of 220L (five fish per tank) and each tank was marked following the assessed time points, being: 15, 30, 180, 360, and 1440 min post-stress. All fish were anesthetized (eugenol; 150 ppm) to facilitate handling and blood extraction. Previous methodology was based on Arkert *et al.* (2020) and Linares-Cordova *et al.* (2024).

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The time-course experiment started at 08:00 h. At this time, all fish were subjected to a three-minute stress condition, which consisted of pursuing fish (without capturing them), with a nylon net, simultaneously in all tanks, in a zig-zag movement (Samaras *et al.,* 2015). At the end of the stress exposure, blood extraction was performed in each of the tanks, respecting the established times. Blood samples were centrifuged at 3000 g for 15 min at 4 °C (1580R GYROZEN®), supernatant plasma was collected, and two aliquots (250 µL for cortisol and 150 µL for glucose) were obtained and stored at -80 °C (Haier Bio-medical) until analysis. Cortisol was measured in triplicate with a commercial enzyme-linked immunosorbent assay kit (Cayman Chemical®, USA Cortisol ELISA Kit Item No 500360), with a detection range of 6.6 to 4,000 pg·mL^{-1}, an inflection point (50 % B/B0) of 150 to 210 pg·mL^{-1}, a sensitivity (80 % B/B0) of 35 pg·mL^{-1} and absorbance was read at 410 nm. Glucose was measured in triplicate with a commercial colorimetric assay kit (Cayman Chemical®, USA; detection range: 0 – 25 mg/dL), and absorbance was determined at a wavelength of 520 nm. Both concentrations were quantified through a spectrophotometer (Thermo Scientific®, Multiskan Skyhigh), following the protocol described by Ibarra-Zatarain *et al.* (2016) and Jiménez-Rivera *et al.* (2023).

Stress coping styles assessment.

These assays were performed one month after finalizing the cortisol and glucose time course trial. All fish were submitted to a single group test and three individual stress coping style tests, as defined ahead.

Group test.

The risk-taking test assessed flathead grey mullet disposition to cross from a safe area (isolated from light) to a risky area (exposed to light). For this, a rectangular fiberglass tank (77 cm length, 50 cm high, 48 cm width), with a plastic barrier dividing the tank into two equal parts, was used. The barrier comprised a small window (10 x 10 cm) in the middle to allow fish to cross between both areas. Risk-taking tests were performed five times independently, one run per experimental tank (n = 5). Trials started by placing fish in the safe area for one hour to allow acclimatization of the fish, and the window was thus opened for two hours. Two video cameras (Swann, Smart Security System®) were used to record fish behaviour and corroborate the results. Individuals that crossed from the safe to the risky area during the 2h-trial were classified as proactive (or bold), while fish that remained in the safe area were identified as reactive (or shy). Previous methodology and characterization of fish was based on Ferrari *et al.* (2020) and Jiménez-Rivera *et al.* (2023).

Individual tests

***a) Restraining*:** Consisted in capturing fish individually with a nylon net and in maintaining it inside the water, during a period of 3 min, while 2 behavioural variables were evaluated: 1) number of escape attempts in restraining (NEAR), considered as each sudden movement or contortion made by fish to free itself from the net and 2) total activity time in restraining (TATR), defined as the total time (in seconds) that fish spent moving in forward direction, following criteria

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of Jiménez-Rivera *et al.,* (2023); ***b) Confinement*:** This test was performed in a small plastic tank (40 cm length, 29 cm high, 25 cm width), and the bottom of the tank was divided into quadrants (10 cm length, 4.5 cm width). Two behavioural variables were evaluated during a period of 3 min: 1) total activity time in confinement (TATC), defined as the total time (in seconds) that fish presented forward locomotion and 2) distance travelled in confinement (DTC) defined as the total distance (in cm) travelled by fish. Quadrants were counted focally, only considering movement when fish's entire body crossed from one quadrant to another in a straight line forward, horizontally, vertically, or diagonally. Two video cameras (Swann, Smart Security System®) were used to corroborate the results. Previous methodology was based on Ibarra-Zatarain *et al.* (2015) and Jiménez-Rivera *et al.* (2023); ***c) New environment:*** This test was performed in a rectangular tank (77 cm length, 50 cm high, 48 cm width), which bottom was divided into quadrants (15.5 cm length, 12 cm width), and three behavioural variables were measured for 3 min: 1) number of escape attempts in new environment (NEANE), represented as each fish movement aiming to escape from the tank; 2) total activity time in new environment (TATNE), defined as the total time (in seconds) of fish forward locomotion and 3) distance travelled in new environment (DTNE), quantified by counting focally the quadrants (in cm) crossed by fish vertically, horizontally and diagonally. Two video cameras were used to corroborate the results. Methodology was adapted from Ibarra-Zatarain *et al.* (2015) and Jiménez-Rivera *et al.* (2023).

