Selecting suitable behavioural tests to identify proactive and reactive stress coping styles in flathead grey mullet (*Mugil cephalus*) juveniles

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Abstract

Identifying Stress Coping Styles (SCS) in new species of interest for aquaculture has important implications for its future domestication and adaptation to captivity. Individual variability allows to select the potential positive characteristics for fish production. The main aim of this study was to identify phenotypic individual differences and characterize proactive and reactive SCS in flathead grey mullet (*Mugil cephalus*) juveniles by exposing fish to different stress situations and evaluating their individual and group responses to level behavioural and physiology. Juveniles were subjected to one group test (risk-taking) and five individual tests (predator, first feeding after stress, restraining, new environment and confinement). All assays were repeated twice, with a one-month interval between tests. Blood samples were taken from each individual (before and after stress) to quantify cortisol and glucose plasma concentrations. Flathead grey mullet juveniles exhibited a high inter-individual variability with two extremes of behaviours: proactive and reactive profiles that were characterized by opposed behavioural (activity time and escape attempts) and physiological (levels of cortisol and glucose) responses to stress and were consistent over time and across contexts. The flathead grey mullet juveniles showed differences in their predisposition for risk taking. Likewise, the Principal Component Analysis showed that three individual stress tests (predator, restraining and confinement tests) were reliable to characterize SCS in this fish species. This work reported for the first time the existence of stress coping styles in *M. cephalus* juveniles and the selection of a set of reliable behavioural tests to identify phenotypic profiles in flathead grey mullet. These results might be of interest for the aquaculture industry to improve fish welfare and health and to adjust management protocols for rearing this fish species in captivity.

Keywords:

Mugil cephalus, aquaculture, stress coping styles

Abbreviation list

PreLat = Latency time to move in the predator test

PreEsc = number of escape attempts in the predator test

PreAct = Total activity time in the predator test

FeedLat = Latency time to first feeding after a stress test

FeedCap = Time to capture the first pellet in first feeding after a capture event test

ResLat = Latency time to move in the restraining test

ResEsc = Number of escape attempts in the restraining test

ResAc = Total activity time in the restraining test

NewLat = Latency time to move in the new environment

NewAct = Total activity time in the new environment

ConLat = Latency time to move in the confinement test

ConEsc = Number of escapes attempts in the confinement test

ConAct = Total activity time in the confinement test

1. Introduction

The ability of animals to cope with stress is essential for them to survive, although some responses to counteract stressful situations can have detrimental effects on animal health and welfare (Galhardo and Oliveira, 2009). In this sense, it is recognized that poor welfare is reflected by characteristic behaviours, such as reduced feeding, alterations in locomotion, increase of aggressive acts and avoidance responses (for example, freezing or escaping), among others (Johansen *et al.,* 2020). Physiologically, stress response promotes cortisol production through the activation of the hypothalamus-pituitary-interrenal axis (HPI) that regulate the endocrine and nervous systems as a stress control mechanism (Clements and Schreck, 2004; Gesto et al., 2013). Important metabolic changes also occur, such as glucose and lactate release in blood circulatory system to cover the energy demand needed to restore homeostasis (Huntingford *et al*., 2006). Regarding aquaculture production parameters, these behavioural and physiological responses might represent a decrease in growth rates, a loss of reproductive capacity, a low immune resistance and a reduction in survival (Ashley, 2007).

The concept of welfare is closely linked to that of stress, since a lack of welfare means that organisms may have a reduced capacity to face environmental challenges (Brown and Dorey, 2019). It has also been suggested that fish, as other vertebrates, exhibit a high inter-individual variability when facing adverse situations (Réale *et al*., 2010). Different definitions have been established to describe the strategies of animals to cope with stress: behavioural syndromes (Sih *et al*., 2004), temperament (Réale *et al*., 2007) or Stress Coping Styles – SCS - (Koolhaas *et al*., 1999). Stress coping styles are a combination of behavioural and physiological responses exhibited by individuals when facing stressful situations and SCS are consistent over time and across contexts (Koolhaas *et al.,* 1999). It is generally recognized that SCS is a continuum that range within two extreme behavioural responses: proactive (also termed bold) and reactive (also termed shy) (Koolhaas *et al*., 1999). Proactive behaviour is characterized by an active risk taking, an explorative behaviour, a high ability to fight against danger and a low production of corticoid hormones and stress metabolites, while the opposite is true for reactive behaviours (Øverli *et al*., 2007; Höglund *et al.*, 2017). Additionally, proactive and reactive SCS have been demonstrated to be associated to differences in characteristics of interest for the aquaculture industry, such as: growth, survival, reproductive success and immune response (Castanheira *et al.,* 2017; Vargas *et al*., 2018). Nevertheless, proactive individuals also have a shorter life expectancy, since proactive individuals are more susceptible to die by higher exposition to threatening situations (Øverli *et al*., 2007). In turn, reactive individuals, present a higher flexibility and adaptation to changing environments and are more cautious when facing dangerous situations, so they tend to live longer (Réale *et al*., 2010; Höglund *et al*., 2017; Geffroy *et al*., 2020).

Likewise, it is important to select appropriated behavioural tests to characterize SCS adapted to each species of interest, considering specific biological and ecological characteristics (Castanheira *et al*., 2017; Burns, 2008). Performing both individual and group tests has been recommended to identify stress coping styles, since animals in groups have been shown to exhibit different patterns of responses to stress compared to when individuals are isolated on their own (Beckamnn and Biro, 2013). Group tests to characterize stress coping styles are: risk taking in new environments (Huntingford *et al*., 2010; Alfonso *et al*., 2020), food intake assessment (Pottinger, 2006; Gesto, 2019) and hypoxia (Castanheira *et al*., 2013a; Ferrari *et al*., 2015; Vindas *et al*., 2017). Regarding individual tests, those that have proved to be effective to characterize SCS are: feeding test (Castanheira *et al*., 2013b; Ferrari *et al*., 2015), restraining test (Castanheira *et al*., 2016; Höglund *et al*., 2020), novel object test (Champneys *et al*., 2018; Skov *et al.*, 2019), new environment test (Castanheira *et al*., 2013a; Ibarra-Zatarain *et al*., 2016) and confinement test (Barreto and Volpato, 2011; Fatsini *et al*., 2020).

To our knowledge, there are no studies related to the characterization of behavioural responses to stress in flathead grey mullet (*Mugil cephalus*). Thus, this study aimed to select appropriate and reliable behavioural tests to prove the existence of proactive and reactive SCS in flathead grey mullet juveniles. Flathead grey mullet is a cosmopolitan fish species, that contributes to food security in some regions of the world, as a product from fisheries and aquaculture (Crosseti, 2016; Ramos-Júdez *et al.*, 2021, 2022). Hence, the identification of SCS in flathead grey mullet juveniles will be of interest for taking it into consideration in selective breeding programs in the aquaculture of this fish species.

2. Materials and methods

2.1. Ethic statement

The number of organisms and the handling procedures used in the present study were in accordance with the guidelines for the Ethical Treatment of Animals according the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs, U.K.) and were authorized by the Bioethics Commission of the State of Nayarit, Mexico (permit number CEBN/05/2017).