Statistical analyses

IBM SPSS V24 was used for statistical analysis. Data is presented as mean ± standard deviation of the mean. Normality and homoscedasticity of data were checked the Shapiro-Wilks and Levene tests. A 95 % confidence interval ($p > 0.05$) was set for all analyses. A one-way analysis of variance (ANOVA) was performed on cortisol or glucose blood concentrations to compare levels between the different time points of blood extraction (0, 15, 30, 120, 360, and 1440 min after exposure to an acute stressor), with a Bonferroni post-hoc test when significant differences were observed. A Student *t*-test was performed to identify differences in cortisol and glucose blood levels between proactive and reactive fish, in response to an acute stress at each different time points of blood extraction. A Multivariate Analysis of Variance (MANOVA) was performed to compare the seven behavioural variables of the three individual tests, as well as weight, length and specific growth rate (SGR) between proactive and reactive fish. Lastly, a Pearson correlation analysis was performed between fish behavioural (NEAR, TATR, TATC, DTC, NEANE, TATNE, and DTNE) and physiological (cortisol and glucose blood concentrations) variables on one hand and fish morphological (weight, length, and SGR) variables on the other hand.

Results

Morphologic parameters

Flathead grey mullet significantly increased their weight and length along the experiment, with final weight, length and SGR of 394.03 ± 120.00 g, 32.75 ± 3.50 cm, and 0.51 ± 0.2 % respectively.

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Time-course of cortisol and glucose plasma concentrations

Basal levels of plasma cortisol and glucose of unstressed flathead grey mullet were 38.00 ± 7.81 ng·mL^{-1} and 25.54 ± 3.06 mg·mL^{-1}, respectively (Figure 1 A and B). Cortisol plasma concentration presented a significant 3.8-fold-increase from its basal level at 15 min after exposure to acute stress, reaching its maximum value (p = 0.001, Figure 1A). Then, cortisol concentration started to decline at 30 min post-stress, showing a significant decrease at 120 min post-stress, where basal levels were significantly restored at 1440 min post-stress and maintained until the end of the trial at 1440 min. Time-course of glucose plasma concentrations followed the same pattern, with significant 1.5 and 1.6-fold-increases from its basal level at 15 and 30 min after exposure to stress, respectively (p = 0.001; Figure 1B). Then, glucose concentration significantly decreased at 120 min post-stress, reaching initial basal levels and maintaining it at 360 min post-stress. However, glucose concentration increased at 1440 min post-stress, being significantly higher than basal levels and similar to levels at 15 and 30 min post-stress.

Stress coping styles assessment.

In the group risk-taking test, 10 of 25 fish (42.31 %) crossed from the safe area to the risky area (proactive) and 15 of 25 fish (57.69 %) did not cross (reactive). No statistical differences were detected in weight and length (MANOVA; p > 0.05) between proactive and reactive fish (Table 1). Four of the 7 (57 %) behavioural variables registered in individual tests presented significant differences between proactive and reactive animals characterized according to group risk-taking test being: number of escape attempts (p = 0.001) and total activity time in the restraining test (p = 0.001); total activity time (p = 0.006) and distance travelled in the confinement test (p = 0.026; Table 1).

Comparison of time-course of cortisol and glucose plasma concentrations between fish with proactive and reactive stress coping styles.

Mullets identified as reactive showed significantly higher cortisol levels than proactive individuals at 15 (p < 0.002) and at 30 (p = 0.010) min post-stress (Figure 2A) and significantly higher glucose levels than proactive fish after at 30 (p = 0.046) and at 120 (p = 0.044) min post-stress (Figure 2B). No significant differences (p > 0.05) were detected in cortisol or glucose plasma concentrations between proactive and reactive fish at any other time points of the trial. It was observed that cortisol levels in proactive fish returned to basal levels after at 30 min post-stress, while this happened after at 120 min post-stress in reactive fish. As for glucose levels, they returned to basal levels at 120 min post-stress in proactive animals, whereas they remained significantly higher than basal levels in reactive ones during the 24h of the experiment. Finally, a significant increase in cortisol plasma concentration was observed after at 1440 min in reactive fish, but not in proactive ones (p =0.001).

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Correlations.

Morphological variables (weight, length, SGR) were not significantly correlated neither with the 7 behavioural variables, nor the physiological variables (cortisol and glucose concentrations) ($p > 0.05$; Table 2).

Discussion

Time-course of cortisol and glucose plasma concentrations

Basal level of cortisol in *Mugil cephalus* registered in the present study was similar to other studies performed on the same fish species at juvenile stage (Mohamadi *et al.,* 2014; Akbary & Jahanbakhshi, 2016; Jiménez-Rivera *et al.,* 2023) and on other fish species, such as rainbow trout (*Oncorhynchus mykiss*) (Sadoul & Geffroy, 2019) and dusky kob (*Argyrosomus japonicus*) (Arkert *et al.,* 2020), with values ranging from 30 to 100 $ng \cdot mL^{-1}$. Nonetheless, it is well known that basal levels of cortisol are variable among fish species (Bordin & Freire, 2021), developmental stage (Ibarra-Zatarain *et al.,* 2016), and sex (Cowan *et al.,* 2017).

Cortisol maximum blood level was detected in grey mullet at 15 min post-stress, representing the latency time needed by this fish species to produce an optimal physiological response to an acute stress. This results was similar to what was found in other fish species, such as in dusky kob (Arkert *et al.,* 2020) and zebrafish (*Danio rerio*) (Tudorache *et al.,* 2013), with a maximum cortisol level reached between 30 to 60 min post-stress, as well as in other studies in the same fish species where animals were submitted to acute stress situations (Mohamadi *et al.,* 2014; Akbary & Jahanbakhshi, 2016). Results were also in accordance with other aquaculture fish species such as grass carp (*Ctenopharyngodon idella*) (Jiang *et al.,* 2017) and dusky kob (Arkert *et al.,* 2020) that presented maximum values after an acute stressful stimulus ranging between 70 and 150 $ng \cdot mL^{-1}$. The rapid elevation of cortisol levels observed in *Mugil cephalus* after an acute stress, confirmed that this hormone is the primary response to stressful conditions, as reported in different studies on reared fish, where routine procedures, such as handling or netting were demonstrated to lead to relatively fast and high cortisol release (Sadoul & Geffroy, 2019; Madaro *et al*., 2022).

Regarding glucose, although baseline blood level in unstressed flathead grey mullet in the present study was different from the one observed for the same fish species in other studies (Thomas *et al.,* 1980; Jiménez-Rivera *et al.,* 2023), it was similar to the study of Prakoso *et al.* (2015) and to studies in other aquaculture fish species, such as grass carp (*Ctenopharyngodon Idella*) (Jiang *et al.,* 2017) and silver mojarra (*Eucinostomus argenteus*) (Bordin & Freire, 2021), where it ranged between 20 and 40 $mg \cdot mL^{-1}$. These differences may be due to divergences between feeding protocols, as Thomas *et al*.(1980) reported that feeding fish once a day or comparing fish at different developmental stages may affect glucose production. Specimens used in this study were older than those of the previously cited studies. Indeed, it has been demonstrated