2.2. Collection and rearing conditions of organisms

A total of 300 flathead grey mullet juveniles (averaged weight and length of 17.1 ± 13.4 g and 11.6 ± 3.6 cm, respectively) were captured from the wild on the coast of Mazatlán, México. Subsequently, individuals were acclimated in two cylindrical 6000 L tanks for four weeks, and subjected to a prophylactic procedure to control parasites and microbial diseases (Crespo and Crespo, 2003). Afterwards, fish were transferred at the facilities of the Nayarit Centre for Innovation and Technology Transfer, in Tepic, Nayarit and maintained for five months. A total of 44 organisms (average weight and length of 46.5 ± 2.62 g and 17.0 ± 2.8 cm, respectively) were randomly selected and transferred to a recirculation aquaculture system (RAS) which consisted of four rectangular 220 L tanks (80 x 68 x 48 cm), with a final density of 11 fish per tank (~2.1 $kg.m^{-3}$). Water parameters were monitored daily and maintained as follow: temperature 26.0 ± 0.5 ºC, salinity 29.0 ± 0.5 ppm, pH 6.9 ± 0.3 and dissolved oxygen 5.5 ± 0.3 mg/L. The photoperiod was 12 hours day light: 12 hours night, artificially controlled with a digital timer (MyTouchSmart, General Electric®) turning on-off at 08:00-20:00 hours. Mullets were hand fed three times per day to apparent satiation (09:00, 13:00 and 16:00 hours) with a commercial diet for marine fish (Skretting®, The Netherlands, 35% protein, 9% lipids, 1mm pellet size). Tanks were siphoned daily to remove remains of food and faeces and to maintain an adequate water quality.

2.3. Stress tests

All organisms were subjected to one group test and five individual stress tests. Individual tests were applied in sequence, one after another, fifteen days after the group test to allow fish fully recovering their homeostasis (Huntingford *et al.,* 2010; Ibarra-Zatarain *et al*., 2016; Bensky *et al.*, 2017; Fatsini *et al*., 2020) (Fig. 1). Group and individual tests were applied over two different periods of time (run 1 and 2), with one month of interval between runs, with the same materials and protocols, same participants and same hours of the day to minimize bias (Fig. 1).

2.4. Group test

2.4.1. Risk taking test. This test was performed in a rectangular 200 L tank (80 x 50 x 50 cm) that was divided into two equal sections with a plastic barrier. One section corresponded to a safe zone or shelter (isolated from light; 5 lux) and the other section corresponded to a risky zone (exposed to light; 45 lux) (Digital luxmeter HER-410, Steren). Two video cameras (Swann/2K Series-1080p) were installed inside of each section in the tank and served for identifying fish that crossed and fish that did not cross. Additionally, the plastic barrier had a central window (10 x 10 cm) with an opening and closing mechanism allowing fish to cross from one zone to another. The first test was performed on groups of eleven animals to avoid crowding stress (Fazio *et al.,* 2014). On the day of the test, fish were first placed in the safe zone and after one hour of acclimation, the window was opened for two hours. At the end of the test, two *M. cephalus* groups were obtained and separated into two different tanks: those that crossed from the safe to the risky zone, categorized as proactive and those that did not cross, defined as reactive. To confirm consistency of SCS of both groups, a second risk-taking test was performed 1.5 month later. In this sense and considering mortality, risk taking test in run 2 was applied on groups of 9 animals, separately on fish that crossed in run 1 (proactive) and on fish that did not cross in run 1 (reactive). If consistency would not be respected between run 1 and 2 (i.e., fish that crossed in run 1 (proactive) and did not cross in run 2, or fish that did not cross in run 1 (reactive) and crossed in run 2), fish would be considered as having an intermediate SCS and would be eliminated of the posterior analysis between proactive and reactive SCS, which did not happen in the present study. Risk taking test has been demonstrated to be an effective assay to discriminate stress coping styles in fish (Rey *et al*., 2013; Ferrari *et al*., 2015; Alfonso *et al*., 2019; Carbonara *et al.*, 2022). Methodology, duration of experiment and number of fish were based on previous studies on rainbow trout *Oncorhynchus mykiss,* gilthead sea bream *Sparus aurata*, zebrafish *Danio rerio* and Senegalese sole *Solea senegalensis* (Huntingford *et al.* (2010); Castanheira *et al*. (2013a); Rey *et al*. (2013); Ibarra-Zatarain *et al*. (2020).

2.5. Individual tests

Similar to group test, a video camera (Swann/2K Series-1080p) was mounted outside the tanks to record fish behavioural responses during individual tests and to avoid the impact of observers' presence on responses to stress.

2.5.1. Predator response test. Fish were individually transferred with a net from the holding tank to a 27 L tank (40 cm length x 30 cm tall x 30 cm width) containing a plastic fish, black seabass (*Micropterus salmoides*), simulating a predator, which was hidden from the fish by a removable dark wall. The predator plastic fish was approximately 50% larger than the juvenile mullets, in order to represent a significant risk for fish (Solomon-Lane and Hofmann, 2019). The wall that concealed the predator was removed ten seconds after introducing fish into the tank and three behavioural parameters were evaluated during 180 seconds: 1) latency time to move (*PreLat*): referred to the time (in seconds) required by fish to start movement; 2) number of escape attempts (*PreEsc)*: defined as the total number of movements performed by fish to flee from the tank; 3) total activity time (*PreAct*): defined as the total time (in seconds) that fish were swimming forward. This methodology was adapted from Castanheira *et al*. (2013b) and Ferrari *et al*. (2015).

2.5.2. First feeding after a capture event test. Fish were individually captured with a net and maintained out of the water for 30 seconds. Afterwards, fish were released in a plastic 27 L tank (40 cm length x 30 cm tall x 30 cm width) and the following behavioural responses were assessed during 420 seconds: 1) latency time to move (*FeedLat*): referred to the time (in seconds) until fish first exhibited a first forward movement; 2) time to capture the first pellet (*FeedCap):* defined as the time (in seconds) required by fish to swallow the first pellet, which were introduced by an automatic feeder at a rate of one pellet per minute. This methodology and times of evaluation were adapted from studies performed by Silva *et al*. (2010), Castanheira *et al*. (2013a) and Ferrari *et al*. (2015).

2.5.3. Restraining test. This test was realized by capturing each organism individually with a nylon net and by maintaining fish inside the water for 90 seconds in the holding tank. Three behavioural responses were evaluated: 1) latency time to move (*ResLat*): referred to the time (in seconds) required by fish to start movement; 2) number of escape attempts (*ResEsc)*: defined as the number of twists performed by the fish to free itself from the net and 3) total activity time (*ResAct)*: determined as the total time (in seconds) fish presented movement. This methodology was based on that described by Martins *et al*. (2011), Tudorache *et al*. (2013), Castanheira *et al*. (2016) and Höglund *et al.* (2020).

2.5.4. New environment test. Fish were individually transferred to a plastic 27 L tank (40 cm length x

30 cm tall x 30 cm width) that simulated a new environment and two parameters were analysed during 180 seconds: 1) latency time to move (*NewLat*): referred to the time (in seconds) required by fish to start exploration; 2) total activity time (*NewAct*): defined as the total locomotion time (in seconds). This test was adapted from Castanheira *et al.* (2013a), Ibarra-Zatarain *et al*. (2016); Sánchez *et al.* (2017) and Fatsini *et al.* (2020).

2.5.5. Confinement test. The confinement test consisted in placing fish individually in a plastic tank with reduced dimensions for mullet juveniles (25 cm length x 14 cm tall x 8 cm width). Moreover, the tank was half–filled with water from the rearing tank (1.5 L) to simulate a confined area. Three behavioural parameters were assessed for 180 seconds: 1) latency time to move (*ConLa):* referred to the time (in seconds) until fish first move; 2) number of escapes attempts (*ConEsc):* referred to the number of movements performed by fish to escape from the tank and 3) total activity time (*ConAct):* defined as the total locomotion time (in seconds). The methodology was adapted from studies on other fish species (Barreto and Volpato, 2011; Ibarra-Zatarain *et al*., 2020 and Fatsini *et al.*, 2020).

2.6. Quantification of cortisol and glucose concentrations

Basal levels of cortisol and glucose were analysed in unstressed fish from holding tank (n=7). A second analysis of cortisol and glucose was performed on post-stressed (after finalizing individual tests) in proactive (n = 7) and reactive (n=7) fish. All fish were previously anesthetized in a 15 L water volume with eugenol at a concentration of 30 mg/L (He *et al.*, 2020). When fish exhibited partial loss of equilibrium, blood samples (0.4 to 0.5 ml) were extracted from the caudal vein of each juvenile fish with a 1 ml-syringe (8 nm needle, DL) coated with sodium heparin (5000 IU Inephar, PiSA®) to prevent blood sample coagulation. Immediately after blood extraction, samples were transferred to 2 ml eppendorf tubes with 10 µl of sodium heparin, centrifuged at 3000 g for 15 minutes at 4 °C (centrifuge GZ-1580R, Gyrozen, Korea) and plasma samples in the supernatant phase were aliquoted and stored at

-80 °C (ULT Freezer DW-86L828J, Haier Bio-Medical). Cortisol concentration was quantified in triplicate by Enzyme-Linked ImmunoSorbent Assay (ELISA) method (Cortisol ELISA Kit, item No. 500360, Cayman Chemical, USA) based on the competition between cortisol from samples and acetylcholinesterase-linked cortisol from the kit, for a limited number of monoclonal antibody binding sites. Glucose concentration was measured in triplicate by colorimetric method (Glucose Colorimetric Assay Kit, Item 10009582, Cayman Chemical, USA). Absorbance measurements were performed at 420 nm and 520 nm wavelength, for cortisol and glucose, respectively, by means of a spectrophotometer (xMark™ Microplate Spectrophotometer, Bio-Rad), following the methodology defined by Martins *et al.* (2011) and Ibarra-Zatarain *et al*. (2019).

2.7. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 25. A Chi-square test was carried out to determine whether or not the proportions of fish that crossed and did not cross were significantly different between the two runs of the risk-taking test. Two Multivariate Analysis of Variance (MANOVA) were performed to compare the 13 behavioural variables recorded during the five individual tests and the 2 physiological parameters (cortisol and glucose concentrations), between proactive and reactive fish categorized after the risk-taking test, on run 1 and on run 2. Consistency was assessed by means of two additional MANOVA performed on the 13 behavioural variables and the 2 physiological parameters, between run 1 and run 2, for proactive grey mullet juveniles on one hand and for reactive ones on the other hand. All data were presented as average ± standard error of the mean and checked for normality by means of a Kolmogorov–Smirnov test and for variance homogeneity by means of a Levene´s test. A 95% confidence interval (P = 0.05) was set for all analyses

A Principal Component Analysis (PCA) analysis was performed to reduce dimensions of behavioural data of individual tests (predator response, first feeding response, restraining, new environment and confinement) from both runs, to extract the variables that most explained the total variance. Five criteria were used to confirm the suitability of PCA and to select variables as meaningful: a) Eigenvalues of variables ≥1; b) Kaiser- Meyer-Olkin adequacy ≥ 0.500; c) Bartlett test of sphericity < 0.05; d) Test communalities to analyze multicollinearity, i.e., to identify variables correlated to any other variable or to their corresponding initial value and e) Oblimin rotation method to correlate variables. Behavioural variables selected and extracted from this PCA were submitted to a Principal Regression Analysis, to reduce collinearity and to generate Principal Component Scores (PCs) for each fish and for each selected variable, which represented the response to stress for each individual. Afterwards, PCs assigned to each fish were compared between fish that crossed (proactive) and fish that did not cross (reactive) in the risk-taking test, by means of a one-way analysis of variance (ANOVA). Such comparisons were made in order to support the effectiveness of the risk-taking test to discriminate proactive from reactive juvenile's mullets, within the selected tests, following criteria from Budaev (2010), Ibarra-Zatarain et al. (2015), Höglund et al. (2020) and Friedrich et al. (2021). Finally, a Pearson correlation test, with a Bonferroni correction, was performed on selected variables extracted from the PCA versus cortisol and glucose concentrations and morphological variables (weight and length) of fish.

3. Results

3.1. Morphological parameters

No significant differences were observed in weight (run 1: Student t-test; P = 0.289; run 2: P = 0.452) and length (run 1: Student t-test; P = 0.126; run 2: P = 0.488) between proactive fish (run 1: 49.1 ± 4.8 g and 17.7 ± 0.5 cm; run 2: 60.4 ± 8.61 g and 18.4 ± 0.8 cm) and reactive fish (run 1: 41.7 ± 4.8 g and 16.4 ± 0.6 cm; run 2: 51.9 ± 7.22 g and 17.7 ± 0.7 cm), neither in run 1, nor in run 2.

3.2. Stress tests

3.2.1. Group test

Risk taking. A total of 8 fish died between run 1 (n = 44) and run 2 (n = 36), six of them crossed from safe to the risky area and two of them did not cross, in risk taking of run 1. In the first run, 24 of 44 (54.4%) of grey mullet juveniles tested crossed from the safe to the risky zone and 20 of 44 (45.6%) did not cross. Fish were separated into two groups according their risk taking response from run 1 and in the second run, all fish (n = 18) that crossed in the risk taking test of run 1 crossed in run 2 (100%) and all fish that did not cross in run 1 did not cross in run 2 (100%). Fish that crossed in the risk-taking test were considered as proactive and fish that did not cross as reactive.

3.2.2. Individual tests

No statistical differences were detected between proactive and reactive grey mullet juveniles in any of their behavioural variables evaluated in the predator test (PreLat $F_{1,78} = 1.89$, $P = 0.173$; PredEsc $F_{1,78} = 2.545$, $P = 0.115$; PreAct $F_{1,78} = 2.80$, $P = 0.098$) (Table 1A). After the stress event caused by the restraining test, 93.75% of flathead grey mullet juveniles did not ingest any food pellet and 6.25% consumed at least one pellet 297 s after starting the test (4 reactive fish) and 180 s after starting the test (1 proactive). Furthermore, proactive and reactive fish did not show any significant differences, neither in FeedLat ($F_{1,78} = 0.314$, P = 0.577) nor in FeedCap ($F_{1,78} = 0.322$, P = 0.572) (Table 1B). In the restraining test, proactive flathead grey mullet juveniles showed significant lower ResLat ($F_{1,78} = 23.435$, $P \leq 0.001$) and higher ResEsc ($F_{1,78} = 36.283$, $P \leq 0.001$) and ResAct ($F_{1,78} = 8.942$, P = 0.004) than reactive fish (Table 1C). Proactive and reactive fish did not present significant differences in latency in the new environment test ($F_{1,78} = 4.590$, P = 0.069), but proactive fish showed significantly higher activity time than reactive individuals ($F_{1,78} = 9.835$, P = 0.002) (Table 1D). In the confinement test, proactive and reactive grey mullet juveniles showed significant differences in ConLat ($F_{1,78} = 4.417$, P = 0.039), ConEsc ($F_{1,78} = 12.601$, $P \leq 0.001$) and ConAct ($F_{1,78} = 20.724$, $P \leq 0.001$) (Table 1E).