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that energy demand is greater in growing juvenile fish (Moraes & de Almeida, 2020) and glucose levels differ from one developmental stage to another (Houbrechts *et al.,* 2019). The highest blood glucose levels in this study (15 and 30 min post-stress) occurred at the same time as the rise in cortisol. In threatening situations, the HPI axis is activated and regulates neuro-endocrine processes, like cortisol release into the bloodstream, among others (Schreck *et al.,* 2016). The high level of glucocorticoids observed during a stress response interacts with insulin and increases the expression of different enzymes involved in gluconeogenesis and glycogenolysis metabolic pathways (Henderson & Small, 2019), thus significantly increasing blood glucose concentration (Schreck & Tort, 2016). Glucose metabolic mobilization initiates what is called second stress response (Schreck & Tort, 2016), which helps individuals generate the energy necessary for the organism to face or escape from hazardous situations (Dai *et al.,* 2022). In addition, cortisol converts muscle non-essential proteins into amino acids that would be transferred into the liver for gluconeogenesis, and also mobilizes lipids stored for fast energy demands, in teleost species (Roychowdhury *et al.,* 2024).

About cortisol level regulation back to basal levels, results suggested that flathead grey mullet overcame the process of stress at 120 min post-stress. This result is similar to those reported by Thomas *et al.* (1980) in juvenile mullet and in other fish species, such as Caspian brown trout (*Salmo trutta caspius*) (Kenari *et al.,* 2012) and dusky kob (Arkert *et al.,* 2020). The recovery of cortisol homeostatic levels reflected the gradual adaptation of mullets to the induced stress performed, as a compensatory strategy of individuals to aversive situations, in accordance with Schreck & Tort (2016). Similar to cortisol, glucose returned to basal levels at 120 post-stress, agreeing with other studies in this species (Thomas *et al.,* 1980) and other fish species, such as Caspian brown trout (Kenari *et al.,* 2012) and silver mojarra (Bordin & Freire, 2021), showing a regulation of their levels from two to three hours after exposure to an acute stress, respectively. This decrease may probably occur since the body is recovering its homeostasis and does no longer require large amounts of energy to keep response mechanisms activated (Jiang *et al.,* 2017; Kristians *et al.,* 2020). Although basal levels of glucose were recovered at 120 and 360 min post-stress, a slight but significant increase was detected at 1440 min post-stress, certainly related to the fact that fish were maintained in a fasting state for the whole period of the trial. This could have triggered a reaction to compensate for the energy needed through the mobilization of body reserves to maintain normal physiological functions, as suggested by James & Tomas (2023).

Time-course of cortisol and glucose plasma concentrations between proactive and reactive fish

This study highlighted, for the first time, the differences in the amplitude of variation of cortisol and glucose blood levels between proactive and reactive fish. Specifically, reactive fish presented significantly higher plasma levels cortisol at times 15 and at 30 min post-stress than proactive fish, in response to the same acute stressful situation. Hence, the present study confirmed the higher regulation of the HPI axis in proactive fish than in reactive individuals, supporting the hypothesis formulated by Réale *et al.* (2010), who suggested that SCS may differ in a set of metabolic and hormonal traits that evolved with the particularities of the life history of individuals and genetics.

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Furthermore, the results of the present study were in agreement with those reported in Senegalese sole (*Solea senegalensis*) (Ibarra-Zatarain *et al.,* 2016) and seabream (*Sparus aurata*) (Alfonso *et al.,* 2020), by demonstrating differences in cortisol production between proactive and reactive individuals in response to acute stress situations. Furthermore, it was observed that proactive individuals recovered basal levels faster than reactive fish, which may provide additional insight into behavioural and physiological aspects of SCS.

The differential pattern of glucose level elevation after stress between proactive and reactive fish was not as marked as for cortisol levels, with a slight trend to be higher in proactive organisms than in reactive ones at 15 min post-stress and being significantly higher in reactive specimens than in proactive ones at 30 min post-stress. This differential profile suggested a contrast between SCS in the time needed after an acute stress to reach a maximum response in terms of available blood glucose (15 min for proactive and 30 min for reactive), being again in agreement with the concept of SCS (Koolhaas *et al.,* 1999).