3.2.3. Consistency of proactive and reactive behaviour

Group test

Risk taking. Proportions of fish that crossed from the safe to the risky zone and those that did not cross were similar (χ^2 = 10148, P = 0.284) between the two runs, meaning that fish predisposition to take risks was consistent over time.

Individual tests

Consistency in behavioural responses between runs 1 and 2 were of 70% (9 of 13 analyzed behavioural parameters) for proactive mullet juveniles and 85% (11 of 13 analyzed behavioural parameters) for reactive fish. In the predator test, proactive fish presented consistency in PreEsc (F_{40} = 7.747, P = 0.375) between runs 1 and 2, but significant variations in PreLat (F_{40} = 14.739 P ≤ 0.001) and PreAct (F_{40} = 6.579, P = 0.014) (Fig. 2, A1). In contrast, reactive fish showed a high consistency between both runs 1 and 2 in all of the three analyzed variables: PreLat (F_{36} = 5.244 P = 0.178), PreEsc (F_{36} = 1.156 P = 0.688) and PreAct (F_{36} = 0.552 P = 0.892) (Fig. 2A). Regarding first feeding after restraining test, proactive fish exhibited consistency in both FeedLat (F_{40} = 0.472, P = 0.618) and FeedCap (F_{40} = 3.529, P = 0.393) between runs 1 and 2 (Fig. 2, B1), while reactive fish presented consistency in their FeedLat (F_{36} = 7.330 P = 0.193), but a significant variation in FeedCap (F_{36} = 29.378 P = 0.043) between runs 1 and 2 (Fig. 2, B2). In restraining test, both proactive and reactive fish showed consistency in all of the analyzed variables between runs 1 and 2 (ResLat, F_{40} = 0.302, P = 0.785 and F_{36} = 1.920 P = 0.686; ResEsc, F_{40} = 0.41, P = 0.669 and F_{36} = 0.107 P = 0.429 and ResAct, F_{40} = 0.408, P = 0.194 and F_{36} = 0.814 P = 0.227, for proactive and reactive fish, respectively) (Fig. 2, C1 and C2). The new environment test, both proactive and reactive fish presented consistency between runs 1 and 2 for both analyzed variables (NewLat, F_{40} = 0.128, P = 0.543 and F_{36} = 3.780 P = 0.179; and NewAct, F_{40} = 7.975, P = 0.217 and F_{36} = 1.148 P = 0.378, for proactive and reactive fish, respectively) (Fig. 2, D1 and D2). Finally, in confinement test, proactive fish showed consistency in their ConLat (F_{40} = 0.864, P = 0.128), but exhibited significant differences in their ConAct (F_{40} = 11.185 P ≤ 0.001) and ConEsc (F_{40} = 10.618 P ≤

0.001) between runs 1 and 2 (Fig. 2, E1), while reactive fish were consistent in their ConLat (F_{36} = 6.767 P = 0.055) and ConAct (F_{36} = 0.107 P = 0.206), but presented significant differences in their ConEsc (F_{36} = 1.133 P = 0.004) between runs 1 and 2 (Fig. 2, E2).

3.2.4. Plasma cortisol and glucose quantification

Basal levels of plasma cortisol and glucose of unstressed flathead grey mullet juveniles were of 10.58 ± 3.31 ng/mL and 69.20 ± 8.71 mg/dL, respectively (Fig. 3). Cortisol levels were significantly increased compared to basal levels in both proactive and reactive fish after applying behavioral tests (Student t-test; $P \leq 0.001$), while glucose levels after stress tests were remained similar for proactive fish (Student t-test; P = 0.011) but significantly higher than basal levels for reactive fish. After behavioural tests, proactive fish were shown to produce significantly less cortisol (Student t-test; $P \leq 0.001$) and glucose (Student t-test; P = 0.011) than reactive fish.

3.2.5. Test selection

The adequacy of the PCA to reduce dimensions and extract variables that most variance explained was confirmed in the present study (KMO = 0.649, χ^2 = 245.31, gl = 105, P < 0.001). The PCA test reduced at 5 Principal Component (PC) axis the 13 behavioural variables analyzed and these 5 PC explained more than 64.9% of total variance: PC1 explained 21.8% of total variance, PC2 15.5%, PC3 10.3%, PC4 8.8% and PC5 8.5% (Table 2). From the 13 behavioural variables that composed each PC, the one that showed the highest loading coefficient was selected as the most representative for this PC: activity in restraining for PC1 (0.814), activity of mullets in front of a predator for PC2 (0.837), activity in confinement for PC3 (0.779), latency time in confinement for PC4 (-0.665) and latency in restraining for PC5 (-0.809) (Supplementary Figure). All those variables exhibited eigenvalues higher than 1 (Table 2). Lastly, significant differences between fish that crossed (proactive) and did not cross (reactive) in the risk-taking test were detected for PC1 ($F_{1,78}$ = 71.98, P < 0.001), PC3 PC1 ($F_{1,78}$ = 5.46, P < 0.019) and

PC5 ($F_{1,78} = 3.84$, $P < 0.047$). Comparison of the 5 selected behavioural variables from individual tests between mullets that crossed and did not cross in the risk-taking test confirmed the suitability of this group test to differentiate proactive from reactive fish.

3.2.7. Correlation between fish weight and behavioural and physiological responses

Variables of behavioural responses that most explained the variability to identify proactive and reactive SCS in mullet juveniles (PC1, PC2 and PC3) were not significantly correlated ($P > 0.05$) to growth parameters (weight and length) for this species (Table 3). Neither were physiological parameters of response to stress (cortisol and glucose) ($P > 0.05$).

4. Discussion

4.1. Exploring SCS in mullet juveniles under stress tests

Inter-individual behavioural variation defined by Koolhaas *et al*. (1999) as SCS was supported by this study, since behavioural and physiological differences found between fish screened in the risk-taking test allowed to propose this grouping test as the one that is able to validate proactive (also named bold) and reactive (also named shy) SCS in flathead mullet. This result confirmed the effectiveness of this group test to characterize SCS, based on boldness characteristic of proactive fish (higher overall activity and willingness to take risk) and on shyness for reactive fish (lower overall activity and willingness to take risk), as it has been previously demonstrated in other fish species, such as gilthead seabream (Castanheira et al., 2013a), European sea bass *Dicentrarchus labrax* (Alfonso *et al.*, 2019), and Senegalese sole (Fatsini *et al.*, 2020).

Moreover, individual tests allowed exploring SCS profiles in *M. cephalus* by evidencing similar responses to those reported by previous SCS studies in other aquatic organisms, such as higher activity time in Senegalese sole (Ibarra- Zatarain *et al*., 2020), higher number of escape attempts in gilthead seabream (Castanheira *et al.*, 2013a) and lower reaction times in brown trout *Salmo trutta* (Adriaenssens and Johnsson, 2011) in proactive than in reactive fish when subjected to stress tests. These responses were likely related to behavioural traits, as activity (Bass and Gerlai 2008), boldness (Martins *et al.*, 2011; Ferrari *et al.*, 2015) and exploration (Fatsini *et al.*, 2020) induced in this study by facing the presence of a predator, restraining, new environment and confinement situations. Toms and Echavarria (2014) also reported four personality axis: aggressiveness, fear, boldness and exploration, for zebrafish subjected to five individual stress tests inducing different behavioural responses between proactive and reactive fish regarding locomotion and stress response indexes. These differences among behavioural profiles reported for proactive and reactive mullet have been already documented in species of interest for aquaculture, such as rainbow trout (Øverli *et al.*, 2004), Atlantic salmon *Salmo salar* (Kittilsen *et al.*, 2009), gilthead sea bream (Castanheira *et al.*, 2013) and Senegalese sole (Fatsini *et al.*, 2020) and have been related to behavioural traits as fear, exploration and boldness. In this context, it can be said that a stress test allowed to evaluate behavioural responses and can be related to different behavioural traits, not only to one specifically (Roche *et al.*, 2016). Besides, some stressful situations can be faced differentially among species, since scenario perceived as "aversive" for a species can be perceived differently for others (Huntingford and Adams, 2005). Thus, the combination of group and individual tests performed in the present study to characterize stress coping styles in flathead grey mullet juveniles was reliable, as both behavioural approaches were taken into consideration to analyze patterns of behavioural responses to stress in fish juveniles. Indeed, some authors have reported that evaluating fish behaviour individually may compromise their response to stress by the absence of the naturally present shoal and of the interaction with conspecifics, while evaluating stress response in a group of fish may create a psyco-social context that promotes exploration and reduce anxiety (Galhardo and Oliveira, 2009; Castanheira et al., 2013).

This study also confirmed how cortisol and glucose levels differ within a same organism before (basal state) and after a stressful situation. These results were comparable to those reported as basal by Wanshu (1992), Mohamadi *et al.* (2014) and Akbary and Jahanbakhshi (2016) in this same fish species and these levels increased significantly when a chemical stress, a handling stress, a salinity stress and a starvation stress were induced, as observed in this study. In addition, differences in the activity of HPI axes between proactive and reactive SCS were highlighted in the present study, being higher in reactive fish than in proactive mullets juveniles when subjected to stress tests and coinciding with results reported by Schjolden *et al*. (2005) and Øverli *et al*. (2007) in rainbow trout and Silva *et al*. (2010) in Senegalese sole. As well, glucose levels were significantly higher in reactive fish than in proactive fish, confirming the divergence between behavioural profiles of mullets to cope with stress, as reported by Carbonara *et al*. (2019) and Arechavala- Lopez *et al*. (2020) in gilthead sea bream and Gesto (2019) in rainbow trout. Furthermore, the increase in glucose blood concentrations after exposure to stress was not significantly different from the basal level in proactive fish. In this sense, Huntinford *et al*. (2010) and Careau *et al*. (2008) reported that proactive carp *Cyprinus carpio* presented higher metabolic rates than reactive fish, even at resting, as probably happened in this study.

4.2. Consistency of SCS over time and across contexts

Stress coping styles, characterized in mullet by its ability to cross or not from the safe to the risky area in the group risk-taking test, were consistent over a short period of time, since both proportions of proactive and reactive fish exhibited repeatability in their behavioural predisposition to take risks in both runs. This allowed to confirm the validity of the group test to identify stress coping styles in juveniles' individuals of *M. cephalus*as, short-time consistency had been previously documented in other fish species, such as *S. aurata* (Castanheria *et al.*, 2013a) and *D. rerio* (Rey *et al.*, 2013). Besides, the present study demonstrated that proactive and reactive behaviours identified in the group risk-taking test were associated to proactive and reactive SCS observed in the individual behavioural tests. Since the divergence in behavioural responses to take or avoid a risk, the activity times and the escape attempts were maintained across contexts, highlighting a short-time consistency of behaviour, as it has been documented by Wong *et al.* (2012) and Ibarra-Zatarain *et al*. (2016).

Regarding repeatability of individual tests over time, results did not show the same consistency over time depending on proactive or reactive behaviours, on variables and tests, which might be attributed to underlying characteristics of SCS, such as behavioural flexibility (Coppens *et al.,* 2010; Koolhaas *et al.,* 2010) or behavioural plasticity (Wund *et al.,* 2015). This could be due to the fact that proactive fish might base their conduct on routines and learning from previous experiences, paying little attention to environmental changes, thus responding quickly to aversive situations, but with no precision in their responses (Ruiz-Gomez *et al.,* 2011). On the contrary, reactive fish might be more flexible and might adapt more easily to stressful situations than proactive fish, so they may exhibit more precise behaviours in response to stimulus and could represent an important adaptive characteristic of SCS for fish farming (Wong *et al.,* 2012; Wund *et al.,* 2015). Additionally and considering that only short-term consistency was evaluated in this study, it would be interesting to explore long-term consistency of SCS over time in mullet, in order to examine more stable behavioural responses to stress and to provide more robust explanations on consistency over time in this species (Budaev and Brown, 2011). Finally, the fact that consistency was not demonstrated for all behavioural variables could be linked to the evolution of behavioural responses as time passes and organisms become more experienced or by the differences in the perception of individuals to the environmental stimuli, which moulded the stress responses in this fish species at juvenile stage, as suggested Castanheira *et al*. (2016). In this context, Budaev and Zworykin (2002) showed that behavioural consistency in the lion-headed cichlid became stronger as individuals' growth (*Steatocranus cassuarius*).

4.3. Test selection

Behavioural responses exhibited by animals when facing adverse situations can vary due to a differential inter-individual perception of stress (Janczak *et al.,* 2003; Koski, 2014). In this sense, stress tests investigated the scenarios in which mullet juveniles exhibited the highest behavioural variability to identify proactive and reactive SCS, as conducted in other studies (Castanheira *et al.*, 2013; Fatsini *et al.*, 2020). Budaev (2010), exposed that methods to validate stress tests focus on exploratory analysis as principal component analysis (PCA), since 98% of 51 studies on animal behaviour used PCA as statistical analytical approach, considering this method as the most robust and adequate for this type of studies. In this study, PCA was shown to be an efficient method to select behavioural tests, by identifying the variables globally with a higher variability index, as previously realized on Senegalese sole by Ibarra-Zatarain *et al.* (2016). It also helps confirming what was proposed by Costello and Osborne (2005) on the inflexion point of PCA screen plot, observing that an additional PCA supported unanimously the selection and validity of stress tests through the reduction from 13 evaluated variables (behavioural response parameters) to 5 variables, similarly to what was performed by Carter and Feeney, (2012). In the present study, the PCA resulted in five principal components (PC) representing three different individual tests, of the five tests evaluated. Predator, restraining and confinement tests were the most suitable to describe behavioural variability in *M. cephalus* juveniles, as selected in previous studies and other species (Silva *et al.*, 2010; Ibarra-Zatarain *et al.,* 2016). Principal component scores in this study were suggested to exhibit five principal behavioural traits for this species such as fear, represented by the latency time to move in confinement and restraining, and boldness/exploration, represented by the total activity of individuals in predator, restraining and confinement tests. Results were similar to what was found by Wilson and Godin (2009) in bluegill sunfish *Lepomis macrochirus*, where principal components of three stress tests represented personality traits, such as exploration and risk taking. These behavioural traits can be of interest since they represent fish SCS responses to conditions they are normally exposed to in captivity and in farms, such as handling, transport and size grading (Castanheira *et al.,* 2016). Additionally, the three selected tests were confirmed to not be correlated to size variables (length and weight), meaning that behavioural and physiological responses corresponding to the three selected tests were intrinsic to organisms, whatever their size and weight.