On the other hand, this study also underlined clear differences between proactive and reactive mullets in the regulation of their HPI axis to recover homeostatic basal state of cortisol and glucose blood concentrations, after physiological stress response. Regarding cortisol levels, proactive fish were able to return to homeostasis 30 min post-stress, while it took 120 min after stress exposure in reactive fish. For glucose, proactive fish were able to recover basal levels at 120 min post-stress, while reactive fish never returned to basal levels in the 24h that lasted the trial. This faster regulation of cortisol exhibited by proactive individuals may be due to the higher control of hypothalamus, which in turn regulates the secretion of ACTH hormone and other metabolites. This assumption is in accordance with Tudorache *et al.* (2013), who reported the same pattern in zebrafish: proactive fish regulated faster cortisol blood concentration after stress response and reached faster their homeostatic basal state back than reactive individuals.

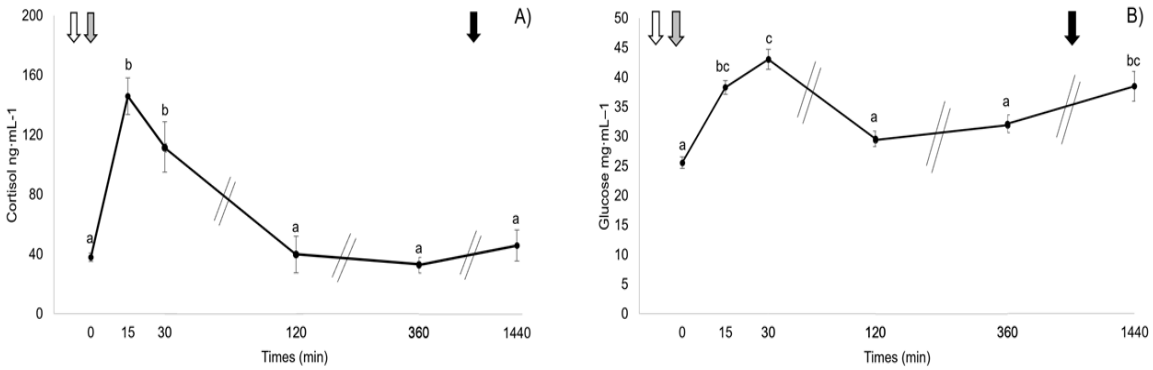

Figure 1. Time-course of cortisol (A) and glucose (B) plasma concentrations of flathead grey mullet.

The (white) arrow indicates that the light turned on, the (gray) arrow indicates stress, and the (black) arrow indicates when the light is turned off. Data is presented as mean ± error of the mean.

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Table 1. Morphological and behavioural variables in flathead grey mullet, characterized by proactive and reactive stress coping styles. Data is presented as mean ± error of the mean. * Indicated significant differences.

Parameters	Variables	Behavioural profiles		MANOVA (*P* < 0.05)	
		Proactive	Reactive	F	*P*
Morphology	Weight (gr)	412.91±161.20	380.19±105.40	0.393	0.537
	Length (cm)	32.90±4.10	32.64±3.20	0.320	0.86
	SGR (gr)	0.66±0.17	0.84±0.24	1.979	0.176
Restraining	NEAR	52.91±16.30	27.73±10.70	19.851	0.001*
	TATR (s)	65.00±8.90	29.60± 10.0	87.589	0.001*
Confinament	TATC (s)	30.18±10.80	17.47±10.60	8.950	0.006*
	DTC (cm)	112.37±57.10	67.03±40.05	5.620	0.026*
New environment	NEANE	1.00±0.90	2.00±2.00	4.971	0.136
	TATNE (s)	24.27±15.36	17.33±12.80	2.380	0.085
	DTNE (cm)	390.24±134.12	285.73±130.50	3.975	0.058