Selected individual tests, plus de addition of the group test, in this study for this fish species represent viable techniques to be implemented in mullet aquaculture production, since they are low-cost operative practices and are operational behavioural tests (OBT) of easy application and interpretation for both scientific and technical staff, as reported previously by Ibarra-Zatarain *et al.* (2016). Moreover, they were validated according to species particularities, being one of the most important considerations to improve knowledge on management of organisms of interest (Burns, 2008; Koski, 2014).

5. Conclusion

Findings of this study demonstrated, for the first time, the existence of phenotypic individual differences corresponding with stress coping styles (SCS) in *M. cephalus* juveniles. Reliable and specific stress tests (predator test, restraining test and confinement test) that induced differential inter-individual behavioural and physiological (cortisol and glucose blood concentrations) responses in mullet juveniles were selected. Short-term consistency over time and across contexts was confirmed in this fish species at juvenile stage. Therefore, these tests were suggested to be implemented in fish farming for the selection of phenotypes exhibiting behaviours with favorable mechanisms in response to stress, higher flexibility to respond to changes, which could improve productive and welfare variables in *M. cephalus* juveniles in captivity.

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Author contribution

J.A. Jimenez-Rivera: Conceptualization, Investigation, Analysis and interpretation of data, writing original draft. **A Boglino**: Conceptualization, Validation, Writing-Review and Editing, Visualization. **J.F. Linarea-Cordova**: Investigation, Review and editing. **N. Duncan**: Conceptualization, Methodology, Review and Editing, Visualization. **S. Rey Planellas**: Conceptualization, Methodology, Review and Editing, Visualization. **Z. Ibarra-Zatarain**: Conceptualization, Investigation, Analysis and interpretation of data, Supervision, Project coordination.

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Figures

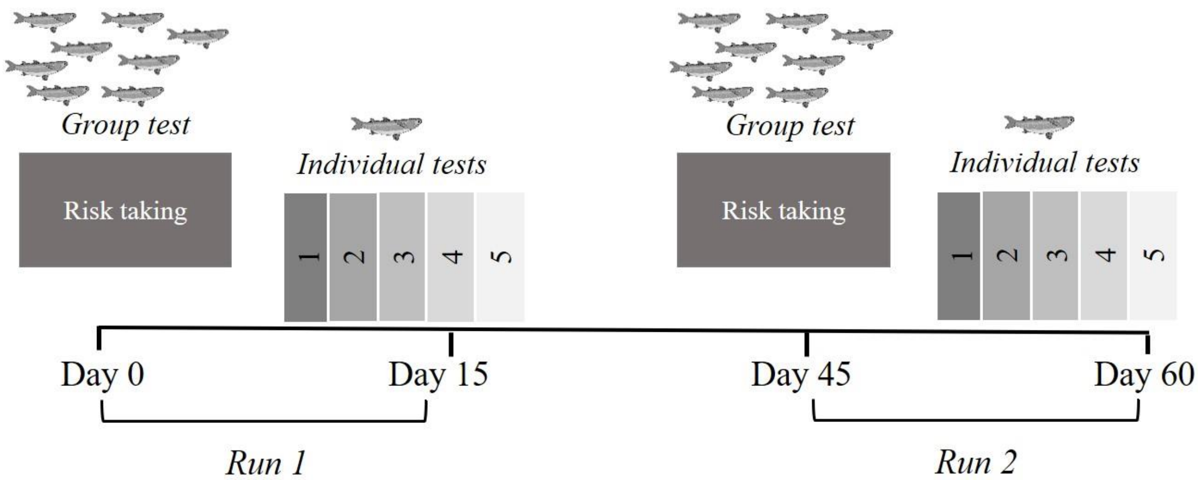

Figure 1. Time line diagram of the group test (risk taking) and individual tests (1= predator, 2= feeding, 3= restraining, 4= new environment, 5= confinement) to characterize the flathead grey mullet juveniles stress coping styles. Tests were applied twice and individual tests were applied in sequence on the same day.

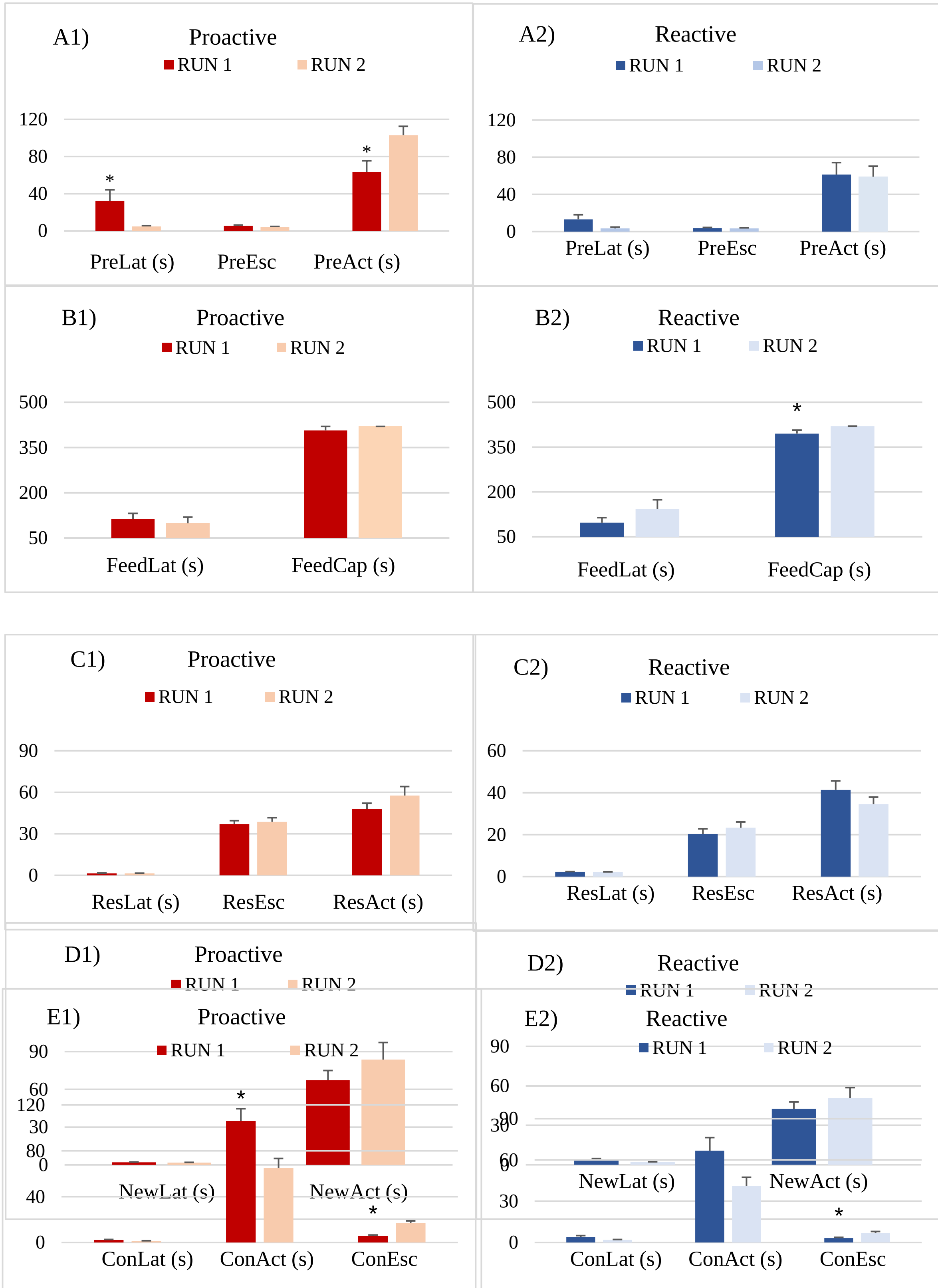
A1) Proactive
RUN 1 RUN 2
120 80 40 0
*
*
PreLat (s) PreEsc PreAct (s)
A2) Reactive
RUN 1 RUN 2
120 80 40 0
PreLat (s) PreEsc PreAct (s)
B1) Proactive
RUN 1 RUN 2
500 350 200 50
FeedLat (s) FeedCap (s)
B2) Reactive
RUN 1 RUN 2
500 350 200 50
*
FeedLat (s) FeedCap (s)
C1) Proactive
RUN 1 RUN 2
90 60 30 0
ResLat (s) ResEsc ResAct (s)
C2) Reactive
RUN 1 RUN 2
60 40 20 0
ResLat (s) ResEsc ResAct (s)
D1) Proactive
RUN 1 RUN 2
90 60 30 0
NewLat (s) NewAct (s)
D2) Reactive
RUN 1 RUN 2
90 60 30 0
NewLat (s) NewAct (s)
E1) Proactive
RUN 1 RUN 2
120 80 40 0
*
*
ConLat (s) ConAct (s) ConEsc
E2) Reactive
RUN 1 RUN 2
90 60 30 0
*
ConLat (s) ConAct (s) ConEsc

Figure 2. Behavioural responses of proactive and reactive juveniles in run 1 and 2 for each test realized: (A) predator test, (B) first feeding test after a capture event, (C) restraining test, (D) new environment test and (E) confinement test. * Asterisks represent significant differences between runs.

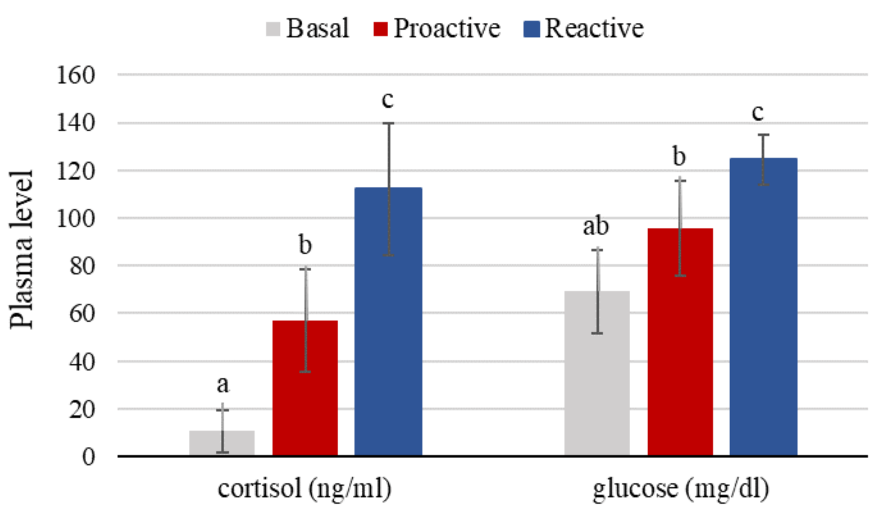

Figure 3. Comparison of basal levels (grey bars) and post-stress levels of cortisol (ng/ml) and glucose (mg/dL), in proactive (red bars) and reactive (blue bars) flathead grey mullet juveniles. Superscript letters represented significant differences.