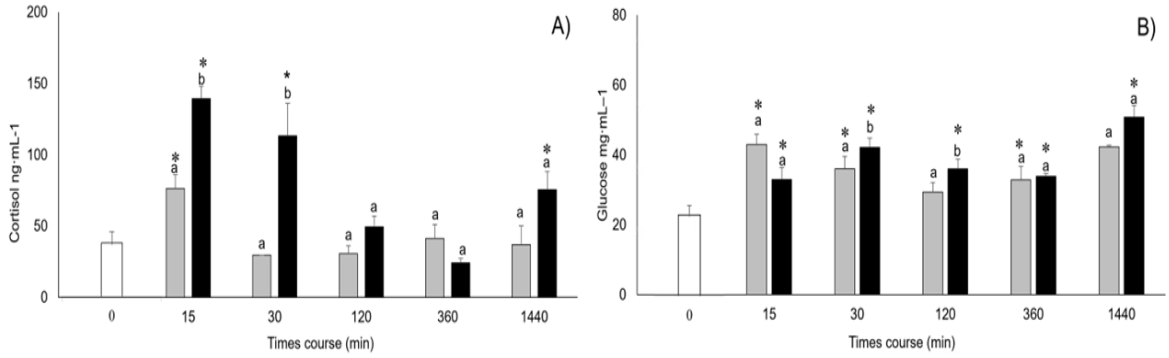

Figure 2. Variation in cortisol (A) and glucose (B) plasma levels in *Mugil cephalus* characterized as proactive (grey bars) and reactive (dark bars) SCS over a 24 h period.

Superscript letters indicated differences in cortisol and glucose levels, between proactive and reactive fish, at each time point; * Showed differences between basal level (white bar) and post-stress levels.

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Table 2. Pearson correlations between morphological, behavioural and physiological variables of flathead grey mullet juveniles.

Variables										
Variables	**SGR (%)**		**NEAR**		TATR (s)		TATC (s)		DTC (cm)	
	r	P	r	***P***	***r***	***P***	***r***	***P***	***r***	***P***
Weight (gr)	-0.30	0.13	-0.06	0.75	0.06	0.76	0.10	0.60	0.16	0.43
Length (cm)	-0.27	0.17	-0.09	0.64	0.00	0.97	0.08	0.67	0.09	0.64
SGR (%)			-0.38	0.05	-0.30	0.13	0.11	0.57	0.20	0.32

Continuation

Variables										
Variables	NEANE		TATNE (s)		DTNE (cm)		Cortisol ($ng \cdot mL^{-1}$)		Glucose ($mg \cdot mL^{-1}$)	
	r	***P***	***r***	***P***	***r***	***P***	***r***	***P***	***r***	***P***
Weight (gr)	-0.15	0.44	-0.29	0.14	-0.07	0.70	-0.29	0.14	0.07	0.72
Length (cm)	-0.11	0.58	-0.36	0.06	-0.02	0.91	-0.34	0.08	0.11	0.58
SGR (%)	0.01	0.94	-0.39	0.06	-0.08	0.68	0.17	0.38	-0.27	0.17

Conclusion

The present study investigated the time-course of cortisol and glucose plasma concentrations for 24 h after an acute stressor exposure in flathead grey mullet juveniles. The highest cortisol and glucose blood concentrations were found at 15 and 30 min post-stress, respectively and both cortisol and glucose concentrations returned to basal homeostatic levels at 120 min post-stress. The most noticeable result was the physiological differences evidenced between proactive and reactive fish, categorized according to their SCS, which were assessed by means of group and individual stress tests. Proactive fish were found to present lower cortisol and glucose blood levels and recovered their homeostatic state faster than reactive fish over the 24h period, showing

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a significantly more efficient regulation of the HPI axis in proactive than in reactive fish. These results are of high interest to understand the mechanisms of the physiological response to stress and its differential regulation between extremes of SCS, and will be useful to establish handling protocols adapted to this species, impacting as little as possible on fish health and welfare.

Author contribution

Carlos A Martínez-Rodríguez: investigation, writing original draft, data analysis; Anaïs Boglino: experimental design, review, editing and proofreading of the manuscript; Emilio Peña-Messina: review and correct manuscript; Joel F Linares-Cordova: methodology, review of manuscript; Zohar Ibarra-Zatarain: conceptualization, experimental design, supervision, funding acquisition, validation and review of manuscript.

All authors have read and agree to publish the article.

Financial outcome

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Ethic statement

Experimental procedures and handling protocols for fish rearing and testing were approved by the Bioethics Commission of Nayarit, Mexico (CEBN number/05/2017). The criteria of the National Center for Replacement, Refinement and Reduction of Animals in Research (NC3RS, United Kingdom) and ARRIVE guidelines were considered for the present research, by using the minimum number of organisms for tests and analyses (Percie du Sert *et al.,* 2020).

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Conflict of interest

The authors declare no conflict of interest.

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