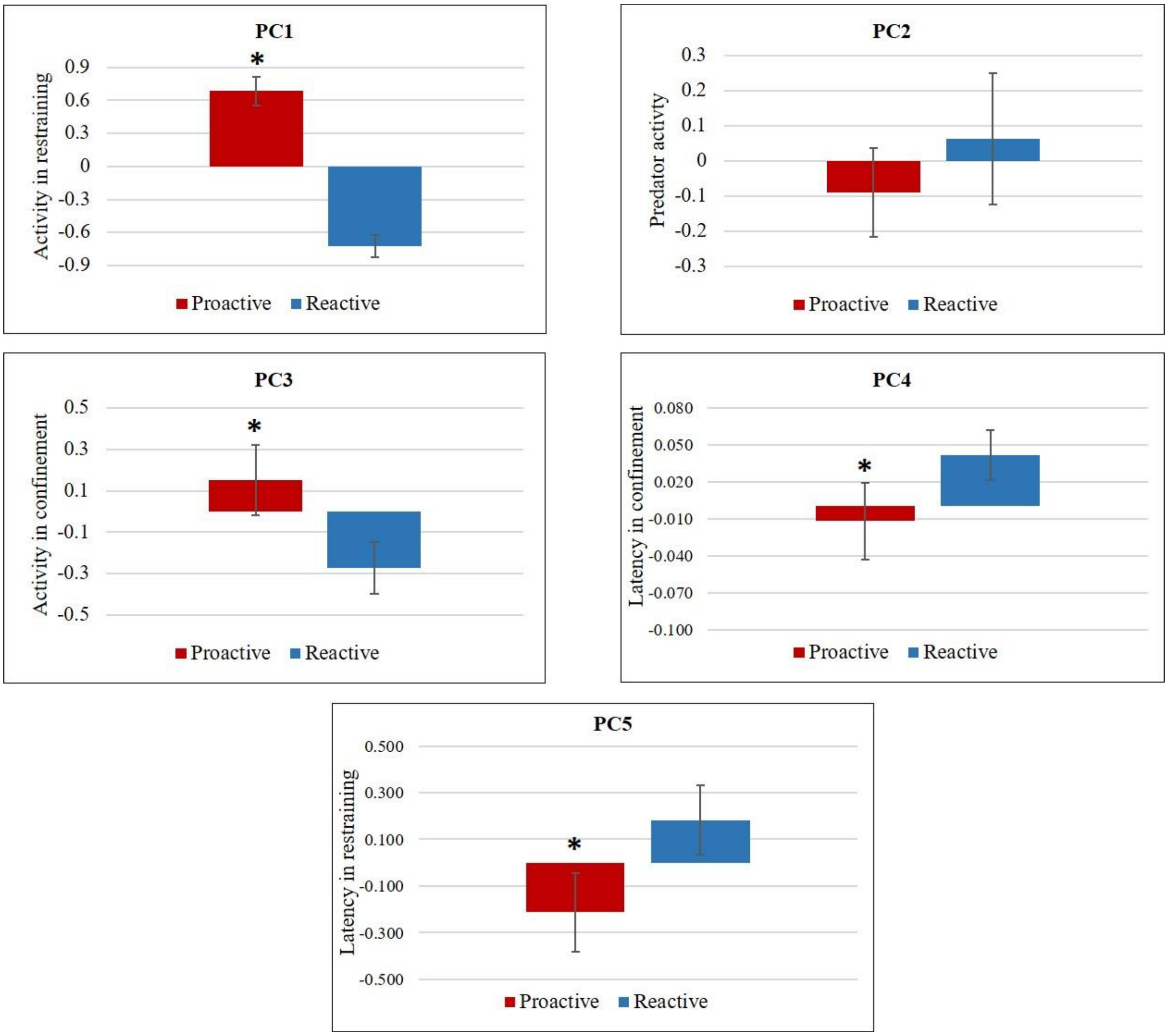

Figure 4. Comparison between proactive (fish that crossed) and reactive (fish that did not cross) mullets according their extracted component in the PCA test (PC1: activity in restraining, PC2: activity in predator, PC3: activity in confinement, PC4: latency in restraining, PC5: latency in confinement). Asterisks represent statistical differences.

Tables

Table 1. General results of individual tests on proactive and reactive juveniles of *M. cephalus*. Averages of the two runs ± standard error and coefficient of variation are presented. Superscript and bold letters reveal significant differences between proactive and reactive for each variable of the test (analysed by MANOVA, $p = 0.05$).

Stress test	Variables	Coping	Means	%CV
A. Predator	Latency time (s)	Proactive	20.55 ± 7.10	231.3
		Reactive	9.66 ± 2.76	
	Escape attempts	Proactive	4.88 ± 0.63	82.4
		Reactive	3.63 ± 0.44	
	Total activity time (s)	Proactive	80.38 ± 8.48	76.8
		Reactive	60.24 ± 8.50	
B. First feeding after restraining test	Latency time (s)	Proactive	106.76 ± 13.71	85.9
		Reactive	118.95 ± 17.12	
	Capture time the first pellet (s)	Proactive	412.57 ± 7.43	10.7
		Reactive	407.00 ± 6.26	
C. Restraining	**Latency time (s)**	Proactive	1.43 ± 0.12[a]	45.4
		Reactive	2.21 ± 0.10[b]	
	Attempts escape	Proactive	37.71 ± 1.90[b]	47.4
		Reactive	21.71 ± 1.85[a]	
	Total activity time (s)	Proactive	52.12 ± 3.67[a]	48.2
		Reactive	38.13 ± 2.78[b]	
D. New environment	Latency time (s)	Proactive	1.95 ± 0.17	110.1
		Reactive	2.89 ± 0.59	
	Total activity time (s)	Proactive	74.26 ± 7.32[a]	68.3
		Reactive	46.50 ± 4.59[b]	
E. Confinement	**Latency time (s)**	Proactive	1.86 ± 0.27[a]	108.8
		Reactive	3.08 ± 0.54[b]	
	Attempts escape	Proactive	10.40 ± 1.34[a]	94.1
		Reactive	4.95 ± 0.66[b]	
	Total activity time (s)	Proactive	88.36 ± 7.73[a]	66.1
		Reactive	46.95 ± 4.30[b]	

Table 2. PCA analysis of behavioural variables from the five individual tests. First five components represented the highest variance and higher eigenvalues.

Component	**Initial Eigenvalues**			**Extraction sums of Squares Loading**			**Rotation sum of square loading**	**Component definition**
	Total	**Variance (%)**	**Cumulative variance (%)**	**Total**	**Variance (%)**	**Cumulative variance (%)**	**Total**	
Restraining activity	**2.834**	**21.8**	**21.8**	**2.834**	**21.8**	**21.8**	**2.232**	**PC1**
Predator activity	**2.017**	**15.518**	**37.318**	**2.017**	**15.518**	**37.318**	**2.175**	**PC2**
Confinement activity	**1.341**	**10.315**	**47.633**	**1.341**	**10.315**	**47.633**	**1.575**	**PC3**
Confinement latency	**1.145**	**8.811**	**56.444**	**1.145**	**8.811**	**56.444**	**1.611**	**PC4**
Restraining latency	**1.102**	**8.479**	**64.923**	**1.102**	**8.479**	**64.923**	**1.388**	**PC5**
Predator escape	0.966	7.43	72.353					
Predator Latency	0.848	6.521	78.874					
New environment activity	0.728	5.598	84.473					
Feeding capture	0.669	5.144	89.616					
Restraining escape	0.504	3.875	93.491					
New environment latency	0.398	3.06	96.552					
Feeding latency	0.284	2.185	98.737					
Confinement escape	0.164	1.263	100					

Table 3. Correlation of weight and length versus extracted behavioural variables, from PCA analysis, and physiological (cortisol and glucose) parameters, in proactive and reactive *Mugil cephalus* juveniles.

	Proactive				**Reactive**			
	Weight		Length		Weight		Length	
Variable	***P***	**r^2**	***P***	**r^2**	***P***	**r^2**	***P***	**r^2**
Restraining, *Total activity*	0.299	0.050	0.288	0.051	0.211	0.075	0.383	0.064
Predator, *Total activity*	0.404	0.058	0.717	0.008	0.318	0.162	0.384	0.048
Confinement, *Total activity*	0.690	0.027	0.370	0.061	0.305	0.065	0.444	0.043
Confinement, *Latency time*	0.376	0.069	0.093	0.414	0.447	0.057	0.343	0.056
Restraining, *Latency time*	0.130	0.138	0.635	0.167	0.237	0.086	0.071	0.189
Cortisol	0.272	0.233	0.389	0.151	0.561	0.072	0.775	0.018
Glucose	0.473	0.107	0.190	0.315	0.585	0.064	0.343	0.180

Declaration of Competing Interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

J.A. Jimenez-Rivera: Conceptualization, Investigation, Analysis and interpretation of data, writing original draft. **A Boglino**: Conceptualization, Validation, Writing-Review and Editing, Visualization. **J.F. Linarea-Cordova**: Investigation, Review and editing. **N. Duncan**: Conceptualization, Methodology, Review and Editing, Visualization. **S. Rey-Planellas**: Conceptualization, Methodology, Review and Editing, Visualization. **Z. Ibarra-Zatarain**: Conceptualization, Investigation, Analysis and interpretation of data, Supervision, Project administration.

Highlights

- *M. cephalus* exhibited individual differences linked to stress coping styles (SCS)
- Proactive mullets had higher total activity than reactive ones in the three selected tests
- Proactive mullets showed lower cortisol and glucose post-stress levels than reactive fish
- PCA analysis identified three suitable tests to characterize SCS in *M. cephalus*